Regulation of Vascular Contractility and Blood Pressure by the E2F2 Transcription Factor

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Background—Recent studies have identified a polymorphism in the endothelin-converting enzyme (ECE)–1b promoter (−338C/A) that is strongly associated with hypertension in women. The polymorphism is located in a consensus binding sequence for the E2F family of transcription factors. E2F proteins are crucially involved in cell-cycle regulation, but their roles in cardiovascular function are poorly understood. Here, we investigated the potential role of E2F2 in blood pressure regulation.

Methods and Results—Tail-cuff measurements of systolic and diastolic blood pressures were significantly higher in E2F2-null (E2F2−/−) mice than in their wild-type littermates, and in ex vivo ring assays, aortas from the E2F2−/− mice exhibited significantly greater contractility in response to big endothelin-1. Big endothelin-1 is activated by ECE-1, and mRNA levels of ECE-1b, the repressive ECE-1 isoform, were significantly lower in E2F2−/− mice than in wild-type mice. In endothelial cells, chromatin immunoprecipitation assays confirmed that E2F2 binds the ECE-1b promoter, and promoter-reporter assays indicated that E2F2 activates ECE-1b transcription. Furthermore, loss or downregulation of E2F2 led to a decline in E1b-1 levels, to higher levels of the membranous ECE-1 isoforms (ie, ECE-1a, -1c, and -1d), and to deregulated ECE-1 activity. Finally, Sam68 coimmunoprecipitated with E2F2, occupied the ECE-1b promoter (chromatin immunoprecipitation), and repressed E2F2-mediated ECE-1b promoter activity (promoter-reporter assays).

Conclusion—Our results identify a cell-cycle–independent mechanism by which E2F2 regulates endothelial function, arterial contractility, and blood pressure. (Circulation. 2009;120:1213-1221.)

Key Words: blood pressure ■ endothelium ■ endothelium ■ E2F transcription factors ■ Sam68 protein, mouse

Endothelin-1 (ET-1) is a potent vessel-constricting peptide. It is synthesized in endothelial cells (ECs) from a larger preproET-1 precursor that is cleaved into an inactive 38–amino-acid peptide, big ET-1 (BigET-1), and then further processed by endothelin-converting enzyme-1 (ECE-1) into the active, 21–amino-acid ET-1. Subsequently, ET-1 acts on the smooth muscle cells through 2 receptors of the G-protein–coupled receptor family, ETA and ETB. Ample evidence from genetic mouse models and from the blockade of ET-1 receptors in humans has demonstrated that the ET-1/ECE-1 system plays a critical role in the regulation of blood pressure (BP) homeostasis.

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ECE-1 is a membrane-bound zinc metalloendopeptidase expressed predominantly in the vascular endothelium and catalyzes the rate-limiting step during biogenesis of ET-1.

The physiological importance of the conversion of BigET-1 to mature ET-1 is demonstrated by observations in ECE-1−/−, ETA−/−, and ETB−/−knockout animals, which exhibit virtually identical cardiac and craniofacial abnormalities during embryonic development, and by the 140-fold greater vasoconstrictive potency of ET-1. There are 4 isoforms of ECE-1: ECE-1a, -1b, -1c, and -1d, which are expressed via alternative promoters from the same gene located on human chromosome 1 (1p36). (Figure IA of the online-only Data Supplement). These isoforms exist primarily as homodimers and localize to different subcellular regions because of their dissimilar N-termini; ECE-1a, -1c, and -1d are located on the plasma membrane, and ECE-1b is intracellular (Figure IB of the online-only Data Supplement). It has been shown that...

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that ECE-1b is located intracellularly in the late endosomes/multivesicular bodies, and its N-terminal leucine-based motifs are involved in this intracellular retention. Interaction of a plasma-membrane ECE-1 isoform with ECE-1b results in its intracellular localization and decreases its extracellular activity. Therefore, the targeting signals specific for ECE-1b constitute a regulatory domain that modulates the localization and activity of other ECE-1 isoforms.

Recently, 2 independent clinical studies have revealed a polymorphism in the 5′-regulatory region of the ECE-1b gene (ECE1 C-338A, 338 bp upstream from the translation start site) that is strongly correlated with increases in systolic, diastolic, and mean BP levels in women. Moreover, the EDN1 K198N polymorphism in the coding region of the preproET-1 gene, previously known to be associated with BP in overweight people, interacts with the ECE1 C-338A variant to influence BP levels. Interestingly, the ECE1 C-338A polymorphism is located in a consensus site for the E2F family of transcription factors and alters its binding affinity specifically to E2F2. However, it is unknown whether E2F2 plays a role in the regulation of ECE-1b gene expression and the pathogenesis of hypertension.

The E2F family of transcription factors regulate cell growth, differentiation, and survival. The classically described mechanism by which E2F family members regulate transcription involves binding to DNA as heterodimers with members of the DP protein family and the recruitment of other regulatory elements. Accumulating evidence indicates that the 9 known E2F proteins (E2F1 through E2F8) target both common and unique genes in the genome and that each E2F family member has diverse physiological functions that are specific to the tissue type and biological context. The functional diversity of E2F members is exemplified by the strikingly different phenotypes found in the corresponding knockout animals. The specific roles of E2F family members in regulating the vasculature, however, are poorly characterized.

Recently, we have shown that E2F1 is involved in the regulation of angiogenesis. Genetic deletion of E2F1 enhanced blood flow recovery after ischemic injury in mice. To determine whether other E2F family members are involved in angiogenesis, we performed experiments in E2F2-null (E2F2−/−) mice. Angiogenesis was unchanged; however, the E2F2−/− mice were hypertensive with altered vasomotion. Here, we provide genetic evidence that E2F2 regulates BP via specific regulation of endothelial ECE-1b expression, subcellular localization, and hence vascular reactivity. Furthermore, our studies reveal that Sam68, an RNA-binding protein and oncogene, acts as a novel transcription cofactor of E2F2 in the regulation of ECE-1b.

**Methods**

**Mice**
The heterozygote E2F2+/− mice were obtained from Dr Gustavo Leone’s laboratory (Ohio State University) and were bred, maintained, and operated on in the Center for Comparative Medicine of Northwestern University following protocols approved by the Institutional Animal Care and Use Committee. All animals were genotyped by polymerase chain reaction (PCR) of tail DNA. Age- and sex-matched E2F2−/−, E2F2+/−, and their wild-type (WT) littermates were used.

**BP Measurement**
Arterial BP and heart rates from WT and E2F2−/− mice were measured by the standard noninvasive tail-cuff method (CODA System, Kent Scientific, Torrington, Conn). Measurements were performed during the day after 5 days of training. On each day of BP determination, 20 measurements were obtained and averaged for each mouse.

**Aortic Ring Assay**
The contractile properties of aortic arteries were analyzed by the ring assay as described previously and as summarized in the Methods section of the online-only Data Supplement.

**Measurement of ET-1 Peptides**
Levels of ET-1 in mouse plasma and in cell culture medium were measured with a commercially available ELISA kit (Biomedica, Vienna, Austria) by following the manufacturer’s instructions.

**Tissue RNA Isolation and Real-Time Reverse-Transcription PCR**
Tissue RNA was extracted and real-time PCR was performed as described previously. Primer sequences are listed in Figure II of the online-only Data Supplement.

**Plasmids and Small Interfering RNA**
Human pECE-1b/C-alkaline phosphatase (AP) and pECE-1b/A-AP plasmids were provided by Dr Benoît Funalot (INSERM, France). pRC/CMV-E2F1 and pRC/CMV-E2F2 plasmids were provided by Dr Farbio Martelli (IRCSS, Italy). Myc-Sam68 plasmid was provided by Dr Chi Wai Eric So (Institute of Cancer Research, Sutton, UK). Human and bovine E2F1 small interfering RNA (siRNA) and E2F2 siRNA were synthesized by Dharmaco, Inc (Lafayette, Colo). The mouse ECE-1b promoter–AP reporter plasmid was constructed by following standard cloning techniques (see the Methods section of the online-only Data Supplement for details).

**Cell Culture, Plasmid and siRNA Transient Transfection, and Reporter Assays**
Human umbilical vein ECs (HUVECs) and bovine aortic ECs (BAECs) were obtained from ATCC (Manassas, Va), cultured as described, and used within passage 5. For plasmid transfection of BAECs, TransFast (Promega, Madison, Wis) or Arrest-In (Open Biosystems, Huntsville, Ala) was used. For siRNA transfection of HUVECs, Arrest-In or RNAiFect (QIAGEN, Valencia, Calif) was used. Control β-Gal–expressing plasmid (pCMV–β-Gal) was co-transfected to normalize for transduction efficiency. Chemiluminescent reporter gene assays for the detection of AP and β-Gal activities were performed as described previously.

**Isolation of Mouse Primary Lung ECs**
Primary ECs were isolated from mouse lung tissues by following the published protocol (see the Methods section of the online-only Data Supplement for details). The cells were used before passage 5, and the EC identity was confirmed before each experiment by staining with FITC-conjugated anti-CD31 antibody.

**Subcellular Fractionation, Western Blotting, and Immunoprecipitation**
Plasma membrane and cytosolic proteins were fractionated by using the Pinpoint Cell Surface Protein Isolation Kit (Pierce Biotechnology Inc, Rockford, Ill) (see the Methods of the online-only Data Supplement for details) and then analyzed by Western blotting. The primary antibodies used were anti-E2F2, anti-E2F1, anti-Sam68, anti-Myc, and anti-AP.
anti-HA, anti–Tie-2, anti-actin (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), anti-human ECE-1 (R&D Systems, Minneapolis, Minn), anti-Camxin (Streptagen, Ann Arbor, Mich), and anti-Myc (Cell Signaling Technology, Danvers, Mass). For immunoprecipitation, cell lysates were incubated overnight at 4°C with the appropriate antibody, then incubated with protein A/G plus agarose (Santa Cruz) for 1 hour at 4°C. After washing, the immunoprecipitates were eluted by boiling for 5 minutes, and extracts were analyzed by immunoblotting as described above. Band intensities were determined densitometrically with Image J software.

**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation (ChIP) assays were performed in HUVECs with a ChIP assay kit (Upstate, NY) (see the Methods section of the online-only Data Supplement for details). Primer sequences are listed in Figure II of the online-only Data Supplement.

**Mass Spectrometry**

Experiments followed a standard protein identification strategy as described in the Methods section of the online-only Data Supplement.

**Fluorescent Immunohistochemistry**

Fluorescent immunohistochemical staining was performed by following standard techniques (see the Methods section of the online-only Data Supplement for details), and the slides were examined under a confocal microscope (Zeiss LSM 510 META).

**ECE-1 Enzymatic Activity in Intact Primary ECs**

Conversion of the exogenous substrate BigET-1 (Alexis, Lausen, Switzerland) into mature ET-1 was determined in primary lung ECs after incubation with $10^{-6}$ mol/L BigET-1 for 60 minutes in a 96-well plate at 37°C. The reaction was stopped by the addition of 1 mmol/L NaEDTA, and the ET-1 generated was measured in the culture medium with an ELISA kit (Biomedica, Vienna, Austria). The value obtained from the well without cells was considered the ELISA cross-reactivity for BigET-1 and subtracted from all the measurements in which BigET-1 had been added. The ET-1 level in each well was normalized to the quantity of cells as determined with a crystal violet staining method that we have described previously.

**Statistical Analysis**

All values are expressed as mean ± SEM. Comparisons between 2 means were performed with an unpaired Student t test, whereas ANOVA with Fisher protected least significant differences and Bonferroni-Dunn posthoc analysis were used for comparisons of >2 means. Comparisons between concentration-response curves were performed with ANOVA for repeated measures. Significance was defined as $P < 0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Loss of E2F2 Expression in Mice Results in Elevated BP and Exaggerated Arterial Contraction in Response to BigET-1**

We measured BP in E2F2$^{-/-}$ mice and their WT littermates and found that despite similar heart rates, the E2F2$^{-/-}$ mice displayed significantly higher systolic and diastolic BPs (Figure 1A). Using ex vivo aortic ring assays, we compared the contractility of aortas isolated from E2F2$^{-/-}$ mice and their WT littermates in response to BigET-1 and a variety of vasoactive substances. Contracture induced by KC1 (30 mmol/L) and phenylephrine ($1 \times 10^{-9}$ to $3 \times 10^{-6}$ mol/L) and relaxation in response to acetylcholine ($1 \times 10^{-9}$ to $3 \times 10^{-6}$ mol/L) were similar in the 2 strains, but aortas from the E2F2$^{-/-}$ mice exhibited significantly greater contractility.
in response to serial doses of BigET-1 (2.7 × 10⁻⁷ to 2.1 × 10⁻⁶ mol/L) (Figure 1B and 1C).

Because BigET-1 is converted to active ET-1 by ECE-1, thereby increasing vessel contractility, our observations suggest that the influence of E2F2 on contractility could be generated, at least in part, through the regulation of ECE-1 activity. If so, the elevated BP in E2F2 mice may be attributable to an increase in ET-1 level and activity. Plasma ET-1 levels were significantly higher in E2F2 mice than in WT mice (Figure 1D), and selective antagonism of the ETA receptor with BQ123 dramatically reduced BP in E2F2 mice to levels that did not differ significantly from those in WT mice (Figure III of the online-only Data Supplement). In addition, we found that E2F2 mice express a higher level of ETA protein in the lung tissues (Figure IV of the online-only Data Supplement). Collectively, these findings indicate that enhanced ET-1 biosynthesis and activity, resulting from increased ECE-1 activity, contribute to the BP elevations observed in E2F2 mice.

**E2F2 Regulates ECE-1b Transcription**

Because recent clinical studies have identified a strong association between an E2F binding site polymorphism on the ECE-1b promoter (C-338A) and hypertension in women, our findings may be relevant to human disease. Accordingly, we evaluated whether E2F2 regulates ECE-1b mRNA expression in vivo via real-time reverse-transcription PCR analyses. In lung tissues, the levels of ECE-1b mRNA were 50% lower in E2F2 mice than in their WT littermates (Figure 2A), suggesting that endogenous E2F2 levels maintain basal ECE-1b expression in WT tissue; the levels of ECE-1a, -1c, and -1d mRNA, however, were similar between E2F2 mice and WT controls (Figure V of the online-only Data Supplement). Interestingly, ECE-1b mRNA levels were similar in the lung tissues from E2F1 and E2F2, as was the case with WT mice (Figure VI of the online-only Data Supplement), indicating that the reduced ECE-1b expression observed in E2F2 mice was an E2F2-specific effect.

To investigate whether E2F2-regulated ECE-1b mRNA expression is allele specific for the ECE-1b promoter, BAECs were cotransfected with an E2F2-expressing plasmid and a reporter plasmid that expressed AP from either the native (−338C) ECE-1b promoter (pECE1b/C-AP) or the polymorphic (−338A) ECE-1b promoter (pECE1b/A-AP) that has been linked to hypertension in women (Figure 2B and Figure IA of the online-only Data Supplement).
ment); cells were harvested 24 hours after transfection for AP activity assays. In the absence of E2F2 overexpression, the activity of the polymorphic promoter was significantly higher than native promoter activity (Figure 2B). Overexpression of E2F2 significantly increased the activity of both the polymorphic and native ECE-1b promoters, but the enhancement of native promoter activity was significantly greater (native, 28.7-fold; C-338A, 1.9-fold; Figure 2B). These observations suggest that E2F2 activates ECE-1b expression, but this activation is impaired by the C-338A polymorphism. Results from similar experiments indicate that the human ECE-1b promoter is not regulated by E2F1 (Figure 2B). To determine whether endogenous E2F2 regulates ECE-1b promoter activity, we performed promoter-reporter assays in BAECs after knocking down endogenous E2F2 expression with siRNA. Both polymorphic and native ECE-1b promoter activity was significantly reduced in the presence of E2F2 siRNA, but the activity of the polymorphic promoter was reduced to a greater extent (Figure 2C).

To correlate our observations from experiments in E2F2−/− mice with the results from our human ECE-1b promoter-reporter assays, we investigated whether regulation of the mouse ECE-1b promoter is disrupted in E2F2−/− mice. BAECs were cotransfected with a plasmid that expressed AP from a murine ECE-1b proximal (1.6 kb) promoter, which contains 3 putative E2F binding sites, and a plasmid expressing either E2F2 or E2F1. Overexpression of E2F2, but not E2F1, significantly upregulated murine ECE-1b promoter activity (Figure 2D), confirming that E2F2 regulates ECE-1b expression in both mice and humans.

To determine whether E2F2 physically interacts with the ECE-1b promoter, we performed ChIP assays with HUVECs. Antibodies to E2F2, but not E2F1, coprecipitated with the ECE-1b promoter, indicating that endogenous E2F2 occupies the ECE-1b promoter region in vivo and that ECE-1b is a direct transcriptional target of E2F2 (Figure 2E).

Sam68 Suppresses E2F2-Mediated ECE-1b Transcription

To identify cofactors that may interact with E2F2 and modulate ECE-1b regulation, we isolated nuclear protein extracts from cultured HUVECs, communoprecipitated the extracts with the E2F2 antibody, resolved the E2F2-complexed proteins on a 1-dimensional SDS-PAGE gel, and identified the proteins via mass spectrometry. One of the proteins identified was Sam68, an RNA-binding protein.32 Both endogenous and overexpressed Sam68 protein communoprecipitated with E2F2 (Figure 3A and 3B), confirming that Sam68 binds E2F2 in vivo and identifying Sam68 as a possible cofactor involved in E2F2 transactivity. We then performed ChIP assays and confirmed that Sam68, like E2F2, occupies the ECE-1b promoter region in vivo (Figure 3C).

To determine whether Sam68 modulates E2F2 transactivity at the ECE-1b promoter, we performed cotransfection

Figure 3. Sam68 suppresses E2F2-mediated ECE-1b transcription. A, Interaction between endogenous Sam68 and E2F2 proteins. HUVEC lysates were immunoprecipitated (IP) with anti-E2F2 antibody, immunoblotted (IB) with anti-Sam68 antibody, and reblotted with anti-HA antibody. B, Interaction between overexpressed Sam68 and E2F2 proteins. The 293T cells were transfected with HA-tagged E2F2 and Myc-tagged Sam68. Cell lysates were immunoprecipitated with anti-HA antibody, blotted with anti-Myc antibody, and reblotted with anti-HA antibody. C, ChIP assay showing Sam68 occupation of the ECE-1b promoter in vivo in HUVECs. D, Effect of Sam68 overexpression on basal and E2F2-induced ECE-1b promoter activity. BAECs were cotransfected with pECE1b/C-AP and with plasmids expressing E2F2 (pE2F2), Sam68 (pSam68), or both; AP activity was assessed 24 hours after transfection and expressed as the fold difference from the level observed in the pECE1b/C-AP–only group. Data are mean±SEM. AP activities were normalized to β-Gal activity (n=9 per treatment; **P<0.01; ***P<0.001).
experiments with plasmids expressing E2F2, Sam68, and the native human ECE-1b promoter–AP construct. Overexpression of Sam68 suppressed E2F2-induced ECE-1b promoter activity in a dose-dependent manner, as well as basal ECE-1b promoter activity (Figure 3D). Collectively, our data indicate that Sam68 is a cofactor that represses E2F2-mediated ECE-1b transcription.

Loss or Downregulation of E2F2 Expression Increases Levels of the Plasma Membrane ECE-1 Isoforms and Reduces Levels of the Intracellular ECE-1 Isoforms

Because E2F2 regulates ECE-1b transcription, we investigated whether ECE-1 protein levels differ in tissues harvested from E2F2−/− mice and their WT littermates. The levels of ECE-1 were determined densitometrically, normalized to the levels of α-actin, and expressed as the fold change from the levels measured in WT lung tissues. Control assessments evaluated ECE-1 levels in HUVECs and HeLa cell lysates, and recombinant human ECE-1 (rECE-1) protein levels (n=6 per group; **P<0.01; NS, not significant vs WT lung tissue; N/A for normalization to α-actin). B, Western blotting analyses of ECE-1 protein levels at the plasma membrane and in the cytosol of the primary lung ECs isolated from E2F2−/− and WT mice. Cells were fractionated with a Sulfo-NHS-SS-Biotin kit. The levels of ECE-1 in the membrane and cytosol were normalized to the levels of Tie-2 and calnexin, respectively, and expressed as the fold change from the levels measured in WT ECs (n=5 per group; **P<0.01 vs WT). C, Confocal microscopy of mouse lung ECs stained with an anti–ECE-1 antibody (red) and counterstained with DAPI (blue) (original magnification ×400); representative results from 4 separate experiments are shown. The anti–ECE-1 antibody recognizes an epitope shared by all 4 ECE-1 isoforms (ECE-1a, -1b, -1c, -1d). D, ET-1 levels in the culture media of WT and E2F2−/− lung ECs after incubation with 10−7 mol/L BigET-1 for 60 minutes (n=6 per group; ***P<0.001). The ET-1 levels were measured with ELISA and normalized to the quantity of cells.

Figure 4. Loss of E2F2 increases levels of the plasma membrane ECE-1 isoforms and reduces levels of the intracellular ECE-1 isoforms. A, Representative Western blotting for ECE-1 (top) and quantification of relative ECE-1 levels (bottom) in the whole-cell lysates from the lung tissues of E2F2−/− mice and their WT littermates. The levels of ECE-1 were determined densitometrically, normalized to the levels of α-actin, and expressed as the fold difference from the levels measured in WT lung tissues. Control assessments evaluated ECE-1 levels in HUVECs and HeLa cell lysates, and recombinant human ECE-1 (rECE-1) protein levels (n=6 per group; **P<0.01; NS, not significant vs WT lung tissue; N/A for normalization to α-actin). B, Western blotting analyses of ECE-1 protein levels at the plasma membrane and in the cytosol of the primary lung ECs isolated from E2F2−/− and WT mice. Cells were fractionated with a Sulfo-NHS-SS-Biotin kit. The levels of ECE-1 in the membrane and cytosol were normalized to the levels of Tie-2 and calnexin, respectively, and expressed as the fold difference from the levels measured in WT ECs (n=5 per group; **P<0.01 vs WT). C, Confocal microscopy of mouse lung ECs stained with an anti–ECE-1 antibody (red) and counterstained with DAPI (blue) (original magnification ×400); representative results from 4 separate experiments are shown. The anti–ECE-1 antibody recognizes an epitope shared by all 4 ECE-1 isoforms (ECE-1a, -1b, -1c, -1d). D, ET-1 levels in the culture media of WT and E2F2−/− lung ECs after incubation with 10−7 mol/L BigET-1 for 60 minutes (n=6 per group; ***P<0.001). The ET-1 levels were measured with ELISA and normalized to the quantity of cells.
These results were confirmed via confocal microscopic analyses of cells immunofluorescently stained for ECE-1 (Figure 4C), and experiments in HUVECs yielded equivalent results: siRNA-mediated knockdown of E2F2 reduced cytoplasmic ECE-1 (ECE-1b) levels and increased plasma membrane ECE-1 (ECE-1a, -1c, -1d) levels (Figure VIII of the online-only Data Supplement). Importantly, the increase in plasma membrane ECE-1 in the E2F2−/− lung ECs was associated with an enhanced conversion of exogenous BigET-1 into ET-1 (Figure 4D). These observations are consistent with a study by Muller et al., who showed that ECE-1b decreases the expression and activity of ECE-1a, -1c, and -1d. Collectively, the results presented here and by Muller et al suggest that the enhanced contractility of E2F2−/− aortic vessels in response to BigET-1 (Figure 1B and 1C) may evolve indirectly through the downregulation of late endosomal ECE-1b levels and a subsequent increase in ECE-1a, -1c, and -1d activity.

**Discussion**

Hypertension is a multifactorial disease in which genetic traits play an important role.33,34 Recent studies indicate that transcriptional regulators in both vascular ECs and smooth muscle cells can critically affect BP control.27,35 Our investigations provide the first evidence that E2F2, a classic cell-cycle regulator, influences BP. Data from our studies suggest that endogenous levels of E2F2 transcriptional activity in nonproliferating endothelium regulate vessel tone through the ECE-1/ET-1 system: E2F2 acts as a transactivator of ECE-1/ET-1 activity, and Sam68 functions as a cofactor that represses E2F2-induced ECE-1b transcription in ECs. Thus, the physiological state could regulate BP, at least in part, by balancing E2F2 and Sam68 activity to fine-tune ECE-1b expression. If so, deregulated E2F2 transcriptional activity (eg, in E2F2−/− mice or in women carrying the C-338A ECE-1b promoter polymorphism) may contribute to the pathogenesis of hypertension.

The ECE-1/ET-1 system has been recognized to regulate BP through multiple mechanisms, including maintenance of the system vessel resistance and renal functions.6,36–38 Increasing evidence emphasizes aberrant vessel tone in the pathogenesis of hypertension.39,40 We observed a striking abnormality of vascular reactivity in E2F2−/− mice. However, increased ECE-1/ET-1 activity has also been shown to increase oxidative stress41 and to mediate angiotensin II–induced hypertension.42 Importantly, the kidney has been shown to be both a source of ET-1 generation and an important target organ of this peptide, where it mediates natriuretic and diuretic effects through the ETb receptor subtype.43,44 Therefore, we cannot rule out a possible contribution of these factors to the hypertension phenotype of E2F2−/− mice.

The (C-338A) ECE-1b promoter polymorphism has been linked to hypertension in women but not in men.16,17 This gender difference may be partially due to the fact that the higher levels of estrogen in women suppress ET-1 synthesis to a greater extent, so women are more sensitive to the increase in ECE-1 activity.45 However, this effect was not seen in a related study of human Alzheimer disease in which E-1 activity also plays an important role28 (see below). In our experimental settings, the elevated BP existed in both female and male E2F2−/− mice.

Higher levels of basal promoter activity were observed for the polymorphic ECE-1b/A promoter than for the ECE-1b/C promoter, and E2F2 siRNA strongly inhibited both promoters, yielding comparable levels of residual activity. Nevertheless, the ECE-1b/A promoter was only marginally activated by E2F2 overexpression. This apparent discrepancy could evolve from intrinsic competition between E2F2 and other cofactors that bind to the ECE-1b promoter. Overexpression of 1 factor (eg, E2F2) could induce conformational changes in other cofactors, and interactions between the cofactor complex and their cognate cis elements in the ECE-1b/A and ECE-1b/C promoters could differ. However, this explanation has yet to be evaluated experimentally.

Funalot et al.28 have recently reported that the E2F2 binding site polymorphism in the ECE-1b promoter is associated with a lower risk of late-onset Alzheimer disease. Because the deposition of β-amyloid in the brain is a pathological hallmark of Alzheimer disease and ECE-1 activity degrades β-amyloid,46 these data corroborate our findings regarding the regulation of ECE-1 activity by this E2F2 binding site in the ECE-1b promoter. In addition, E2F2 may have a role in other pathological conditions that have been linked to ECE-1 activity, including pulmonary hypertension, myocardial infarction, and renal failure.47 Thus, the implications of our findings presented here may extend to a broad range of pathological conditions and normal developmental processes.

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**References**


Hypertension is largely attributed to abnormal renal sodium handling; however, a growing body of evidence now suggests that primary abnormalities in vessels can also cause aberrations in blood pressure. Very often, the source of the abnormality resides in the endothelial cells that regulate the functional state of the entire vessel, and this knowledge has directed our search for new diagnostic and therapeutic targets. To date, the role of transcriptional mechanisms in blood pressure regulation is poorly characterized. In this study, we found that E2F2, a transcription factor involved in cell-cycle control, regulates blood pressure by modulating vessel contractility. This previously unknown function of E2F2 evolves from the unique role of the molecule in endothelial cells: suppression of endothelin-converting enzyme 1 (ECE-1). ECE-1 converts the inactive precursor molecule big endothelin-1 into the potent vasoconstrictor endothelin-1, and genetic deletion of E2F2 in mice was associated with both exaggerated vessel contractility in response to big endothelin-1 stimulation and high blood pressure. E2F2 suppresses ECE-1 activity indirectly by promoting transcription of ECE-1b, the negative isoform of ECE-1, and our results indicate that Sam68 functions as a cofactor that represses E2F2-mediated ECE-1b transcription. These findings are especially provocative because clinical studies have identified an E2F binding site polymorphism on the ECE-1b promoter that is strongly associated with hypertension in women. Collectively, our results and observations from other laboratories may have identified a previously unknown mechanism of blood pressure maintenance that operates through the E2F2/Sam68–ECE-1 pathway, and deregulation of this pathway may contribute to blood pressure disorders, including hypertension in humans.
Regulation of Vascular Contractility and Blood Pressure by the E2F2 Transcription Factor
Junlan Zhou, Yan Zhu, Min Cheng, Deepika Dinesh, Tina Thorne, Kian Keong Poh, Dongxu Liu, Chantal Botros, Yao Liang Tang, Nichole Reisdorph, Raj Kishore, Douglas W. Losordo and Gangjian Qin

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Aortic ring assay

The aortic ring assay was performed as previously described\(^1\). Briefly, segments of thoracic aorta (3 mm) were dissected with the endothelium intact and mounted in organ baths containing 15 mL Krebs buffer; optimal resting tension was determined in baseline experiments performed before treatment with the compounds under investigation. Vessels were submaximally precontracted with phenylephrine (PE), and endothelial function was evaluated by vascular relaxation in response to acetylcholine (Ach). At the end of each experiment, vessels were contracted by the addition of K\(^+\) to verify that baseline contractile function was preserved during the course of the study. Isometric forces were recorded with force transducers connected to a PowerLab/800 Eight-channel 100 kHz A/D converter (AD Instruments, Mountainview, CA).

Mouse ECE-1b promoter-AP reporter plasmid

To clone murine ECE-1b promoter, genomic DNA was isolated from C57BL/6 mouse tail with QIAGEN kit and used for high fidelity PCR amplification with primers listed in Supplemental Figure S2. The PCR product was first cloned into TOPO TA cloning plasmid (Invitrogen) and
subsequently cloned into pSEAP plasmid\textsuperscript{2} by the use of MluI and ClaI restriction enzymes. The murine ECE-1b promoter sequence in the final reporter construct was verified by sequencing.

**Chromatin immunoprecipitation (ChIP) assay**

Cells were lysed and sheared by sonication, then extracts were pre-cleared with protein A agarose/salmon sperm DNA and immunoprecipitated overnight. Precipitates were washed for 5 min with buffer and extracted twice with 1% SDS, 0.1 M NaHCO\textsubscript{3}, then the elutes were pooled and heated at 65°C for 5 h to reverse the formaldehyde cross-linking. DNA fragments were purified via phenol/chloroform extraction, then 2 μL of DNA was added to a standard PCR mixture, and genes were amplified for 23 cycles; primer sequences are listed in Supplemental Figure S2. PCR products were separated on a 2% agarose gel.

**Mass spectrometry (MS)**

Experiments followed a standard protein identification strategy\textsuperscript{3}. Candidate proteins were excised from the gel, reduced with dithiothreitol, and alkylated with iodoacetamide, then the bands were digested overnight with trypsin, and the extracted peptides were speed vacuumed to reduce volume and remove organic. Peptides were chromatographically resolved on-line with a C18 column and a 1200-series high-performance liquid chromatography (HPLC) apparatus, then analyzed by using a 6340 liquid-chromatography ion-trap mass spectrometer (Agilent Technologies, Palo Alto, CA) with an HPLC-chip interface (Agilent Technologies). Raw data was extracted and searched using the Spectrum Mill search engine (Rev A.03.03.038 SR1, Agilent Technologies), and “peak picking” was performed with a signal-to-noise ratio of 25:1 and a maximum charge state of 4 (z<sub>≤4</sub>); the program was directed to “find” a precursor charge
state. Searches were performed in the International Protein Index human database (IPI Human rel. 3.28, 16-APR-2007) with the following criteria: fixed carbamidomethylation modification, trypsin, no more than 1 missed cleavage, precursor mass tolerance ±1.7, product mass tolerance ±0.7, and maximum ambiguous precursor charge = 3. Protein identification was considered significant if the following confidence thresholds were met: 2 or more peptides per protein, individual peptide scores ≥10, protein score >20, and Scored Percent Intensity (SPI) ≥70%; the SPI indicates what percentage of the total ion intensity matches the peptide’s MS/MS spectrum. A reverse (random) database search was simultaneously performed, and the spectra were inspected manually to validate the match of the spectrum to the predicted peptide fragmentation pattern. Quality-control standards were run at the beginning of each day and at the end of each set of analyses.

**Isolation of mouse primary lung ECs**

Primary ECs were isolated from mouse lung tissues as described in a published protocol. Briefly, tissues were minced and digested with collagenase and dispase, then the mixture was passed through a 100-μm cell strainer to obtain single-cell suspensions. Red blood cells, granulocytes, nonvital cells, and cell debris were removed via density centrifugation with Histopaque-1.083 (Sigma), then the ECs were immunostained with CD31-PE, FACS sorted to ≥ 95% purity, and cultured in EBM-2 medium (Lonza, Walkersville, MD).

**Subcellular fractionation**

Plasma-membrane and cytosolic proteins were fractionated by using the Pinpoint Cell Surface Protein Isolation Kit (Pierce) according to the manufacturer’s protocol. Briefly, cells were
labeled with the cell-impermeable and cleavable biotinylation reagent Sulfo-NHS-SS-Biotin (sulfo succinimidyl-2-[biotinamido] ethyl-1, 3-dithiopropionate) for 30 min at 4°C, then lysed on ice and sonicated. Labeled proteins were isolated with immobilized NeutrAvidin Gel and eluted with SDS-PAGE sample buffer containing 50 mM DTT. The reducing agent (DTT) cleaves the disulfide bond in the biotin label, and nearly 100% of the bound proteins are recovered.

**Immunofluorescent staining**

Primary lung ECs were briefly rinsed with buffer A (1 mM MgCl₂ and 0.1 mM CaCl₂ in PBS), fixed in methanol at room temperature for 10 min, rinsed again, then permeabilized for 15 min in Buffer B (Buffer A with 0.3% Triton X-100, 0.1% BSA); nonspecific protein binding was blocked for 30 min with buffer C (Buffer A with 0.3% Triton X-100, 1% BSA). Cells were incubated overnight at 4°C with goat polyclonal anti-ECE-1 antibodies (0.6 µg/ml) diluted in Buffer C, washed 3 times for 5 min (15 min total) in buffer B, then incubated with PE-conjugated anti-goat IgG antibodies (Jackson ImmunoResearch). One hour later, cells were washed 3 times for 5 min (15 minutes total) in Buffer B and once for 10 min in 5 mM NaP₀ (pH 7.5), then mounted with a solution containing 0.1% p-phenylenediamine (Sigma-Aldrich Corp.), 70% glycerol, 150 mM NaCl, and 10 mM NaP₀ (pH 7.4).
Supplemental Figures

Figure S1: ECE-1 promoter regions and N-terminal amino-acid sequences. (A) The four ECE-1 isoforms (top), organization of the upstream regions of the human ECE-1 gene (middle), and the reporter plasmid containing the ECE-1b promoter and alkaline phosphatase (AP) (bottom); each isoform-specific promoter is identified by a dark bulb, and the E2F binding-site polymorphism (-338C/A) is identified with inverted red triangles. P denotes promoter; d, splicing donor; a, splicing acceptor. (B) The N-terminal amino acid sequences of the ECE-1 isoforms, which determine their subcellular location. TMD denotes transmembrane domain.
Figure S2: Primer sequences used for real-time RT-PCR, for cloning the murine ECE-1b promoter, and for ChIP assays.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Real-Time RT-PCR for gene expression assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>murine ECE-1b</td>
<td>CATCGGGACCGTGTGGTC</td>
<td>AAGAAGTCCTGGCACGGGTC</td>
</tr>
<tr>
<td>murine ECE-1a</td>
<td>TGGTCTCATGGTCTCGCTGC</td>
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<td>murine ECE-1c</td>
<td>GAGCCCTAGCAGGAGGAGTGC</td>
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</tr>
<tr>
<td>murine ECE-1d</td>
<td>TGGAGACGCTGAGGGAGTCC</td>
<td>AAGAAGTCCTGGCACGGGTC</td>
</tr>
</tbody>
</table>

| **Cloning of murine ECE-1b promoter (1.6 kb)** |
| murine ECE-1b | CTCTTACCGCTGCCAGGACTCAAATCCCTATTGTA | CGATTGAAGCTTttgcacccgcgcacgc |

| **Chromatin immunoprecipitation (ChIP) assays** |
| human ECE-1b | CTTTTTTTGTCTGGCTCTTCTAGCA | CCAGTGGCAGATAAAGGTATCA |
| human DHFR | TTCTGCTGTAACGGAGGCTGGGA | CTACAAGTTAGAGAAAACACGATTACTGAA |
| human 3’ end DHFR | CTGATGTCCAGGAGGAAGGG | AGCCCGACAAATGTCAAGGACTG |
Figure S3: The selective ETA-receptor antagonist BQ123 reduces the blood pressure (BP) of E2F2−/− mice to WT levels. Mice were allowed to rest for at least 10 min before basal measurements were recorded, then BQ123 (Alexis USA, San Diego, CA) (dissolved in 0.9% NaCl) was administered as a single bolus injection (10 nmol/kg body weight) into the tail vein and measurements were recorded for 30 min; Pre indicates before BQ123 treatment. n=12 per group; **P<0.01, *P<0.05 versus WT; §P<0.01 versus pre-treatment.
Figure S4: $\text{ET}_A$ and $\text{ET}_B$ protein levels in the lung tissues of E2F1$^{-/-}$ and WT mice. $\text{ET}_A$ and $\text{ET}_B$ protein levels in lysates of lung tissues were analyzed by Western blot (n=3).
Figure S5: mRNA levels of ECE-1a, -1c, and -1d in the lung tissues of E2F2−/− and WT mice. The mRNA expression of ECE-1a (left panel), -1c (middle panel), and -1d (right panel) were analyzed by real-time RT-PCR with total RNA isolated from lung tissue and normalized to GAPDH mRNA expression. Data are displayed as means ± SEM. n=4 per group; N.S., not significant versus WT.
Figure S6: ECE-1b mRNA levels in the lung tissues of E2F1\(^{-/-}\) and WT mice. (A) ECE-1b and (B) E2F1 mRNA expression were assessed by real-time RT-PCR with total RNA isolated from lung tissue and normalized to GAPDH mRNA expression. Data are displayed as means ± SEM. n=4 per group; N.S., not significant versus WT.
Figure S7: Primary lung endothelial cells (ECs) isolated from E2F1<sup>−/−</sup> and WT mice. Mouse lung ECs were isolated via enzymatic digestion of tissues, density centrifugation of single-cell suspensions, and FACS sorting for CD31 expression as described in a published protocol<sup>4</sup>. Shown are representative WT and E2F1<sup>−/−</sup> lung ECs after isolation and double-staining with FITC-conjugated anti-CD31 antibodies (green) and DAPI (blue); the cells were seeded in endothelial culture medium (EBM-2, Lonza, Walkersville, MD) and cultured for 2 days (200× original magnification).
Figure S8: Knockdown of E2F2 expression increases plasma-membrane ECE-1 levels and reduces intracellular ECE-1 levels in HUVECs. HUVECs were treated with E2F2 siRNA (+) or non-targeting siRNA (–) for 24 hours, then fractionated with a Sulfo-NHS-SS-Biotin kit. ECE-1 protein levels at the plasma membrane and in the cytosol were analyzed via Western blot, normalized to the levels of Tie-2 and Calnexin, respectively, and expressed as the fold-difference from the levels quantified in cells treated with the non-targeting siRNA. n=4 per treatment; *P<0.05, **P<0.01 versus non-targeting siRNA treatment.
Supplemental References


