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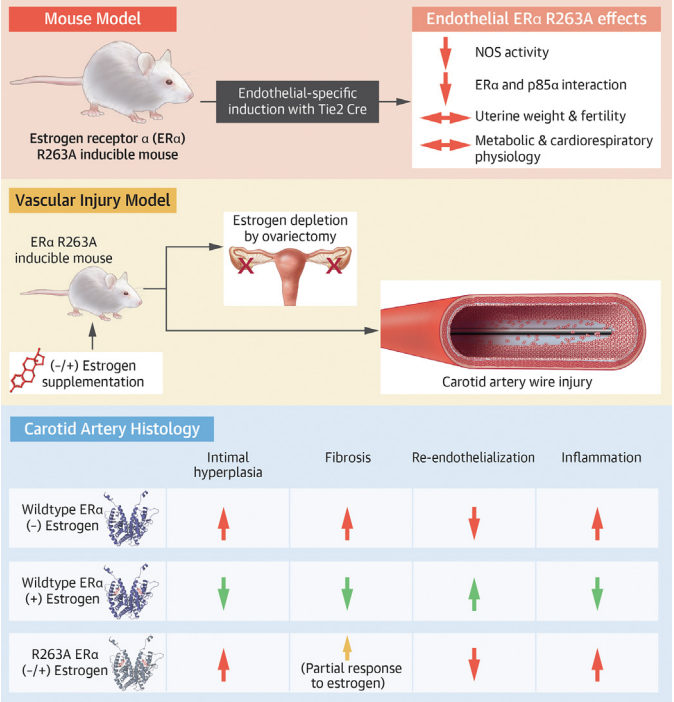
ORIGINAL RESEARCH - PRECLINICAL

Tie2-Cre–Induced Inactivation of Non-Nuclear Estrogen Receptor-α Signaling Abrogates Estrogen Protection Against Vascular Injury

Pang-Yen Liu, MD, PhD,[a,b,*] Nobuaki Fukuma, MD, PhD,[b,*] Yukio Hiroi, MD, PhD,[c,d,*] Akiko Kunita, PhD,[e,*] Hiroyuki Tokiwa, MD, PhD,[b,*] Kazutaka Ueda, MD, PhD,[b] Taro Kariya, MD, PhD,[b,f] Genri Numata, MD, PhD,[b] Yusuke Adachi, MD, PhD,[b] Miyu Tajima, MD, PhD,[b] Masayuki Toyoda, MD, PhD,[b] Yuxin Li, MD, PhD,[d,g] Kensuke Noma, MD, PhD,[d,h] Mutsuo Harada, MD, PhD,[b] Haruhiro Toko, MD, PhD,[b] Tetsuo Ushiku, MD, PhD,[e] Yoshimitsu Kanai, MD, PhD,[i] Eiki Takimoto, MD, PhD,[b,j] James K. Liao, MD,[d,k] Issei Komuro, MD, PhD[b]

VISUAL ABSTRACT



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HIGHLIGHTS

- **Arg 263 on ERα (Arg 259 for human ERα) is a critical amino acid involved in the interaction with the p85α-subunit of PI3K and thus non-nuclear signaling.**
- **Using the Cre-loxP system, a novel mouse model in which ERα non-nuclear signaling was inactivated in endothelial cells with intact nuclear function was generated.**
- **Tie2-Cre–induced inactivation of ERα non-nuclear signaling abrogated the protection of estrogen against mechanical vascular injury, whereas it had a minimal impact on baseline cardiovascular and metabolic phenotypes.**

From the [a]Division of Cardiology, Department of Internal Medicine, Tri-Service General Hospital Penghu Branch, National Defense Medical Center, Taipei, Taiwan; [b]Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [c]National Center for Global Health and Medicine, Tokyo, Japan; [d]Vascular Medicine Research, Brigham and Women's Hospital and Harvard Medical School, Cambridge, Massachusetts, USA; [e]Department of Pathology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [f]Department of Anesthesiology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [g]Nihon University School of Medicine, Tokyo, Japan; [h]Research Institute for Radiation Biology and Medicine,

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ABBREVIATIONS AND ACRONYMS

cDNA = complementary deoxyribonucleic acid

E2 = 17β-estradiol

ECGM = endothelial cell growth medium

eNOS = endothelial nitric oxide synthase

ER = estrogen receptor

ERα$^{KI/KI}$ = estrogen receptor-α$^{knock\text{-}in/knock\text{-}in}$

LVEDD = left ventricular end-diastolic diameter

NOS = nitric oxide synthase

PI3K = phosphatidylinositol 3-kinase

PLA = proximity ligation assay

Vo_2 = oxygen consumption

SUMMARY

Using the Cre-loxP system, we generated the first mouse model in which estrogen receptor-α non-nuclear signaling was inactivated in endothelial cells. Estrogen protection against mechanical vascular injury was impaired in this model. This result indicates the pivotal role of endothelial estrogen receptor-α non-nuclear signaling in the vasculoprotective effects of estrogen. (J Am Coll Cardiol Basic Trans Science 2023;8:55–67)

The risk of cardiovascular disease is lower in women than in age-matched men; however, after menopause the risk difference between sexes decreases.[1] This observation led to a hypothesis that estrogen has protective effects against cardiovascular disease; furthermore, this hypothesis has been supported by several clinical studies of hormone replacement therapy.[2-5] Consistently, studies have revealed beneficial cardiovascular effects of estrogen both at the cellular and molecular levels.[6-9] In addition to classical nuclear function as a transcriptional factor, estrogen receptor-α (ERα) activates the non-nuclear signaling process, which is known as the rapid or membrane-initiated steroid signaling pathway.[9] Upon binding to estrogen, a subpopulation of ERα induces rapid activation of signaling pathways that are coupled to phosphatidylinositol 3-kinase (PI3K) at the cell membrane and mediates part of estrogen's physiological function.[10,11] Prior studies used pharmacologic interventions, such as estrogen-dendrimer conjugate or pathway preferential estrogen, and demonstrated the role of ERα non-nuclear signaling in vasodilatation, endothelial healing, and activation of endothelial nitric oxide synthase (eNOS).[12,13] Furthermore, several genetically engineered mouse models have been developed and reported[14-18]; however, they are global inactivation models, and tissue-specific roles for ERα non-nuclear signaling remain undetermined. A key molecular interaction for the initiation of non-nuclear signaling is the binding of ERα to the p85α subunit of PI3K upon estrogen binding.[10] Amino acids 251 to 260 on human ERα, which have been identified as essential for non-nuclear signaling, critically regulate this molecular interaction.[19] In particular, our preliminary glutathione S-transferase pull-down results suggest the essential role of R259 and/or R260 on ERα in its binding to the p85α subunit of PI3K (data not shown). In Hela cells cotransfected with eNOS complementary deoxyribonucleic acid (cDNA) and mutant forms of ERα, R260A completely abolishes 17β-estradiol (E2)-stimulated eNOS activity and impairs activator protein 1 transcriptional activity.[19] On the other hand, 259A fails to show significant loss of eNOS activity.[19] However, given the simplified and artificial nature of the experiments using Hela cells, in vivo models are necessary to better determine the impact of either residue in the non-nuclear signaling of ERα. Adlanmerini et al[18] recently developed a mouse model with global inactivation of ERα non-nuclear signaling by introducing R264A (human R260A) substitution on ERα and reported the blunted estrogen-stimulated vasodilation in mutant animals without altering fertility. In the present study, we introduced the replacement of Arg263 on mouse ERα with alanine (R263A, which corresponded to R259A on human ERα) using the Cre-loxP system under the control of Tie2 promoter and generated a tissue-specific knock-in mouse model that inactivates non-nuclear signaling in endothelial cells. We examined the baseline cardiovascular and metabolic phenotypes and determined the role of ERα non-nuclear signaling in vascular pathological changes after mechanical injury.

Hiroshima University, Japan; [i]Department of Anatomy and Cell Biology, Wakayama Medical University, School of Medicine, Wakayama, Japan; [j]Division of Cardiology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA; and the [k]Section of Cardiology, Department of Medicine, The University of Chicago Medical Center, Chicago, Illinois, USA. *Drs Liu, Fukuma, Hiroi, Kunita, and Tokiwa contributed equally to this work.
The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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METHODS

Primary antibodies used for histologic analyses are listed in Supplemental Table 1.

ANIMALS. All experimental procedures involving animals were performed in accordance with the principles and guidelines established by the Experimental Animal Care and Use Committee of Harvard Medical School and the University of Tokyo. Mice were housed in cages and maintained in a temperature-controlled facility with a 12-hour light/dark cycle. Estrogen receptor-$\alpha^{\text{knock-in/knock-in}}$ ($ER\alpha^{KI/KI}$) mice were developed on a C57BL/6J background by Ozgene. A floxed mouse line was generated in which a single alteration was inserted into exon 4 of the mouse ERα gene to replace the amino acid R263 (corresponding to R259 of human ERα) with alanine by a codon substitution of CGC with GCC. Endothelial-specific $ER\alpha^{KI/KI}$ mice were generated through crossing the line with Tie2-Cre transgenic mice.[20] We examined female mice because our study focused on the female-specific role of ERα nonnuclear signaling.

ANIMAL SURGERY. Ovariectomy was performed in anesthetized animals with 1% inhaled isoflurane at 8 to 12 weeks of age, as previously described.[21] One week later, a sustained-release E2 pellet (0.25 mg, 60-day release, Innovative Research of America) or placebo pellet was implanted subcutaneously.

The carotid artery wire injury was performed as previously described.[14] Briefly, the left common carotid artery of an anesthetized animal (1% to 2% inhaled isoflurane) was cannulated, and the endothelium was denuded by wire insertion through the external carotid artery. The right carotid artery of each mouse was subjected to a sham operation for the uninjured control in each mouse. On day 7 or 14, the mice were sacrificed, and the vessels were harvested.

PROXIMITY LIGATION ASSAY. A proximity ligation assay (PLA) was performed using the Duolink In Situ Detection Reagents Brightfield (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, formalin-fixed, paraffin-embedded 3-μm sections were dewaxed in xylene and hydrated in ethanol. Antigen retrieval was performed by immersing the sections in citrate buffer (pH = 6.0) for 15 minutes at 98 °C and for an additional 15 minutes at room temperature. Anti-p85α antibody (St John's Laboratory) was conjugated overnight to an activated MINUS oligonucleotide (Sigma-Aldrich) using the Duolink In Situ Probemaker MINUS (Sigma-Aldrich) according to the manufacturer's instructions. After blocking endogenous peroxidase (10 minutes at room temperature) and nonspecific protein binding (1 hour at 37 °C), the sections were incubated overnight at 4 °C with an anti-ERα antibody (1:400, Sigma-Aldrich) and an anti-p85α antibody conjugated with a MINUS probe (1:100). The next day rabbit PLUS secondary PLA antibody (Sigma-Aldrich) was applied, and ligation and amplification reactions were performed. The probes were visualized using a horseradish peroxidase-labeled hybridization probe followed by NovaRED detection (Vector Laboratories). Counterstaining was performed using hematoxylin for 2 minutes. Positive PLA signals in the endothelial cells were counted and normalized to the intraluminal circumference. Images were acquired using an Olympus BX53 microscope.

ENDOTHELIAL CELL ISOLATION AND NITRIC OXIDE SYNTHASE ACTIVITY MEASUREMENT. Endothelial cells were isolated from $ER\alpha^{KI/KI}Tie2^{Cre-}$ and $ER\alpha^{KI/KI}Tie2^{Cre+}$ mice as previously described.[22] Briefly, the brain was removed and dissected into small pieces, which were then centrifuged at 290 *g* for 5 minutes in a 15-mL conical tube containing 8 mL Eagle's minimum essential medium-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The pellet was recovered and incubated in a solution containing papain (0.5 mg/mL, Worthington Biochemical Corporation) and DNase I (Worthington Biochemical Corporation) for 70 minutes in a 37 °C water bath with continuous agitation, which were then centrifuged at 1,360 *g* for 10 minutes after the addition of 7 mL 22% bovine serum albumin/Eagle's minimum essential medium-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The pellet was washed with endothelial cell growth medium (ECGM), which was made with Ham's F12 medium, endothelial cell growth supplement (Sigma-Aldrich), 10% charcoal/dextran treated fetal bovine serum (Cytiva), L-ascorbate, L-glutamine, and heparin. After centrifugation at 290 *g* for 5 minutes, the cells were resuspended in 8 mL ECGM and seeded for culture. The next day the cells were washed 8 times with Ham's F12 medium to remove any debris. After several hours of adding fresh ECGM, 4 μg/mL puromycin was added for 2.5 days to purify endothelial cells. Cells grown to 90% confluence were treated with 10^{-8} mol/L E2 or vehicle (75% ethanol) for 24 hours and harvested.

The nitric oxide synthase (NOS) activity of isolated endothelial cells was measured using an enzyme immunoassay kit (NB78, Oxford Biomedical Research) according to the manufacturer's protocol.[23] Immunofluorescence staining was performed to confirm the purity of endothelial cells.[22] Briefly, cells were seeded on an 8-well chamber slide (Thermo Fisher Scientific) and cultured to 70% to 80% confluence. Cells were

fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes. After blocking with 1% bovine serum albumin, cells were incubated with anti-CD31 antibody (1:100, Abcam) for 1 hour at room temperature. Subsequently, a fluorescent-conjugated secondary antibody (Alexa Fluor Plus 488, Thermo Fisher Scientific) was applied for 1 hour at room temperature. The slides were mounted using the ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Between each procedure, the slides were rinsed 3 times with phosphate-buffered saline with 0.05% Tween 20. Images were acquired using an FSX100 inverted microscope (Olympus).

EVALUATION OF FEMALE MICE FERTILITY. The number of pups was evaluated to assess the reproductive ability of the $ER\alpha^{KI/KI}Tie2^{Cre}$ female mice. Briefly, a 12- to 40-week-old female mouse was housed with a wild-type male mouse. The number of pups for each delivery was counted and averaged, and 3 to 4 female mice of each genotype were used for assessment.

PHYSIOLOGICAL ANALYSIS. Blood pressure was measured 3 times before the carotid artery injury using a tail-cuff plethysmography system (MK-2000ST, Muromachi Kikai), and the values were averaged. Echocardiography was performed in conscious animals using a Vevo2000 imaging system (FUJIFILM Visual Sonics). Transthoracic M-mode echocardiography was performed to measure the cardiac dimensions and function.[21] The midportion of the left ventricle was assessed in the parasternal short-axis view. Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and thickness of the anterior and posterior walls were measured. Fractional shortening of the left ventricle was calculated using the following equation: fractional shortening = ([LVEDD − left ventricular end-systolic diameter (LVESD)]/LVEDD) × 100 (%). Echocardiography and blood pressure data were analyzed by genotype-blinded investigators.

BASELINE METABOLIC MEASUREMENT. Metabolic features, which were assessed by oxygen consumption (V_{O_2}) (normalized to body weight) and locomotor activity, were evaluated using an O_2/CO_2 metabolic measurement system (MK-5000, Muromachi Kikai) as described previously.[17]

MEASUREMENT OF SERUM E2 CONCENTRATION. Serum E2 levels in ovariectomized mice with or without E2 supplementation and in non-ovariectomized mice were determined using an enzyme-linked immunosorbent assay kit (CAY-582251 or CAY-501890, Cayman Chemical) as previously described.[23,24] Serum was collected 2 weeks after ovariectomy and 1 week after E2 pellet implantation.

EVANS BLUE STAINING. Evans blue staining was performed as previously described.[12,25-28] Seven days after wire injury, the mice were anesthetized (1%-2% inhaled isoflurane) and intubated. Evans blue dye (FUJIFILM Wako Pure Chemical Corporation) was dissolved in normal saline to a concentration of 15 mg/mL. Subsequently, 100 μL of this solution was injected into the internal jugular vein. Ten minutes later, the mice were sacrificed, and the carotid arteries were harvested. The carotid arteries were dissected longitudinally, and images of the intimal surface of the vessels were acquired using a MZ10F stereomicroscope (Leica). Areas not stained with Evans blue were regarded as re-endothelialized, and the ratio of these areas to the total intimal surface area was calculated as the re-endothelialization ratio. Images were analyzed using National Institutes of Health ImageJ software.

HISTOLOGY. Carotid arteries were harvested after perfusion fixation with 10% formalin and embedded in paraffin. For histologic analyses, we selected a part of the common carotid artery 200 to 400 μm proximal to the carotid bifurcation, where the injury was induced; 4-μm cross sections were used for analyses.

Serial sections obtained 14 days after wire injury were subjected to elastin staining (Elastica van Gieson stain) and Masson's trichrome staining to investigate intimal hyperplasia, medial thickening, and fibrosis after wire injury. The area between the internal and external elastic laminae was recognized as the medial area, and the area inside the internal elastic lamina was defined as the intimal area. The intima/media ratio was calculated by measuring the intimal and medial areas. Medial fibrosis, recognized as a blue-green area on Masson's trichrome staining, was quantified as previously described.[29]

Sections obtained from mice 7 days after wire injury were subjected to immunohistochemical staining for CD31 and CD45. To assess re-endothelialization, samples were incubated overnight at 4 °C with anti-CD31 antibody (1:200, Abcam), and the signals were detected using an HRP/DAB detection IHC kit (ab64261, Abcam). The sections were counterstained with hematoxylin. Endothelial coverage was evaluated as the CD31+ luminal lining length divided by the total cross-sectional luminal lining length.[30,31]

To evaluate the degree of vascular inflammation,[32,33] 7 days after wire injury, the sections were pretreated with 5% horse serum and incubated overnight at 4 °C with anti-CD45 antibody (1:200, BD

Biosciences). The samples were incubated with ImmPRESS HRP goat antirat IgG (MP-7444-15, Vector Laboratories) for 30 minutes at room temperature, developed with ImmPACT DAB peroxidase (HRP) substrate (SK-4105, Vector Laboratories), and counterstained with hematoxylin. The percentage of areas with positive CD45 signals in the adventitia was measured to assess vascular inflammation. Images were acquired with an Olympus FSX100 inverted microscope and analyzed using National Institutes of Health ImageJ software. All staining procedures and analyses were performed by treatment-blinded investigators.

ANALYSIS OF GENE EXPRESSIONS. The expression of target genes was evaluated by reverse transcription polymerase chain reaction. Total messenger RNA was extracted using TRIzol reagent (Molecular Research Center Inc) and reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems).

The primer sequences were as follows:

- Wild-type *Esr1:* forward ATACGGAAAGACCGCCGAG, reverse TGCCAACCTTTG GCCAAG
- Mutant *Esr1:* forward ATACGGAAAGACGCCCGAG, reverse GCCCTCTTGTGATTAAGCACAC
- *Greb1:* forward GACCGTCTACTACCTCGTCCA, reverse GCCAGGAGCGTAGGAAGAT
- *C3:* forward CGGCATAGAGAAGAGGCAAG, reverse AAGGCAGCATAGGCAGAGC
- *GAPDH:* forward CATGGCCTTCCGTGTTCCTA, reverse CCTGCTTCACCACCTTCTTGAT

cDNA was amplified with THUNDERBIRD qPCR Mix (TOYOBO Inc), and the relative expression levels of target genes were measured using the Light Cycler480 (Roche) as previously described.[23] Each sample was run in duplicate, and the results were normalized to GAPDH.

STATISTICAL ANALYSIS. Data are presented as mean ± SEM. D'Agostino and Pearson normality tests were used to determine whether the data followed a normal distribution. When the values were normally distributed, analysis of variance with the Bonferroni post hoc correction (multiple pairwise comparison for uterine weights, expression of Greb1 and C1, and histologic staining analysis) was applied to compare 3 or more groups, whereas the *t*-test was used to compare 2 groups (expression of Esr1, NOS activity, assessment of PLA, fertility, Vo_2, locomotor activity analysis, and serum 17β-estradiol level). A P value <0.05 was considered statistically significant. GraphPad Prism 8 (GraphPad Software) was used for statistical analyses.

RESULTS

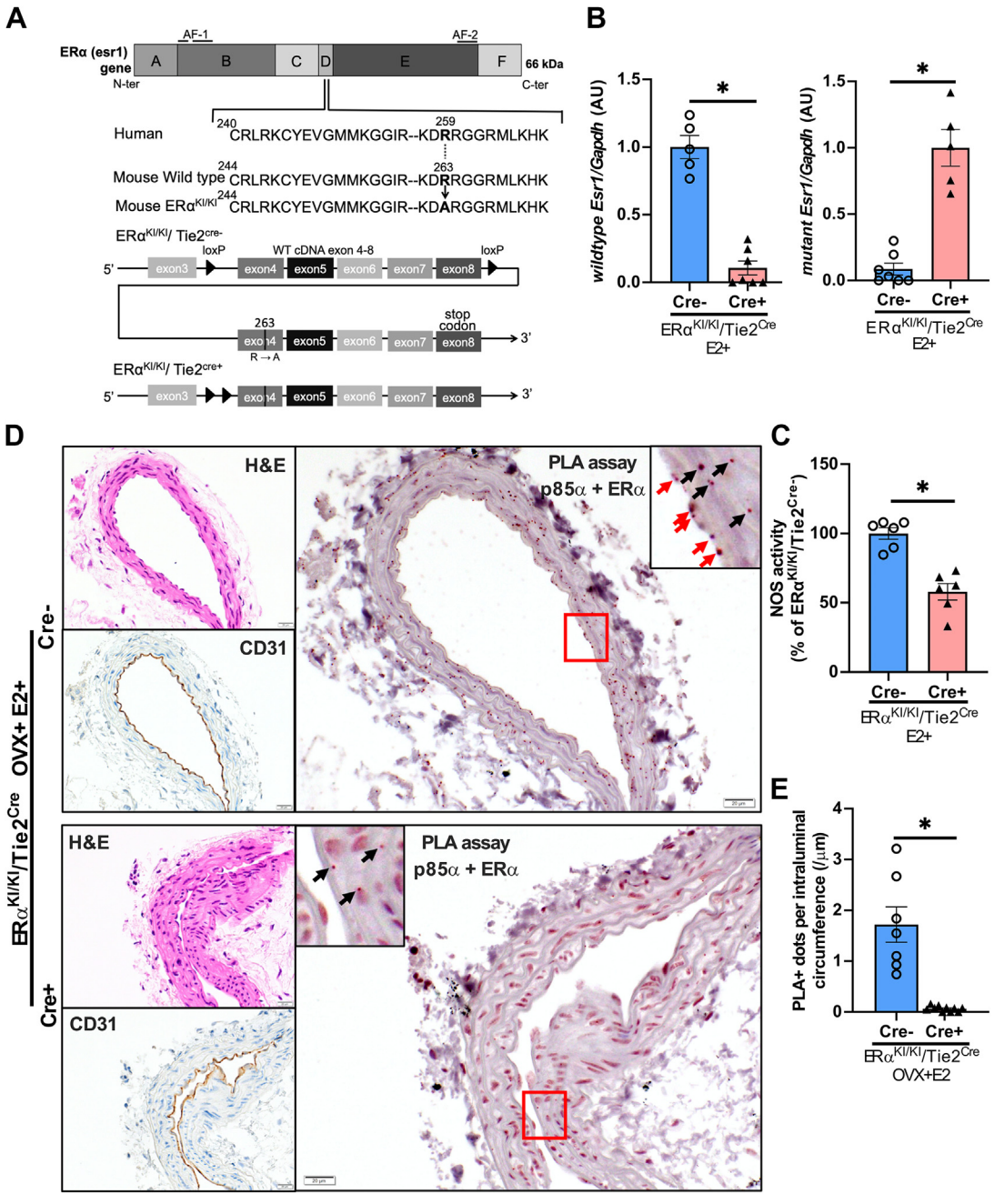
GENERATION OF ERα$^{KI/KI}$Tie2^{Cre+} MICE. We used a Cre-loxP gene-targeting system to generate a mouse model lacking tissue-specific non-nuclear ERα signaling. Site-directed mutagenesis was introduced into exon 4 of the mouse ERα gene to replace Arg263 with alanine (R263A) and floxed (**Figure 1A**). By crossing floxed animals (ERα$^{KI/KI}$) with Tie2 promotor-driven Cre overexpressors (Tie2-Cre), we obtained animals expressing endothelial cell-specific ERα R263A (ERα$^{KI/KI}$Tie2^{Cre+}). In the vascular endothelial cells isolated from ERα$^{KI/KI}$Tie2^{Cre+} mice, the mutated ERα was dominantly expressed, and E2-stimulated NOS activity was significantly reduced by 50% compared with that in ERα$^{KI/KI}$Tie2^{Cre-} mice, which is consistent with the inactivation of ERα non-nuclear signaling (**Figures 1B and 1C,** Supplemental Figure 1).

To confirm that this substitution disrupted the interaction between ERα and the p85α subunit of PI3K, we performed in situ PLA. Although PLA signals for the interaction of ERα and p85α were evident in the endothelium of the wild-type littermate carotid artery with E2 supplementation (ERα$^{KI/KI}$Tie2^{Cre-}), virtually no signals were detected in the endothelium of the carotid artery with E2 of mutant mice (ERα$^{KI/KI}$Tie2^{Cre+}) (**Figures 1D and 1E**), indicating disruption of the ERα-p85α interaction. PLA signals were minimal in the endothelium of ovariectomy mice of both genotypes (data not shown).

NO DIFFERENCE IN BASELINE PHYSIOLOGICAL OR METABOLIC PHENOTYPE BETWEEN ERα$^{KI/KI}$Tie2^{Cre-} AND ERα$^{KI/KI}$Tie2^{Cre+} MICE. ERα$^{KI/KI}$Tie2^{Cre+} mice were apparently indistinguishable from ERα$^{KI/KI}$Tie2^{Cre-} mice at 8 to 12 weeks of age. The body and uterine weights of the mutant mice were not different from those of the wild-type mice at the age of 12 weeks (**Table 1**). Importantly, mutant mice showed preserved uterine growth response to E2 and normal fertility (**Figures 2A to 2C**). ERE-containing genes, including *Greb1* and *C3*, responded to E2 similarly in the endothelial cells of both genotypes (**Figure 2D**). These results suggest that the genomic actions of ERα are preserved in mutant mice.

Baseline metabolic measurements, including Vo_2 and locomotor activity, in ERα$^{KI/KI}$Tie2^{Cre+} mice were not different from those in wild-type mice in both light (day) and dark (night) phases (**Figures 3A and 3B**), with serum estrogen levels comparable to those in wild-type mice (**Figure 3C**). The baseline cardiovascular phenotype of the mutants was unremarkable as assessed by blood pressure, echocardiography, and anatomy (**Table 1**). The serum estrogen

FIGURE 1 Mouse Model With Tie2-Cre-Induced Inactivation of ERα Non-Nuclear Signaling



(A) A schematic diagram of gene targeting strategy. A single alteration was introduced into the mouse estrogen receptor-α (ERα) exon 4 by replacing Arg263 with alanine. **(B)** Wild-type ERα and mutated ERα expressions in isolated estrogen receptor-$\alpha^{\text{knock-in/knock-in}}$ ($ER\alpha^{KI/KI}$)$Tie2^{Cre}$ endothelial cells with 17β-estradiol (E2) stimulation. **(C)** Nitric oxide synthase (NOS) activities in isolated $ER\alpha^{KI/KI}Tie2^{Cre}$ endothelial cells with E2 stimulation. **(D)** Hematoxylin-eosin (H&E) staining, proximity ligation assay (PLA) of p85α and ERα, and CD31 immunohistochemical staining in the carotid arteries treated with E2. Sections were counterstained with hematoxylin. Positive PLA signals are revealed by **red dots**; **red arrow**: positive PLA signals on endothelial cell area, **black arrow**: positive PLA signals on nonendothelial cell area. Scale bars = 20 μm. **(E)** Quantification results for PLA-positive cells. Data in **B**, **C**, and **E** were analyzed by the unpaired Student's *t*-test. $^*P < 0.05$. Data are presented as mean ± SEM.

TABLE 1 Baseline Characteristics and Echocardiographic Parameters in 8- to 12-Week-Old $ER\alpha^{KI/KI}Tie2^{Cre-}$ and $ER\alpha^{KI/KI}Tie2^{Cre+}$ Mice

	$ER\alpha^{KI/KI}Tie2^{Cre-}$	$ER\alpha^{KI/KI}Tie2^{Cre+}$
BW, g	18.9 ± 0.4	19.2 ± 0.4
SBP, mm Hg	103.5 ± 2.1	106.1 ± 3.3
UW, mg	102.0 ± 14.9	113.3 ± 18.7
Echocardiography		
LVEDD, mm	2.62 ± 0.03	2.67 ± 0.02
Wall thickness, mm	0.82 ± 0.02	0.82 ± 0.02
FS, %	55.2 ± 0.9	54.4 ± 0.8

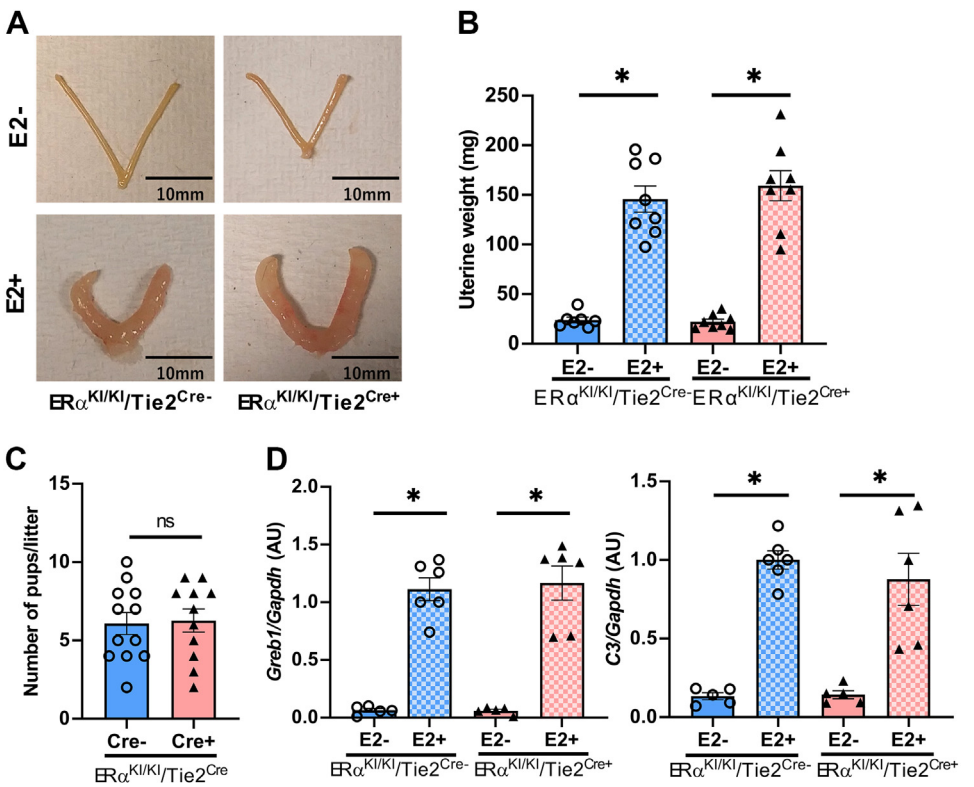
Values are mean ± SD. FS was calculated as ([LVEDD − left ventricular end-systolic diameter (LVESD)])/LVEDD) × 100 (%). Wall thickness was the average of anterior and posterior wall thickness. There was no significant difference between the genotypes in each parameter (*t*-test, $P < 0.05$, n = 5-12).

BW = body weight; $ER\alpha^{KI/KI}$ = estrogen receptor-$\alpha^{knock\text{-}in/knock\text{-}in}$; FS = fractional shortening; LVEDD = left ventricular end-diastolic diameter; SBP = systolic blood pressure; UW = uterine weight.

levels of ovariectomized mice with or without E2 supplementation were also comparable between genotypes (Supplemental Figures 2A and 2B).

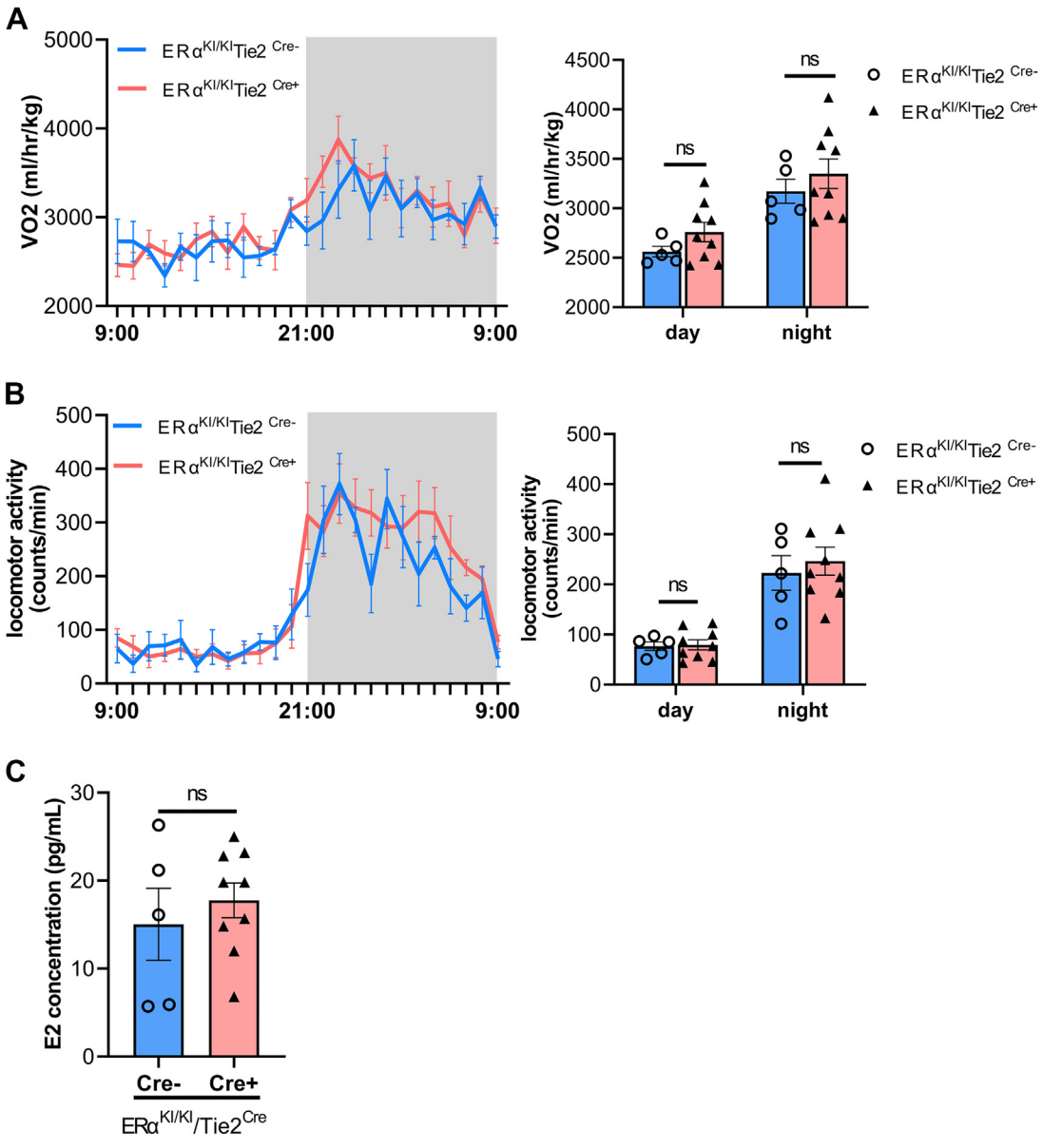
ENDOTHELIAL-SPECIFIC ERα NON-NUCLEAR SIGNALING CRUCIAL FOR THE PROTECTIVE EFFECTS OF E2 AGAINST VASCULAR INJURY. To elucidate the role of endothelial ERα non-nuclear signaling in vascular protection against mechanical injury, we used a 2-week wire injury protocol (Figure 4A) and assessed intimal hyperplasia, medial thickening, and fibrosis in the presence or absence of E2 in ovariectomized animals of both genotypes. Elastica van Gieson staining at 2 weeks after wire injury revealed that the beneficial effects of estrogen on intimal hyperplasia and medial thickening (assessed by the intima/media ratio and medial area) were markedly abrogated in mutant animals, whereas

FIGURE 2 ERα Genomic Signaling Is Preserved in $ER\alpha^{KI/KI}Tie2^{Cre+}$ Mice



(A) Representative uterine morphologies of $ER\alpha^{KI/KI}Tie2^{Cre}$ mice after ovariectomy (OVX) and OVX+E2 supplementation. **(B)** $ER\alpha^{KI/KI}Tie2^{Cre}$ mice uterine weights ($ER\alpha^{KI/KI}Tie2^{Cre-}$, n = 7-8; $ER\alpha^{KI/KI}Tie2^{Cre+}$, n = 8). **(C)** Fertility analysis of $ER\alpha^{KI/KI}Tie2^{Cre}$ female mice. Litter sizes are shown as the number of pups per litter. **(D)** Endothelial estrogen response element-containing gene expressions by E2 stimulation. Data in **B** and **D** were analyzed by 2-way analysis of variance for "genotype × estrogen status" interaction. Data in **C** were analyzed by the unpaired Student's *t*-test. *$P < 0.05$. Data are presented as mean ± SEM. Abbreviations as in Figure 1.

FIGURE 3 Baseline Metabolic Characteristics and Serum E2 Concentration of ERα$^{KI/KI}$Tie2^{Cre} Mice

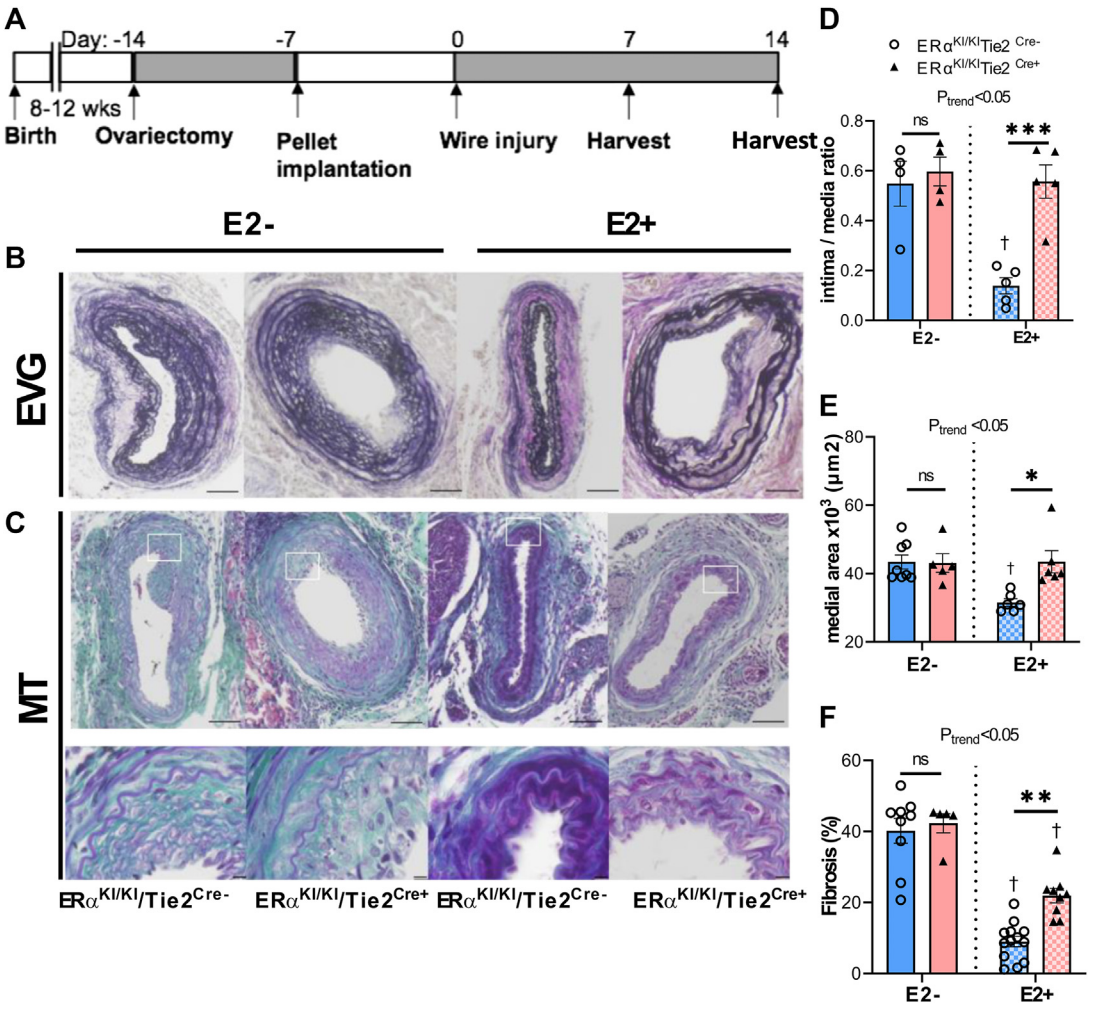


(A) Oxygen consumption (VO2) normalized to body weight per hour for 24 hours. The graph demonstrates average VO2 during the light (day) and dark (night) phases. **(B)** Locomotor activity normalized to body weight per hour for 24 hours. The graph depicts average locomotor activity during the light (day) and dark (night) phases. The unpaired Student's *t*-test was performed separately in each phase. **(C)** Serum E2 concentrations in nonovariectomy ERα$^{KI/KI}$Tie2^{Cre} mice (ERα$^{KI/KI}$Tie2^{Cre-}, n = 5; ERα$^{KI/KI}$Tie2^{Cre+}, n = 9). Data are presented as mean ± SEM. Abbreviations as in Figure 1.

no difference was observed between genotypes in the absence of estrogen (Figures 4B, 4D, and 4E). Masson's trichrome staining revealed more severe fibrosis in mutant animals than in wild-type animals in the presence of estrogen, although estrogen reduced fibrosis in both genotypes (Figures 4C and 4F). These results indicate the pivotal role of endothelial ERα non-nuclear signaling in the vascular protection conferred by estrogen.

ESTROGEN-STIMULATED ENDOTHELIAL REPAIR ATTENUATED WITH EXACERBATED INFLAMMATION 7 DAYS AFTER WIRE INJURY BY DEFICIENT ENDOTHELIAL ERα NON-NUCLEAR SIGNALING. To examine the process of vascular healing at earlier

FIGURE 4 Assessments of Intimal Hyperplasia, Medial Thickening, and Fibrosis After Wire Injury



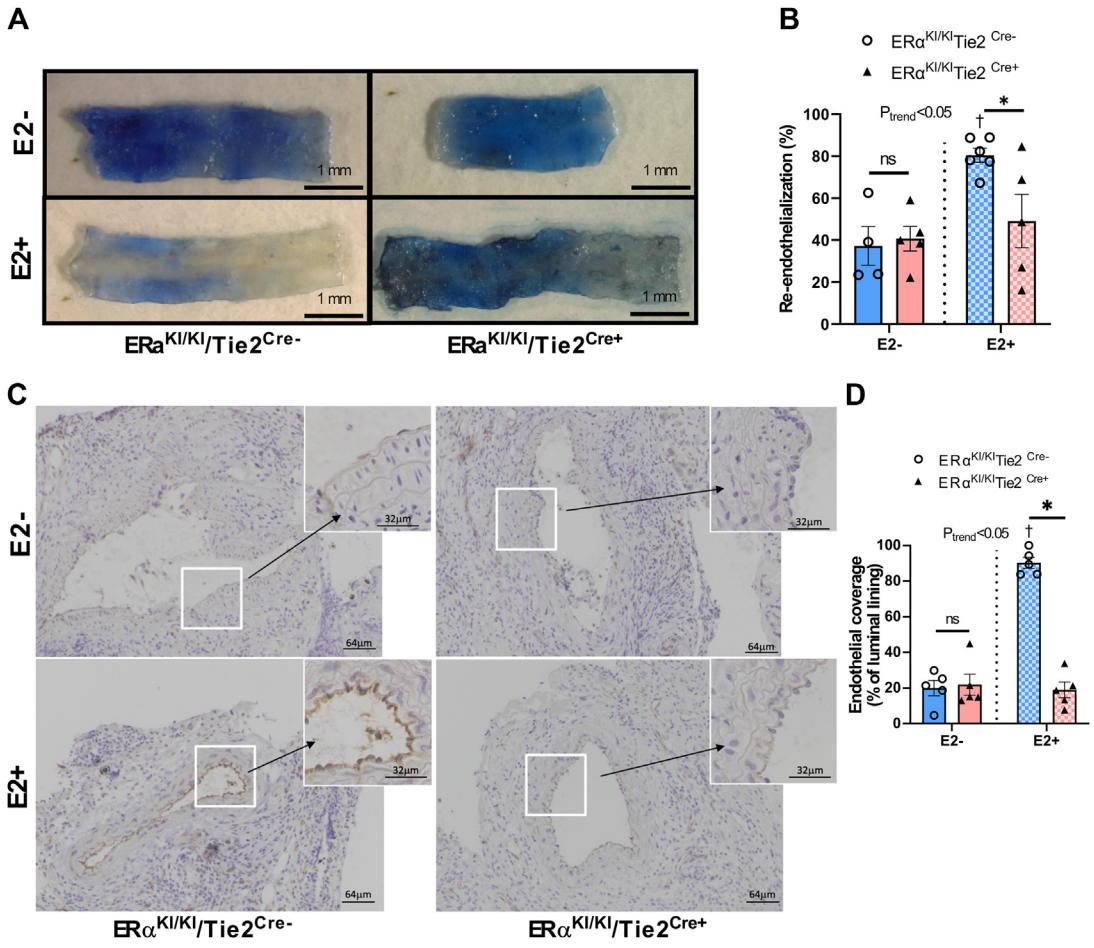
(A) The protocol of the wire injury model. **(B)** Representative Elastica van Gieson (EVG) staining and **(C)** Masson's trichrome (MT) staining images of carotid arteries 14 days after wire injury. Quantification results of **(D)** the intima-to-media ratio, **(E)** medial area ($\times 10^3$ μm^2) (n = 4-8 for each group), and **(F)** fibrosis (area stained with **blue green**) in the medial layer (n = 5-13 in each group). P_{trend} derived from comparison of the trends using 2-way analysis of variance for "genotype × estrogen status" interaction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in comparison between ERα$^{KI/KI}$Tie2^{Cre-} and ERα$^{KI/KI}$Tie2^{Cre+} mice by Bonferroni's post hoc correction for multiple comparisons. †$P < 0.05$ in comparison between E2+ and E2-; scale bar of **upper panel** = 64 μm, **lower panel** = 8 μm. Data are presented as mean ± SEM. E2+/- indicates with/without E2 pellet implantation. Abbreviations as in Figure 1.

stages, we assessed re-endothelialization and inflammation of the carotid arteries 7 days after wire injury. E2 supplementation promoted re-endothelialization after wire injury in wild-type mice, as assessed by Evans blue staining, whereas this effect was attenuated in mutant mice (Figures 5A and 5B). Consistent results were obtained for the assessment of endothelial coverage by CD31 staining (Figures 5C and 5D). This was associated with more CD45-positive inflammatory cells in the adventitia of the mutant vessels than in the adventitia of the wild-type vessels (Figures 6A and 6B). These results indicate that estrogen-mediated early control of endothelial repair and inflammation is critically regulated by endothelial ERα non-nuclear signaling, leading to the subsequent suppression of vascular wall thickening and fibrosis.

DISCUSSION

In addition to functioning as a nuclear receptor, ERα is localized on the plasma membrane and activates non-nuclear signaling, which mediates the beneficial

FIGURE 5 Assessments of Re-Endothelialization in the Carotid Arteries 7 Days After Wire Injury

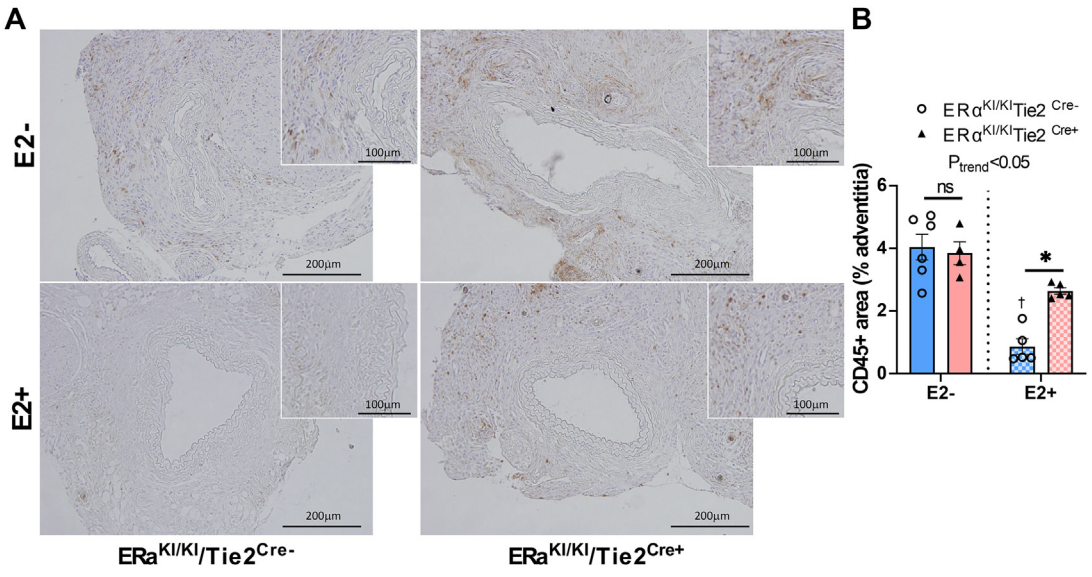


(A) Representative images of Evans blue staining of carotid arteries. **(B)** Quantification results for the re-endothelialization area (%) determined by Evans blue staining (deendothelialized areas are stained with blue) (n = 4-6 in each group). **(C)** Representative images of CD31 immunohistochemical staining. **(D)** Quantification results for the endothelial coverage (% of luminal lining) (n = 5 in each group). P_{trend} derived from comparison of the trends using 2-way analysis of variance for "genotype × estrogen status" interaction. *$P < 0.05$ in comparison between ERα$^{KI/KI}$Tie2^{Cre-} and ERα$^{KI/KI}$Tie2^{Cre+} mice by Bonferroni's post hoc correction for multiple comparisons. †$P < 0.05$ in comparison between E2+ and E2-; scale bar of upper panel = 1 mm, lower panel = 32 μm and 64 μm. Data are presented as mean ± SEM. E2+/- indicates with/without E2 pellet implantation. Abbreviations as in Figure 1.

cardiovascular and metabolic effects of estrogen.[9,34] We generated a conditional knock-in mouse model in which ERα non-nuclear signaling was inactivated by expressing ERα R263A in the endothelium using a Cre-loxP system under the control of the Tie2 promoter. Inactivation of ERα non-nuclear signaling in the endothelium did not alter the baseline cardiovascular or metabolic phenotype but did critically impair the vasoprotective effects of estrogen after mechanical injury.

A decade of research effort has been made to decipher the roles of ERα subfunctions (nuclear vs non-nuclear). In particular, analyses of genetically engineered animals have greatly contributed to our understanding of the in vivo roles of ERα subfunctions. Mice lacking the ERα activation function 2 domain (amino acids 543-549), which is essential for their nuclear function, show neither uterine growth response to estrogen nor the benefits of estrogen, including protection against atherosclerosis, diabetes, and bone demineralization.[35-37] Mice lacking the activation function 1 domain (amino acids 2-148) that is also necessary for ERα nuclear function are deficient in uterine growth and protection against

FIGURE 6 **Assessments of Inflammation in the Carotid Arteries 7 Days After Wire injury**



(A) Representative images of CD45 immunohistochemical staining. **(B)** Quantification results for the ratio of CD45 positive area/adventitial area (%) (n = 4-6 in each group); P_{trend} derived from comparison of the trends using 2-way analysis of variance for "genotype × estrogen status" interaction. *$P < 0.05$ in comparison between ERα$^{KI/KI}$Tie2^{Cre-} and ERα$^{KI/KI}$Tie2^{Cre+} mice by Bonferroni's post hoc correction for multiple comparisons. †$P < 0.05$ in comparison between E2+ and E2-; scale bars = 100 μm and 200 μm. E2+/- indicates with/without E2 pellet implantation. Data are presented as mean ± SEM. Abbreviations as in Figure 1.

atherosclerosis by estrogen administration.[36-38] With regard to non-nuclear function, several global transgenic mouse lines have been generated using a strategy to interfere with the membrane association of ERα[14-18]; however, none of these mouse models are tissue specific. In an early search for the ERα domain critical for its non-nuclear function, Chambliss et al[39] found that amino acids 250 to 274 of human ERα are required for PI3K-Akt and eNOS activation and further identified amino acids 250 to 260 as critical for ERα non-nuclear signaling.[19,40] Our tissue-specific model expressing ERα R263A (human R259A) demonstrates disruption of the ERα-p85α interaction and a significant reduction in NOS activity without affecting genomic function, suggesting the critical role of this arginine residue. Interestingly, Adlanmerini et al[18] recently reported the abrogation of rapid signaling in a global knock-in mouse model harboring an R264A alteration on ERα.[18] Therefore, both arginine residues (R263 and R264) may be required for ERα to physically associate with p85α and activate downstream signaling pathways.

The present results may not seem completely consistent with those of a prior study using Hela cells showing that human R260A but not R259A significantly reduced eNOS activity.[19] This could be attributed to the difference between Hela cells and mouse models; the former is more simplified and artificial than the latter. We observed partial (50%) reduction in the eNOS activity in R263A (human R259A) endothelial cells, and the remaining eNOS activity could be attributed to potential partial disruption of ERα-p85α interaction with R263A; the estrogen-stimulated G protein-coupled estrogen receptor-mediated mechanism[41] and/or estrogen-independent mechanisms could also contribute here.[42]

Our tissue-specific model revealed exacerbated intimal hyperplasia, medial thickening, and fibrosis 14 days after wire injury, which was preceded by early impairment of endothelial repair and exacerbated inflammation. Although the role of ERα non-nuclear signaling in vasoprotection has been consistently reported in prior global transgenic models, the current study provides the first evidence of the predominant role of endothelial ERα non-nuclear signaling. Importantly, fibrosis at later stages (14 days) was partially reduced in mutant mice treated with E2. This could be at least partially mediated by genomic actions given the prior work by Smirnova et al[43] demonstrating the role of the genomic actions of vascular smooth muscle cells in a model of femoral

artery injury. ERα non-nuclear signaling in vascular smooth muscle cells also contributes to controlling vascular smooth muscle cell proliferation.[44]

The mutant animals showed no evident abnormalities in the metabolic or cardiovascular phenotypes at baseline. In contrast, mice with global inactivation of ERα non-nuclear signaling show excessive weight gain and low physical activity.[17] Our results indicate that endothelial cell signaling may not play a major role in metabolic activity.

STUDY LIMITATIONS. First, Tie2-Cre mouse models have Cre recombinase activity in hematopoietic cells, although these remain the most widely used animal models to determine the endothelial-specific effects of gene modification in vivo.[45] Only a small number of circulating cells were reportedly Cre positive in adult animals[45]; however, we could not rule out the potential involvement of ERα alterations in hematopoietic cells.

Second, we did not examine the effects of estrogen receptor-β and G protein-coupled estrogen receptor, other estrogen receptors that are reported to be expressed in various tissues, including arteries. These estrogen receptors may also play a significant role in estrogen non-nuclear signaling.[34,46]

CONCLUSIONS

Our study highlights the tissue-specific role of ERα non-nuclear signaling and provides insights into the beneficial effects of estrogen on the cardiovascular system.

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ADDRESS FOR CORRESPONDENCE: Dr Eiki Takimoto, Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo, Tokyo 113-8655, Japan. E-mail: etakimoto-tky@umin.ac.jp.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In addition to its classical nuclear function as a transcription factor, ERα activates non-nuclear signaling, which is known as rapid or membrane-initiated steroid signaling. Although several transgenic mouse models have revealed the importance of this signaling pathway in the cardiovascular and metabolic benefits of estrogen, they are all global models. Hence, the tissue-specific role of non-nuclear signaling in vivo remains unclear. In this study, we established a novel mouse model for tissue-specific inactivation of ERα non-nuclear signaling and demonstrated that inactivation of ERα non-nuclear signaling in the endothelium critically impairs the beneficial effects of estrogen against vascular injury.

TRANSLATIONAL OUTLOOK: This conditional knock-in mouse model enabled us to decipher the tissue-specific role of ERα non-nuclear signaling in vivo. Clarification of ERα non-nuclear signaling using this model contributes to a better understanding of the physiological function of estrogen and may lead to its better clinical application.

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KEY WORDS endothelial cells, estrogen receptor-α, non-nuclear signaling, tissue-specific regulation

APPENDIX For supplemental tables and figures, please see the online version of this paper.