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ORIGINAL RESEARCH

# Tamoxifen Accelerates Endothelial Healing by Targeting ERα in Smooth Muscle Cells

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**RATIONALE:** Tamoxifen prevents the recurrence of breast cancer and is also beneficial against bone demineralization and arterial diseases. It acts as an ER (estrogen receptor) α antagonist in ER-positive breast cancers, whereas it mimics the protective action of 17β-estradiol in other tissues such as arteries. However, the mechanisms of these tissue-specific actions remain unclear.

**OBJECTIVE:** Here, we tested whether tamoxifen is able to accelerate endothelial healing and analyzed the underlying mechanisms.

**METHODS AND RESULTS:** Using 3 complementary mouse models of carotid artery injury, we demonstrated that both tamoxifen and estradiol accelerated endothelial healing, but only tamoxifen required the presence of the underlying medial smooth muscle cells. Chronic treatment with 17β-estradiol and tamoxifen elicited differential gene expression profiles in the carotid artery. The use of transgenic mouse models targeting either whole ERα in a cell-specific manner or ERα subfunctions (membrane/extranuclear versus genomic/transcriptional) demonstrated that 17β-estradiol-induced acceleration of endothelial healing is mediated by membrane ERα in endothelial cells, while the effect of tamoxifen is mediated by the nuclear actions of ERα in smooth muscle cells.

**CONCLUSIONS:** Whereas tamoxifen acts as an antiestrogen and ERα antagonist in breast cancer but also on the membrane ERα of endothelial cells, it accelerates endothelial healing through activation of nuclear ERα in smooth muscle cells, inviting to revisit the mechanisms of action of selective modulation of ERα.

**GRAPHIC ABSTRACT:** A graphic abstract is available for this article.

**Key Words:** endothelium ■ estrogen ■ receptors, smooth muscle ■ tamoxifen ■ vascular

**Editorial, see p 1488 | In This Issue, see p 1469 | Meet the First Author, see p 1470**

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Cardiovascular diseases have become an increasingly important cause of long-term morbidity and mortality among breast cancer survivors, mainly because of the improvement of survival after the disease and the increase in the duration of life expectancy. The closed link between breast cancer and cardiovascular disease was recently reviewed,[1] demonstrating shared risk factors and cardiovascular toxicity effects of cancer therapy. The treatments of patients with ERα+ (estrogen receptor α) breast cancer are mainly endocrine therapies, such as tamoxifen and aromatase inhibitors that are both very efficient in reducing the risk of cancer recurrence in women[2] but also in men.[3] However, aromatase inhibitors appear to increase the risk of cardiovascular disease through suppression of the cardiovascular protective effects of estrogens,[4–6] whereas tamoxifen seems to prevent acute myocardial infarction.[5,6] Experimental data in mice directly demonstrated several vasculoprotective effects of tamoxifen. Indeed, tamoxifen prevents atherosclerosis[7] and neointimal

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## Novelty and Significance

### What Is Known?

- The main estrogen, 17β-estradiol (E2), accelerates endothelial healing by targeting membrane ER (estrogen receptor)-α in endothelial cells.
- Tamoxifen is a selective ERα modulator that acts as an agonist or antagonist depending on the tissue.
- Tamoxifen is used as an adjuvant therapy against breast cancer, and its effect on the vascular system remains to be better understood.

### What New Information Does This Article Contribute?

- As E2, Tamoxifen is able to accelerate endothelial healing, but in contrast to E2:
- Tamoxifen accelerates endothelial healing indirectly by targeting ERα in smooth muscle cells.
- Tamoxifen accelerates endothelial healing by targeting nuclear ERα, and more specifically the activation function 1.

Cardiovascular diseases have become an increasingly important cause of mortality among breast cancer survivors. Millions of breast cancer patients received tamoxifen as adjuvant treatment for at least 5 years, but its effect on the vascular system remains to be better defined. Indeed, although successfully used for 40 years in medicine, the understanding of its mechanisms of action in vivo is only now beginning to emerge. Tamoxifen acts as a selective modulator of the estrogen receptor (SERM), antagonizing or mimicking 17β-estradiol (E2) action depending on the tissues. We show here that as E2, tamoxifen contributes to vascular protection through acceleration of endothelial healing. Surprisingly, although both E2 and Tamoxifen actions on endothelial healing are mediated by ERα, activation of this receptor occurs in different cell types. In contrast to E2, which activates membrane ERα in endothelial cells, tamoxifen accelerates endothelial healing by targeting nuclear ERα in smooth muscle cells. These results should serve as a paradigm to revisit the action of SERMs not only in arterial pathophysiology but also in angiogenesis and breast cancer. The spectrum of the vascular protective actions of tamoxifen should also be reconsidered in the assessment of benefit/risk ratio in the treatment of breast cancer.

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### Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **AF1** | activation function-1 |
| **AF2** | activation function-2 |
| **E2** | 17β-estradiol |
| **ECs** | endothelial cells |
| **ER** | estrogen receptor |
| **MISS** | membrane-initiated steroid signaling |
| **PCR** | polymerase chain reaction |
| **SMCs** | smooth muscle cells |

hyperplasia after artery injury.[8] These beneficial vascular effects are mediated by ERα.[8,9]

ERα belongs to the nuclear receptor family and can regulate gene transcription through AF (activation functions)-1 and -2. Whereas 17β-estradiol (E2) activates both ERα AFs, tamoxifen exerts antagonist actions on ERαAF2 and agonist actions on ERαAF1.[8–11] In addition to these classical, nuclear, genomic actions, a subpopulation of ERα is present at/or near the plasma membrane where it can elicit rapid, nongenomic, MISS (membrane-initiated steroid signaling) effects.[12] We previously demonstrated that E2 accelerates endothelial healing after electric injury through ERα, but not through ERβ,[13] and more precisely through membrane ERα[14] actions in endothelial cells (ECs).[15] The endothelium has numerous functions, acting as a crucial barrier between the blood and the vessel and regulating vascular tone.[16] In contrast, abnormalities of endothelial function and loss of the integrity of the endothelial monolayer constitute the key step in the onset of atherosclerosis.[17] Furthermore, massive endothelial death/erosion is directly responsible for thrombus formation and cardiovascular events in about one-third of the cases of acute coronary syndromes.[18,19] The therapeutic treatment of both chronic and acute arterial diseases that includes angioplasty followed by stent implantation destroys the endothelium. Finally, radiotherapy and chemotherapies used in cancer treatment also concur to alter endothelium integrity.[20,21] In all these interventions, delayed endothelial healing represents one of the important contributing factors of thrombosis and neointimal hyperplasia.[22]

In the present study, we then sought to delineate the arterial protection conferred by tamoxifen in 3 complementary mouse models of arterial injury, that is, in vivo and ex vivo model of endovascular injury with preserved smooth muscle layers and in vivo model of electric injury in which both endothelium and media are destroyed. Cre-Lox models were further used to distinguish between the endothelial and the smooth muscle cells (SMCs) specific actions of tamoxifen and E2. The molecular mechanisms underlying the effects of tamoxifen were finally characterized using transgenic mice in which the ERαAF1

and the membrane ERα functions have been specifically invalidated.

## METHODS

Materials and data that support the findings of this study are available from the corresponding authors upon reasonable request. For research materials listed in the Methods and Materials please see the Major Resources Table in the Data Supplement.

### Mice

All procedures involving experimental animals were performed in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM) and were approved by the local Animal Care and Use Committee. The investigation conforms to the directive 2010/63/EU of the European parliament. Wild-type female mice with a C57Bl/6J background were purchased from Charles River Laboratories (France), (n=4–12 per group). *Tie2CreERα$^{lox/lox}$*, *αSMACreER$^{T2}$ ERα$^{lox/lox}$*, *ERαAF1$^{0}$*, and *ERα-C451A* mouse line were generated as described previously.[8,14,23,24] *αSMACreER$^{T2-}$ ERα$^{lox/lox}$* (control mice) and *αSMACreER$^{T2+}$ ERα$^{lox/lox}$* mice were injected daily with tamoxifen (1 mg/mouse per day, Sigma, France) during 5 days from 4 weeks of age to induce activation of the Cre recombinase. Throughout all protocols, mice were housed at the animal facility of the faculty of medicine (Toulouse, France). Mice were housed in a temperature-controlled room with a 12:12-hour light-dark cycle and maintained with access to food and water ad libidum. Details about randomization, blinding, and group size are described in Data Supplement.

### Ovariectomy and Treatments

Bilateral ovariectomy was performed at 4 weeks of age after anesthesia with a mixture of ketamine (100 mg/kg, Merial, Lyon, France) and xylazine (10 mg/kg; Sigma-Aldrich; Isle d'Abeau Chesnes, France). After 2 weeks of recovery, mice were subcutaneously implanted with pellets releasing either tamoxifen (5 mg for 60 days, ie, 4 mg/kg per day; Innovative Research of America, Sarasota, FL), E2 (0.1 mg for 60 days, ie, 80 µg/kg per day; Innovative Research of America, Sarasota, FL) or vehicle. These doses of E2 and tamoxifen were previously used effectively to demonstrate their beneficial actions on the arterial wall.[8,9,13,15,24–26] We systematically checked that vehicle-treated ovariectomized mice had an atrophied uterus (<10 mg) and nondetectable circulating levels of E2, whereas those implanted with E2 or tamoxifen releasing pellet had a significant increase of uterine weight. Mice were submitted to carotid artery injury after 3 weeks of drug administration.

### Mouse Carotid Injury and Quantification of Reendothelialization

Endovascular injury of the carotid artery was performed on mice as described previously.[27] Briefly, the left common carotid artery was isolated and the external carotid was incised. A 0.35 mm diameter flexible wire with a 0.25 mm tip was advanced and pulled back 2 times into common carotid artery (5 mm length in total) and blood flow was restored. Five-day postinjury, carotid arteries were stained with Evans blue dye (sc-203736, Santa Cruz) and mounted with Kaiser's Glycerol gelatin (Merck). Electric injury of the carotid artery was performed on mice as described previously.[13,27] Briefly, the left common carotid artery was isolated, and electric injury was applied (3 mm total length) with a bipolar microregulator. Images were acquired using DMR 300 Leica microscope using LAS V3.8 and ImageJ software. The percentage of reendothelialization was calculated relative to the initial deendothelialized area (day 0).

### Ex Vivo Reendothelialization of Mouse Carotid Artery

Ex vivo endovascular injury of the carotid artery was performed as described previously.[28] Briefly, carotid arteries were isolated from mice and a 2 mm portion of artery was deendothelialized with a 0.35 mm diameter flexible wire with a 0.25 mm tip. The carotid arteries were then flushed with endothelial growth cell medium MV (CC-22020, Promocell) supplemented with a supplement Mix (CC-39225, Promocell) and cultured in this medium for up to 5 days at 37 °C, 5% CO2, renewing the medium every 2 days.

### En Face Immunostaining

Carotid arteries were fixed for 20 minutes in 4% paraformaldehyde, opened longitudinally and were then permeabilized for 15 minutes with PBS containing 0.3% TritonX100. After blocking with PBS containing 3% bovine serum albumin (BSA), 1% fetal bovine serum (FBS), and 0.1% tween-20 for 1 hour, carotid arteries were immunostained with primary antibody (CD31 antibody (sc-1506, Santa Cruz, catalog no. sc1506; 1:300), anti-VE-Cadherin antibody (555289, BD Pharmingen, catalog no. 555289; 1:300) for ECs and anti-α SMA (abcam-7817, catalog no. 7817; 1:300) for SMCs were performed overnight at 4 °C. Then, the arteries were incubated with secondary antibodies for 2 hours (anti-mouse [Alexa Fluor 488, catalog no. 715-545-150, 1:300] or anti-rat [Alexa Fluor 594, no. 714-585-150, 1:300] for 1 hour at room temperature). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; 5 µg/mL). Control experiments using the appropriate secondary antibody alone were performed to determine antibody specificity. Microscopy imaging was performed with a Zeiss LSM780 confocal microscope. Blinded imaging and quantification were performed with ImageJ software using identical exposures and matched imaging and processing conditions.

### RNA Sequencing and Analysis

RNA was isolated from carotid arteries through the phenol-chloroform method using TRIzol (Ambion) reagent. The resulting RNA quality was determined using a Fragment Analyzer Instrument and all samples had RNA integrity numbers above 7.4.

RNA sequencing libraries were prepared according to Illumina's protocols using the Illumina TruSeq Stranded mRNA kit (reference no. 20020595). Eleven cycles of polymerase chain reaction (PCR) were applied to amplify the libraries. Library quality was assessed using a Fragment Analyzer Instrument and libraries were quantified by qPCR using the kit KAPA KK4824, on the QuantStudio 6 of Thermo Fisher Scientific. Sequencing was performed in paired-end (2×150

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bp) on an Illumina NovaSeq sequencer at the Integragen company platform (https://www.integragen.com/fr/). All data and materials (Fastq files) have been made publicly available at the GEO portal[29] and can be accessed at GSE154268 accession number.

The reads were first trimmed for adapters and low-quality ends by the Trim Galore! Algorithm (-- t, -q, -e, -- length 20) available on the Galaxy web server (https://usegalaxy.org/). Subsequent informatics processes of the sequenced reads were locally done under python and R environments. Reads were aligned onto the mouse genome (mm 10) by bowtie2 (-- fr, --no-mixed, --no-discordant, -- sensitive).[30] Reads were further processed by the featureCounts tool (-B, -C, -O) to evaluate gene expression levels using the mus_musculus. GRCm38.98 Ensembl version of genes' annotations. All sequencing statistics are provided within the (Table I in the Data Supplement).

Primary evaluation of the 18 samples expression profiles by principal component analysis (PCA; plot within Figure I in the Data Supplement) evidenced that one OVX (ovariectomized) and one tamoxifen libraries were far from being grouped with their corresponding ones. The functional annotations of the genes exhibiting a 2-fold different expression level in these samples as compared to their relative clearly indicated that these 2 samples were contaminated by mRNAs of neural origin (Table II in the Data Supplement), presumably from the vagus nerve located near the carotid. Discarding these 2 samples allowed the rest of the samples to be correctly grouped by the PCA evaluation (Figure I in the Data Supplement). Differential gene expression analysis was next performed using the R-interfaced DESeq2 suite[31] following the removal of genes with no detected expression. We used the HTSfilter[32] to set the threshold values to filter the weakly expressed genes. Genes were declared as differentially regulated when their fold change (FC) was $>1.5$ or $<0.66$ with a BH (Benjamini-Hochberg) adjusted $P<0.05$. Functional annotations were made using the GSEA program (v4.0.3)[33] interrogating MSigDB hallmarks[34] and the EnrichR web platform (https://amp.pharm.mssm.edu/Enrichr/).[35]

## Gene Expression Analysis

Tissues (carotid artery, aorta, and enriched SMCs fraction) were homogenized using a Precellys tissue homogenizer (Bertin Technol, France), and total RNA from tissues was prepared using TRIzol (Invitrogen, Carlsbad, CA). Five hundred nanograms of RNA was reverse transcribed at 25 °C for 10 minutes and then at 37 °C for 2 hours in 20 μL final volume using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems). For gene expression, qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) on the StepOne instrument (Applied Biosystems). Primers were validated by testing the PCR efficiency using standard curves (95% < efficiency < 105%). Gene expression was quantified using the comparative Ct (threshold cycle) method; tumor protein, translationally controlled 1 (Tpt1) was used as reference. Details about qPCR conditions are described in the Data Supplement.

## Statistical Analysis

Data are shown as means ± SEM (SEM). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA; https://www.graphpad.com). A difference of $P<0.05$ was considered significant. Gaussian (normal) distribution was determined using the Shapiro-Wilks normality test ($n\geq5$). For normally distributed populations, Student *t* test (2 groups) or 1-way ANOVA followed by Tukey post-test (3 or more groups) was conducted. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. In case of significant interaction, Tukey post-test was subsequently performed. For data that failed normality testing (including $n\leq5$), Mann-Whitney test (2 groups), or Kruskal-Wallis with Dunn post-test (3 or more groups) was performed. No corrections for multiple testing were made across assays. Exact number (n), precise *P* values and statistic test used in each experiment are described in Data Supplement. When representative images are shown, the selected images were those that most accurately represented the average data obtained in all the samples.

# RESULTS

## Tamoxifen Accelerates Endothelial Healing and This Action Requires the Presence of Underlying SMCs

To evaluate the effect of tamoxifen treatment on endothelial healing, we first used a mouse model of endovascular injury of the carotid in vivo.[27] To this aim, ovariectomized wild-type female mice were chronically treated with either vehicle or tamoxifen (Figure 1A). E2 was used as a positive control of full ERα activation. As expected, both chronic treatment with E2 or tamoxifen led to uterine hypertrophy, confirming the agonist effect of tamoxifen alone in this organ (Figure 1B). Indeed, tamoxifen and E2 both led to an increase in uterine weight gain (100 mg ± 7.93 and 50 mg ± 2.46, respectively). Endothelial healing rates were analyzed and quantified after Evans Blue (Figure 1C) or CD31 (Figure 1D) en face staining 5 days after arterial endovascular injury in these mice. Quantification of reendothelialized area showed 30% of endothelial regeneration in vehicle-treated mice compared with about 70% in both E2- and tamoxifen-treated female mice (Figure 1C and 1D). Interestingly, tamoxifen also accelerated endothelial healing in intact or castrated male mice with the same efficacy than in female mice (Figure II in the Data Supplement). In ovariectomized female mice, combination of E2 and tamoxifen treatment induced a similar acceleration of endothelial healing than E2 or tamoxifen alone (Figure 1E). This contrasts with the partial antagonist action of tamoxifen on uterus. Indeed, as previously described,[36] animals treated with a combination of E2 plus tamoxifen had significantly smaller uterine weights than the ones treated with E2 alone (64.2 mg ± 4.1 versus 100 mg ± 7.93; Figure III in the Data Supplement). To evaluate the regenerated endothelium after complete reendothelialization, we performed VE-cadherin staining of the arteries 10 days after injury. The VE-cadherin staining pattern of reendothelialized area was similar in vehicle and treated mice, suggesting the efficiency of functional integrity of the endothelium after healing, regardless of the treatment (Figure 1F and 1G).

These findings are in contrast with our previous work reporting that tamoxifen failed to accelerate endothelial

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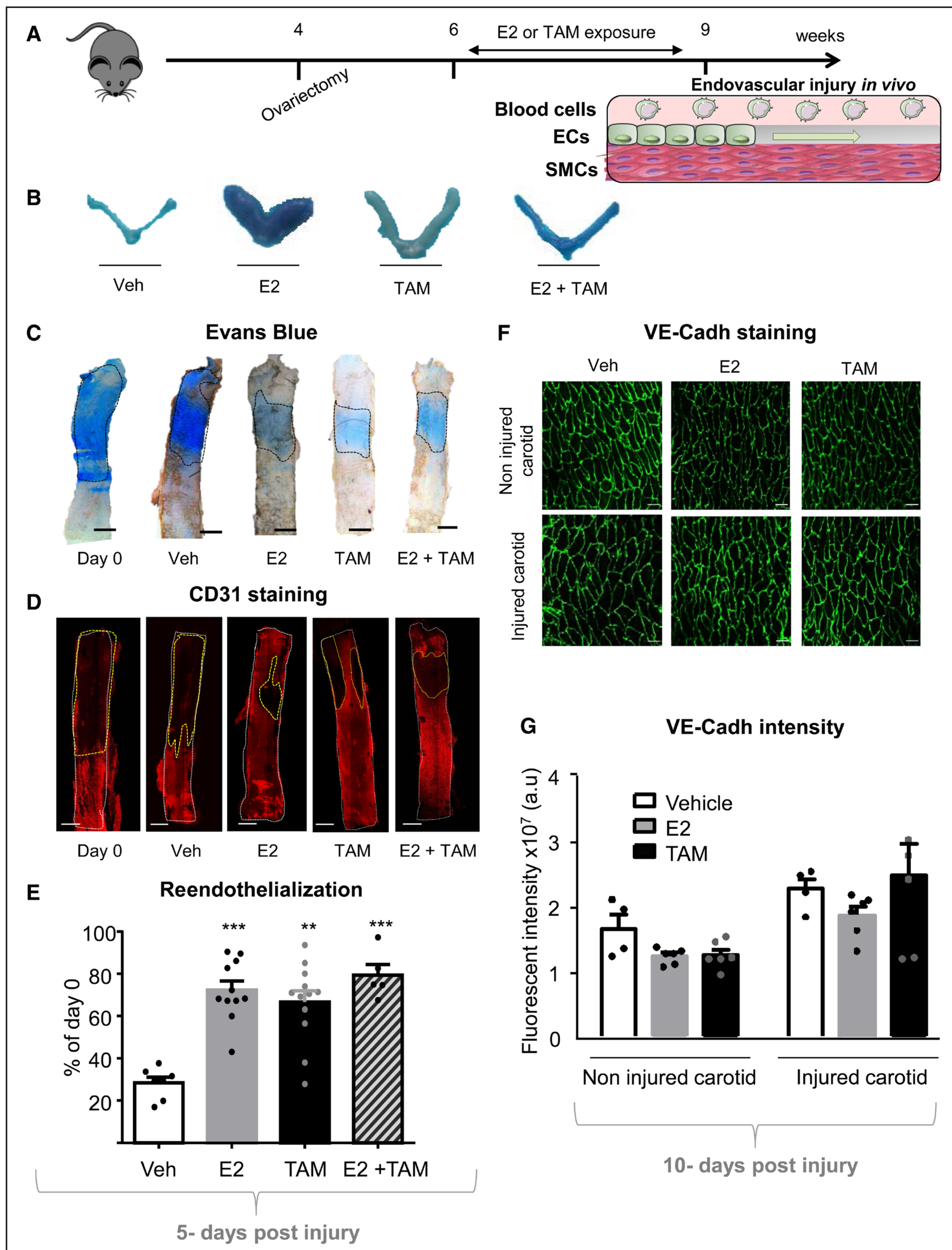


**Figure 1. As 17β-estradiol (E2), tamoxifen (TAM) induces acceleration of endothelial healing after endovascular injury in vivo.** **A**, Four-week-old female mice were ovariectomized and 2 wk after received either Veh (vehicle), E2 (80 µg/kg per day), TAM (4 mg/kg per day), or combination of both E2 and TAM. Three weeks later, mice were submitted to endovascular injury of the carotid artery. Carotid reendothelialization was analyzed 5 days after injury. **B**, Representative photography of uterus. **C**, Representative Evans blue or **D**, CD31+ staining of carotids (Scale Bar, 500 µm) and **E**, quantitative analysis of reendothelialization, expressed as a percentage of reendothelialized area compared with day 0. The outlined zones correspond to the deendothelialized area. Results are expressed as means ± SEM (n=5–12 per group). Carotids were also analyzed 10 d after the injury: **F**, Immunostaining of endothelial cells with anti-VE-cadherin antibody (scale bar, 20 µm) and **G**, quantitative analysis for VE-cadherin fluorescent intensity. Results are expressed as means ± SEM (n=4–6 per group). To test the effect of the different treatments, Kruskal-Wallis test was performed (**$P<0.01$, ***$P<0.001$).

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healing after perivascular electric injury.[9] We first confirmed that tamoxifen, unlike E2, does not accelerate endothelial healing after electric injury of the carotid artery (Figure 2A and 2B). We previously demonstrated that E2 effect on endothelial healing after electric injury is mediated by membrane ERα.[14] The fact that tamoxifen was not able to elicit this membrane ERα action raises 2 hypotheses: (1) either tamoxifen fails to bind membrane ERα or (2) tamoxifen is able to bind but does not activate membrane ERα. To address this question, we coadministrated E2 and tamoxifen and found no acceleration of endothelial healing after electric injury of the carotid from these mice compared with vehicle. Thus, tamoxifen has an antagonist activity on membrane ERα signaling pathway.

In addition, endovascular and perivascular injuries of the mouse carotid artery are similar in their efficiency to destroy the endothelium, but they differ since endovascular, but not perivascular injury preserves the medial SMCs[13,27,37,38] (Figures IV and V in the Data Supplement). Our result thus demonstrates that tamoxifen accelerates endothelial healing but, at variance to E2, its action requires SMCs integrity. Recently, 2 studies highlighted the importance of SMCs-ECs paracrine communication during this regenerative process.[28,39] Depending on the context, SMCs could either promote reendothelialization through CXCL7 (C-X-C motif ligand 7)-mediated recruitment of ECs[39] or on the contrary prevent this process via CXCL10 secretion.[28] However, no difference in either Cxcl7 or Cxcl10 mRNA levels was observed in tamoxifen or E2 treated mice (Figure VI in the Data Supplement).

## Tamoxifen, in Contrast to E2, Is Still Able to Accelerate Endothelial Healing After Endovascular Injury Ex Vivo

Endothelial healing also involves the coordinated action of circulating immune cells.[28,40] To investigate the possible contribution of circulating factors in tamoxifen action during reendothelialization process, we used a model of ex vivo endothelial healing (Figure 2C).[28] Deendothelialization was performed ex vivo, and endothelial healing was evaluated after 5 days of culture in an EC-specific medium. α-SMA immunostaining and contractile response to KCl (potassium chloride) revealed that SMCs are viable and functional at the end of the procedure (Figure VII in the Data Supplement). Experiments performed on carotid arteries from mice treated with either vehicle, E2, or tamoxifen indicated that in vivo preconditioning with tamoxifen, but not with E2, was sufficient to accelerate endothelial healing ex vivo (Figure 2D). This in vivo pretreatment of the mice with tamoxifen was necessary since ex vivo treatment of the carotid artery with 4-hydroxytamoxifen, the main active metabolite of tamoxifen, was unable to accelerate endothelial healing (Figure VIII in the Data Supplement). Altogether, these results concur to demonstrate that (1) the cellular actors involved in E2 and tamoxifen endothelial healing are different and (2) a period of in vivo tamoxifen exposure before the injury is necessary and sufficient to subsequently accelerate endothelial healing, suggesting long-term genomic actions as explored in the next paragraph.

## Chronic E2 and Tamoxifen Treatment Induce Different Gene Expression Profiles in the Carotid Artery

We, therefore, reasoned that performing RNA sequencing on noninjured carotid from ovariectomized mice treated chronically with E2 or tamoxifen may help to identify a subset of genes involved in this effect. Gene regulations by E2, tamoxifen, and vehicle in the carotid artery were compared and presented in the heatmap (Figure 3A). The number of significantly regulated genes at a fold change >1.5 over vehicle level was higher after E2 treatment than after tamoxifen exposure, especially for upregulated genes. Importantly, the patterns of genes regulated by E2 and tamoxifen (absolute fold change >1.5; $P<0.05$) were substantially different (Figure 3B). Indeed, only 68 genes were commonly regulated by these 2 ER ligands from totals of 717 and 227 genes regulated by E2 and tamoxifen, respectively.

Independent reverse transcribed-qPCR quantification confirmed the changes indicated by RNAseq analysis, except for some genes (Tgm3, Tent5b, and Aqp5) which were found to be regulated by tamoxifen but not by E2 (Figure IX in the Data Supplement). Of note for these genes, RNAseq analysis indicated a more pronounced effect of tamoxifen (log2fold changes: 3.2, 1.6, and 4.5) than of E2 (log2fold changes: 0.7, 0.7, and 1.8). Moreover, the use of $ER\alpha^{-/-}$ mice highlighted that most of the regulated genes were ERα dependent since transcriptional regulation in response to tamoxifen was totally abolished (Figure X in the Data Supplement).

Functional annotation of the gene subsets regulated by both E2 and tamoxifen by the GSEA method[33] revealed MSigDB[34] hallmarks for early and late estrogen response including classical estrogen-responsive genes such as Pgr. In addition, as revealed by previous large scale approaches performed in the aorta in response to E2, we found that Grem2 was upregulated by E2[41,42] and also by tamoxifen. Interestingly, hedgehog signaling was the only hallmark specifically enriched for tamoxifen-regulated genes in noninjured carotid arteries (Figure 3C). Furthermore, mRNA expression of Gli1/2 and Ptch1/2, the main readouts of hedgehog signaling activity was upregulated in the carotid arteries of tamoxifen-treated mice compared with vehicle-treated mice during the reendothelialization process, that is, 1, 3, or 5 days after endovascular injury (Figure XIA in the Data Supplement). Since hedgehog pathway is well described to be involved in angiogenesis,

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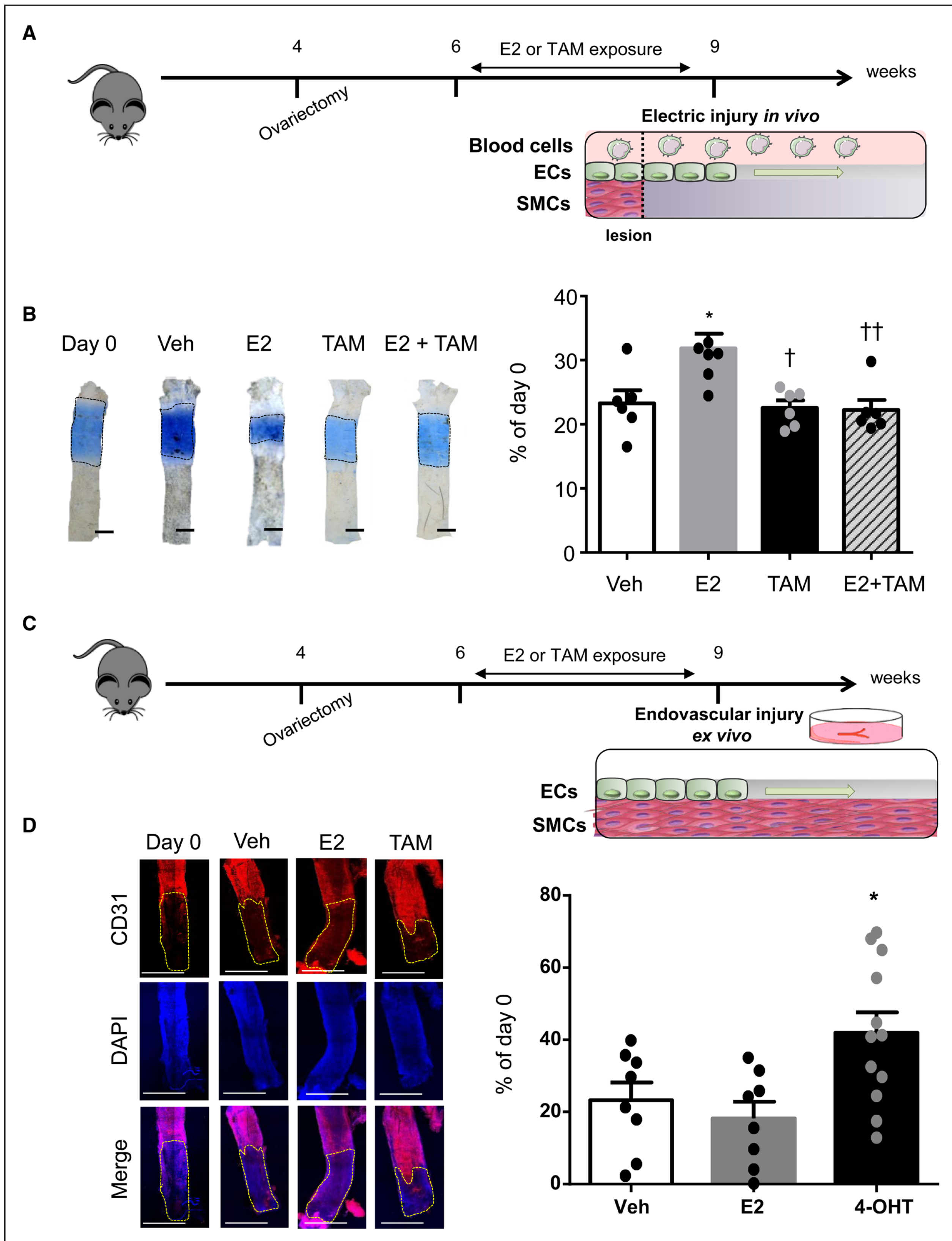


**Figure 2. The cellular actors involved in the acceleration of endothelial healing in response to tamoxifen (TAM) and 17β-estradiol (E2) are different.**

Four-week-old female mice were ovariectomized and 2 wk after were given either Veh (vehicle), E2 (80 μg/kg per day), TAM (4 mg/kg per day), or combination of both E2 and TAM. **A**, Three weeks later, mice were submitted to electric (perivascular) injury of the carotid artery. **B**, Representative image of Evans blue-stained en face carotid arteries from indicated treatment (**left**) and quantitative analysis of (*Continued*)

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in particular through Vegf upregulation,[43–45] we analyzed genes linked to this pathway during endothelial healing process. Interestingly, we found that Vegfa, Vegfd, and Flk1 mRNA levels were higher in the carotid from tamoxifen-treated mice compared with those from the control group (Figure XIB in the Data Supplement). We then took advantage of the published transcriptome single-cell analysis obtained on mouse aorta,[46] and comparison of these data with ours indicated that a majority of the genes that we identified as regulated by E2 in the carotid arteries were mainly expressed in monocytes and fibroblasts, while those responding to tamoxifen are representative of genes expressed in SMCs and fibroblasts (Figure XII in the Data Supplement).

Altogether, these results revealed that gene expression profiles in carotid arteries from mice treated by tamoxifen and E2 substantially differ both in terms of regulated pathways and of cellular targets.

## In Contrast to E2, Tamoxifen Effect on Endothelial Healing Requires SMCs ERα but Not Endothelial/Hematopoietic ERα

Thus, we used the Cre-lox strategy (*Tie2Cre ERα$^{lox/lox}$* and *αSMACreER$^{T2}$ ERα$^{lox/lox}$* mice) to directly evaluate the role of ERα in endothelial/hematopoietic cells versus SMCs, respectively. The efficiency of treatment with either E2 or tamoxifen was verified by the uterine response in each mouse model (Figure XIII in the Data Supplement). First, we showed that E2 regenerative effect on endothelium after endovascular injury was abolished in *Tie2Cre+ERα$^{lox/lox}$* mice, demonstrating the crucial role of ERα in hematopoietic/endothelial compartment in response to E2 in this model of endovascular injury, similar to that observed for perivascular electric injury.[15] In contrast, tamoxifen effect on endothelial healing was fully preserved in *Tie2Cre+ERα$^{lox/lox}$* mice (Figure 4A). Then, we used a mouse model expressing the inducible Cre-ER$^{T2}$ fusion gene under the control of the α-SMA promoter with *ERα$^{lox/lox}$* mice to evaluate whether SMCs ERα is involved in endothelial healing after mechanical injury in response to tamoxifen. In both genotypes, vehicle-treated mice displayed a similar low percentage of reendothelialization, close to 40% of reendothelialization. As expected, both E2 and tamoxifen treatments increased endothelial healing in control mice, raising 80% of reendothelialization. In *αSMACreER$^{T2}$+ ERα$^{lox/lox}$* mice, tamoxifen failed to have such an effect, by contrast to the fully preserved effect of E2 (50% of reendothelialization in tamoxifen and vehicle-treated mice as compared to 80% in E2-treated mice; Figure 4B). Altogether, these results identified SMCs as the main target cells for the accelerative effect of tamoxifen on endothelial healing. Importantly, this result highlights that, although the accelerative action on endothelial healing by E2 and tamoxifen are both mediated through ERα, the cellular targets involved in this process are completely different.

## Whereas E2 Action on Endothelial Healing Requires ERαMISS, This Effect Is Mediated by Nuclear ERα in Response to Tamoxifen

We previously showed that membrane ERα mediates acceleration of reendothelialization after electric injury in response to E2[14] independently of nuclear ERα.[47] Using a mice model harboring a mutation of ERα cysteine 451, recognized as the key palmitoylation site required for ERα plasma membrane location (*ERα-C451A*), we confirmed the crucial role of membrane ERα in response to E2 in this process using the model of endovascular injury. Indeed, we observed no effect of E2 on endothelial healing in *ERα-C451A* mice. By contrast, the effect of tamoxifen was fully preserved in this mouse model of loss of membrane localization of ERα (Figure 5A). Because ERαAF1 is necessary to mediate actions of tamoxifen in vitro[48,49] and in vivo,[50,51] we then sought to evaluate the role of this subfunction on endothelial healing. To this aim, ovariectomized *ERαAF1$^{+/+}$* and *ERαAF1$^{0}$* mice were treated with E2, tamoxifen or a vehicle, and were submitted to mechanical injury of the carotid. E2 treatment increased endothelial healing in both *ERαAF1$^{+/+}$* and *ERαAF1$^{0}$* control mice, extending the previous results obtained in the perivascular model.[24] Tamoxifen failed to have such an effect using *ERαAF1$^{0}$* mice, highlighting the crucial role of ERαAF1 in the vascular response to tamoxifen (Figure 5B). Accordingly, reverse transcribed-qPCR analysis performed in the aorta of these mice revealed the crucial role of ERαAF1 in the transcriptional action of tamoxifen since the induction of the most regulated ERα target genes in response to tamoxifen was abrogated in *ERαAF1$^{0}$* mice (Figure 6A). Importantly, analysis of this gene regulation profile in the SMC enriched-fraction (media) further reinforces the importance of SMCs in the transcriptional response of tamoxifen in the vessel (Figure 6B). Altogether, our data indicate that E2 and tamoxifen act via the same receptor: ERα to induce acceleration of endothelial healing but these 2 ligands target different subfunctions of the

**Figure 2 Continued.** reendothelialization expressed as a percentage of reendothelialized area compared with day 0 (**right**). Scale bar, 500 μm. **C**, Three weeks later, carotid arteries were isolated and deendothelialized ex vivo. Subsequently, they were placed in an endothelial cell-specific medium for 5 d. **D**, CD31 staining of en face carotid arteries. The zone outlined in yellow represents the area that has not been recovered with endothelial cells. Scale bar, 450 μm. Quantification of reendothelialization expressed as the percentage of CD31+ area compared with day 0. Results are expressed as means ± SEM (n=6–12 per group). To test the effect of the different treatments, Kruskal-Wallis test (abnormal distribution; **B**) or 1-way ANOVA (normal distribution; **D**) were performed (*$P<0.05$). † indicates differences between E2 and TAM or E2+TAM (†$P<0.5$, ††$P<0.01$).

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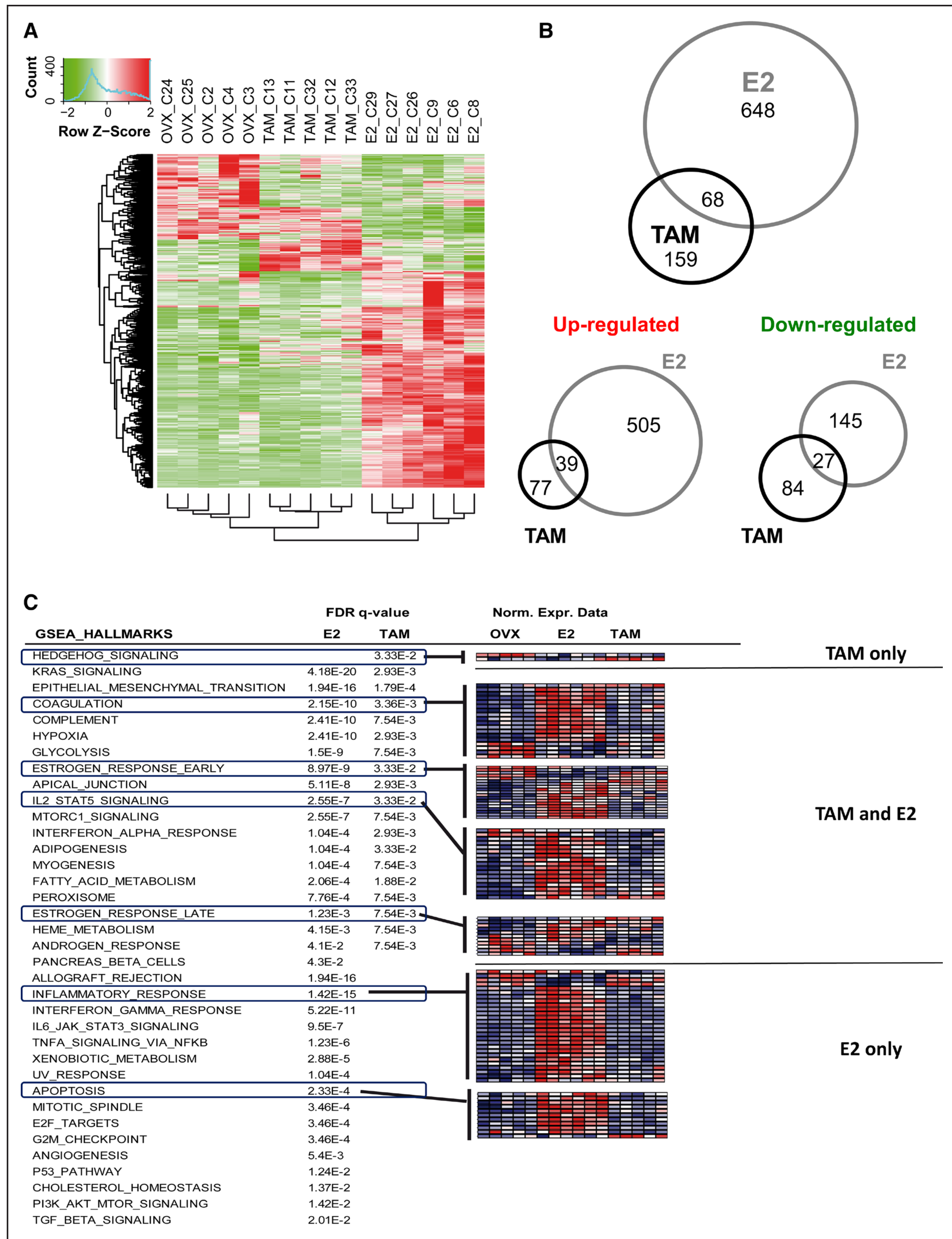


**Figure 3. 17β-estradiol (E2) and tamoxifen (TAM) regulate a different subset of genes in carotid arteries.**
**A**, RNAs were isolated from carotid arteries of ovariectomized mice (n=6 per group) treated by E2 (80 μg/kg per day) or TAM (4 mg/kg per day) for 3 wk and sequenced. The heatmap shown illustrates the relative expression values of all genes significantly regulated following E2 and TAM treatment (threshold of fold change of expression >1.5 over control with a BH (Benjamini-Hochberg) corrected $P$<0.05). HCL (Hierarchical Clustering) clustering regroups each sample with its corresponding treatment group. **B**, Venn diagram representing the overlap of genes regulated by E2 and TAM in carotid arteries. **C**, GSEA analysis representing the different hallmarks pathways regulated by E2 and TAM. Calculated false discovery rate (FDR) $Q$ value is given for each term, as well as the heatmap of the normalized expression values (Norm.Expr.data) of associated genes in each sample. Notably, the hallmarks specifically enriched in the E2 condition involve mainly genes regulated only by E2.

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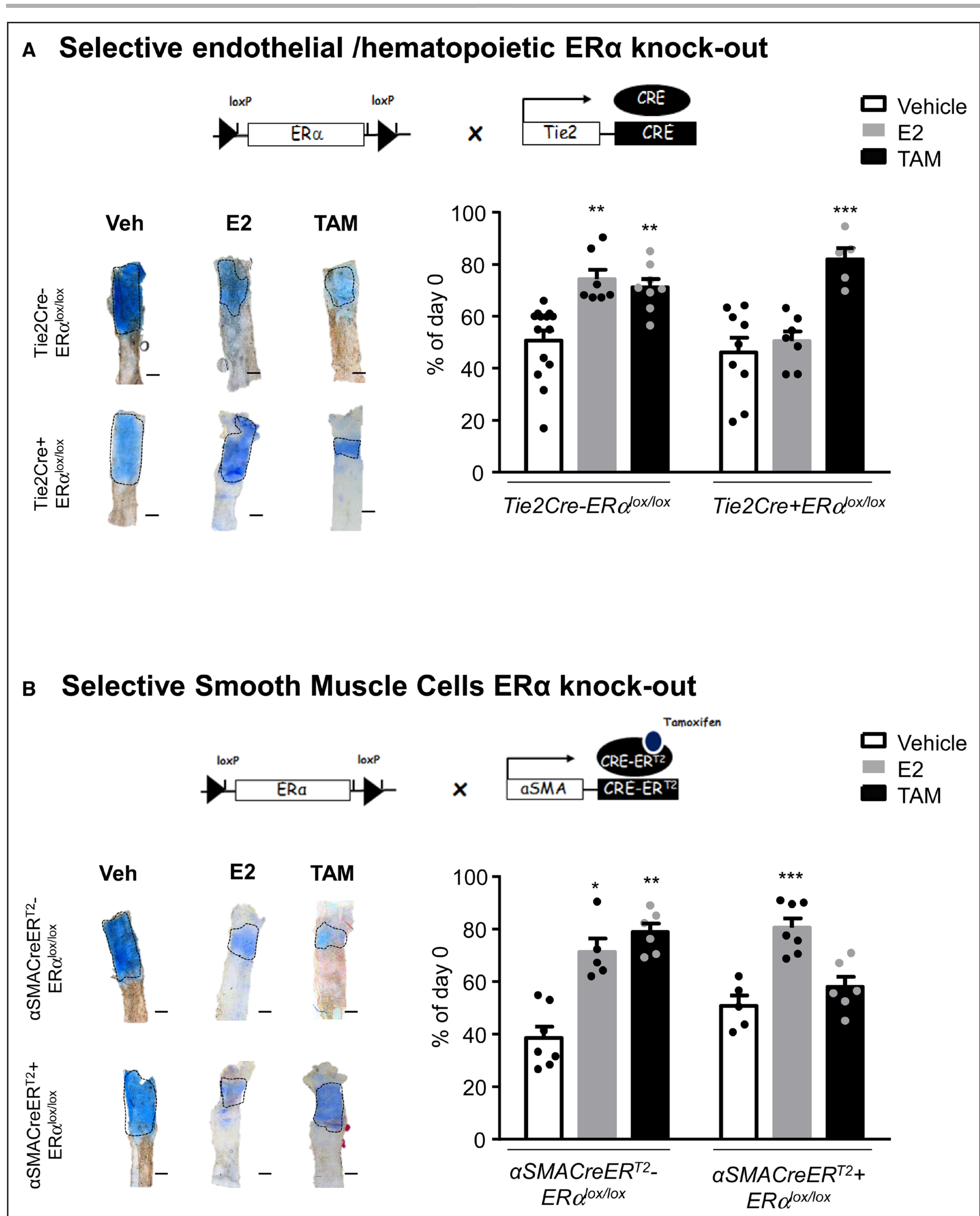


**Figure 4. ER (Estrogen receptor)-α in smooth muscle cells is necessary to induce endothelial healing in response to tamoxifen (TAM) but not to E2.**

Schematic representation of **A**, *Tie2CreERα$^{lox/lox}$* and **B**, *αSMACre$^{ERT2}$ ERα$^{lox/lox}$* mouse models. Four-weeks-old mice were ovariectomized and received E2 or TAM treatment for 3 wk. Mice were then submitted to endovascular injury of the carotid artery (day 0). Deendothelialized area was evaluated after Evans blue staining. Quantitative analysis of reendothelialization was expressed as a percentage of reendothelialized area at day 5 compared with day 0. The outlined zones correspond to the deendothelialized area. Scale bar, 500 µm. Results are expressed as means ± SEM (n=5–14 per group). To test the effect of E2 or TAM treatment in each genotype, Kruskal-Wallis test was performed (*$P$<0.05, **$P$<0.01, and ***$P$<0.001).

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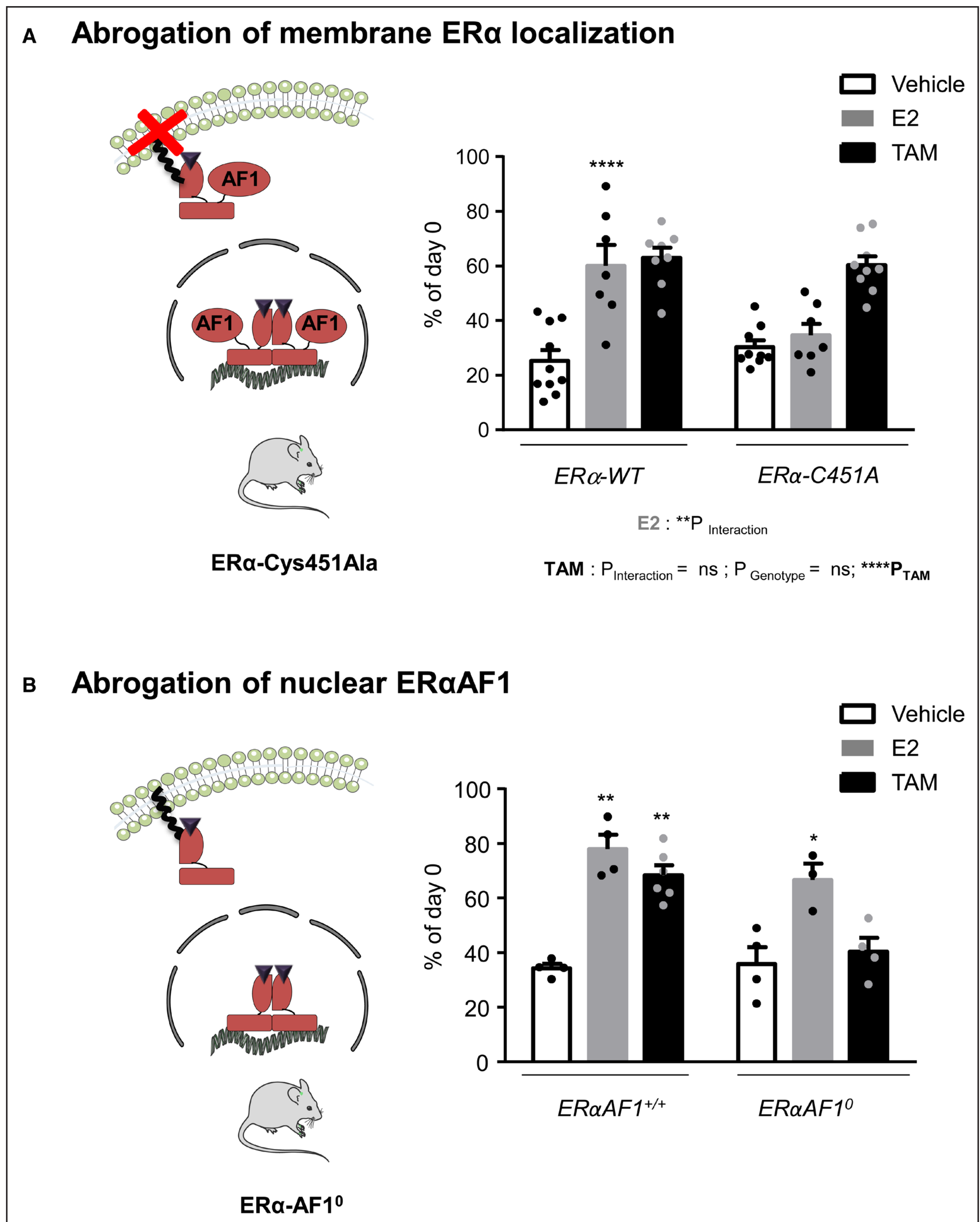


**Figure 5. Nuclear but not membrane ER (estrogen receptor)-α is necessary to induce endothelial healing in response to tamoxifen (TAM).**
Schematic representation of **A**, *ERαC451A* and **B**, *ERαAF1⁰* mouse models. Four-weeks-old mice were ovariectomized and received E2 or TAM treatment for 3 wk. Mice were submitted to endovascular injury of the carotid artery (day 0). Deendothelialized area was evaluated after Evans blue staining. Quantitative analysis of reendothelialization was expressed as a percentage of reendothelialized area at day 5 compared with day 0. Results are expressed as means ± SEM (n=4–8 per group). To test the effect of E2 or TAM treatment in each genotype, a 2-way ANOVA (**A**) or a Kruskal-Wallis test (**B**) was performed (*$P<0.05$, **$P<0.01$, and ****$P<0.0001$).

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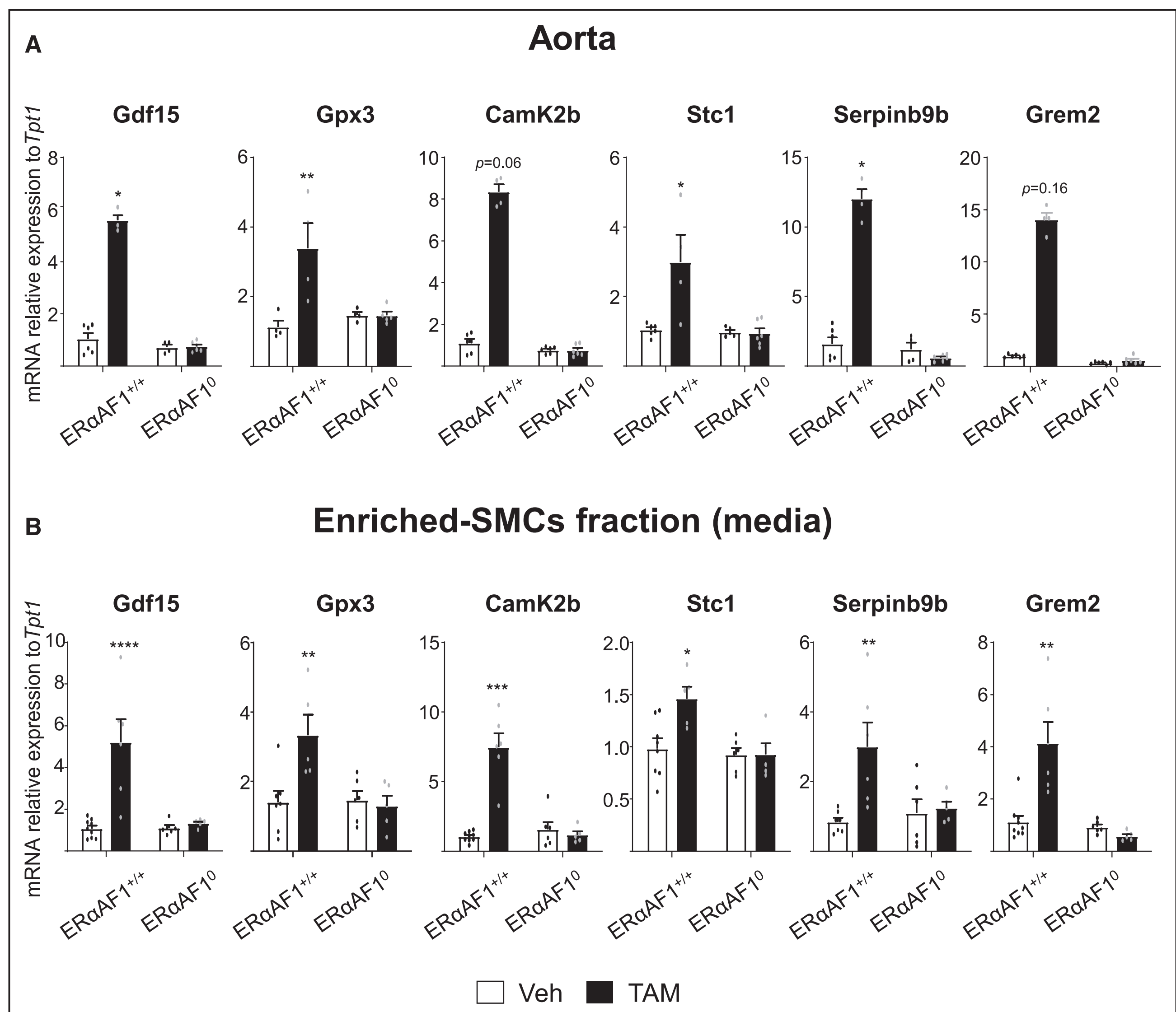


**Figure 6. ERαAF1 (estrogen receptor α activation function-1) is necessary for the transcriptional response to tamoxifen (TAM) in the aorta and in the enriched smooth muscle cells (SMCs) fraction (media).**
mRNA levels relative to Tpt1 mRNA level from **A**, aorta **B**, and SMC enriched-fraction from *ERαAF1*$^{+/+}$ and *ERαAF1*$^{0}$ mice treated by E2 (80 µg/kg per day) or TAM (4 mg/kg per day) during 3 wk were quantified by reverse transcribed quantitative polymerase chain reaction (RT-qPCR). Results are expressed as means ± SEM (n=4–9/group). To test the effect of TAM treatment in each genotype, Kruskal-Wallis test was performed (*$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$).

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receptor: membrane ERα for E2 and nuclear ERαAF1 in response to tamoxifen.

## DISCUSSION

Clinical studies report a protective action of tamoxifen on cardiovascular diseases but mechanisms underlying the beneficial agonist action of this drug remain poorly understood. The main aims of this study were to evaluate the action of tamoxifen on endothelial healing and to explore the cellular and molecular targets involved. Here, we demonstrated for the first time that tamoxifen accelerated endothelial healing after endovascular injury in both female and male mice. By contrast, in the model of perivascular electric injury, in which medial SMCs are destroyed, tamoxifen failed to accelerate endothelial healing, suggesting an important role of this cell type in tamoxifen action. Accordingly, we found that ERα in SMCs is necessary to mediate this vascular action. Medial SMCs could be critical in orchestrating vascular injury response through paracrine action. Indeed, SMCs continuously dialogue with ECs. However, whether and how SMCs may influence endothelial repair remains largely unknown. Recently, it was reported that SMCs promote reendothelialization through CXCL7-mediated recruitment of ECs from uninjured endothelium.[39] Lupieri et al[28] also demonstrated that SMCs through CXCL10 prevent endothelial healing. Thus, depending on the context, SMCs paracrine

factors can accelerate or slow endothelial healing. However, no change in the profile of expression of these cytokines could be detected under E2 or tamoxifen treatment in both intact and injured carotids.

The major findings of this study are that tamoxifen can mimic estrogen vasculoprotective effects through the activation of different/divergent cellular and molecular targets. Indeed, both E2 and tamoxifen can accelerate endothelial healing, but this beneficial action is dependent on the experimental model (ie, dependent on the cell type environment of the model used). Indeed, tamoxifen but not E2 required the presence of the underlying medial SMCs (comparison of perivascular versus endovascular injuries). In addition, the in vivo pretreatment with tamoxifen, but not with E2, is sufficient to induce acceleration of endothelial healing after ex vivo injury. Accordingly, Cre-Lox models reinforce this conclusion since we demonstrated that tamoxifen action is mediated by ERα in SMCs, whereas E2 action relies on endothelial/hematopoietic ERα. Hence, transcriptomic analysis reveals differential gene expression profiles in the carotid artery from mice treated chronically with E2 or tamoxifen. Functional annotation of the gene subsets regulated by either ER ligand indicated that inflammatory response was the major MSigDB[34] hallmark enriched for E2 regulated genes but not in response to tamoxifen. The hedgehog signaling pathway was specifically enriched for tamoxifen-regulated genes in intact carotid arteries. The expression of several genes involved in the hedgehog signaling pathway were also significantly higher in carotid from tamoxifen-treated mice compared with control mice during the endothelial healing process (1, 3, or 5-day postinjury). This is in accordance with previous works in adult blood vessels, showing in several experimental models that activation of this pathway promotes endothelial wound healing and angiogenesis through an indirect action on ECs exerted by SMCs and fibroblasts.[43–45,52,53] Interestingly, the comparison of genes selectively regulated by tamoxifen with the single-cell data on mouse aorta[46] revealed that these genes are mainly expressed in SMCs. By contrast, genes regulated by E2 are reminiscent of the expression pattern of immune cells.

Selective estrogen receptor modulators, such as tamoxifen, are defined as molecules mimicking the actions of E2 in some tissues and antagonizing the actions of E2 in others. The main mechanism underlying this tissue-specificity was so far attributed to differential cofactor expression/recruitment in each tissue. In the breast, where tamoxifen is an antagonist, tamoxifen induces the recruitment of corepressors instead of coactivators to ER target promoters.[11] In contrast, in the uterus where tamoxifen acts as an agonist, it recruits coactivators instead of corepressors to ER target genes.[11] The present results highlight, for the first time, to our knowledge, that the cell/tissue specificity of selective estrogen receptor modulator may also involve different cell types of ERα, shedding a new light on the mechanisms of action of selective modulation of ERα. This new dimension will, therefore, have to be taken into account when examining the mechanisms underlying the benefit/risk profile of tamoxifen for the treatment of breast cancer. Among other interrogations raising from our finding, since ERα regulates the immune system, it appears crucial to (re)consider the action of tamoxifen on immune surveillance in cancer.[54] Interestingly, estrogens and tamoxifen, but not pure antiestrogens, were also recently shown to partially protect against severe acute respiratory syndrome in a mouse-adapted severe acute respiratory syndrome-CoV model.[55]

We have developed for 2 decades several mouse models of carotid artery injury. Thanks to the combination of (1) genetically models targeting ERα or its subfunctions and (2) pharmacological tools, we were able to demonstrate that acceleration of endothelial healing in response to E2 is entirely dependent on membrane ERα.[14,47,56] In line with our previous work,[9] we first confirmed that tamoxifen failed to activate membrane ERα, raising the question of whether this is due to the failure of tamoxifen to bind to membrane ERα or the failure of membrane ERα to become activated by tamoxifen binding. Coadministration of tamoxifen and E2 validated the later hypothesis, as it failed to accelerate endothelial healing after electric injury, showing that tamoxifen is not only devoid of ERαMISS, but tamoxifen is also able to antagonize these E2 MISS effects. The role of this membrane/extranuclear action of tamoxifen in another pathophysiological context, in particular, breast cancer angiogenesis[57] should be studied in future works. The inhibition of the migration and invasion of breast cancer cells induced by extranuclear signaling in response to estrogen would be of peculiar interest.[58]

The use of mice harboring mutated specific functions of ERα revealed that tamoxifen acts through nuclear ERα activation, and more precisely through the AF1 to accelerate endothelial healing. It is highly likely that the nuclear ERαAF1 activation by tamoxifen occurred prominently in SMCs, but in the absence of cell-specific invalidation of this function does not allow to affirm that. In vitro experiments previously demonstrated the crucial role of AF1 to the agonist action of tamoxifen.[48,49] More recently, we showed that ERαAF1 is also necessary in vivo to relay the effects of tamoxifen on (1) the proliferation of uterine epithelial cells,[50] (2) the prevention of atherosclerosis,[9] and (3) the prevention of high-fat diet–induced metabolic disorders,[51] since all these beneficial actions are abrogated using mouse model selectively deficient for ERαAF1 (*ERαAF1*$^0$). Interestingly, ERαAF1 is dispensable to mediate these 2 latter actions in response to E2.[24,59]

To conclude, the present study demonstrates that tamoxifen and E2 both accelerate endothelial healing through an ERα-dependent effect. However, the cellular (ECs versus SMCs) and molecular (ERα subfunctions) target accounting for this protective action are quite dissimilar. These results should serve as a paradigm to revisit the action of estrogens and selective estrogen receptor modulators in breast cancer initiation and growth, angiogenesis,

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and immune surveillance but also in arterial pathophysiology. Although successfully used for 40 years in medicine, the understanding of the mechanisms of action of tamoxifen is still in their beginning. Its arterial protective actions should be seriously considered in the evaluation of benefit/risk ratio in the treatment of breast cancer.

## ARTICLE INFORMATION

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### Disclosures

None.

### Supplemental Materials

Online Supplemental Materials include:
Statistics Data set
Supplemental Methods
Online Figures I–XVIII
Online Tables I–II
Major Resources Table

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