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Tamoxifen Accelerates Endothelial Healing by Targeting ER α in Smooth Muscle Cell

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ABSTRACT

Rationale: Tamoxifen prevents the recurrence of breast cancer and is also beneficial against bone demineralization and arterial diseases. It acts as an Estrogen Receptor (ER) α antagonist in ER-positive breast cancers, whereas it mimics the protective action of 17 β -estradiol (E2) in other tissues such as arteries. However, the mechanisms of these tissue-specific actions remain unclear.

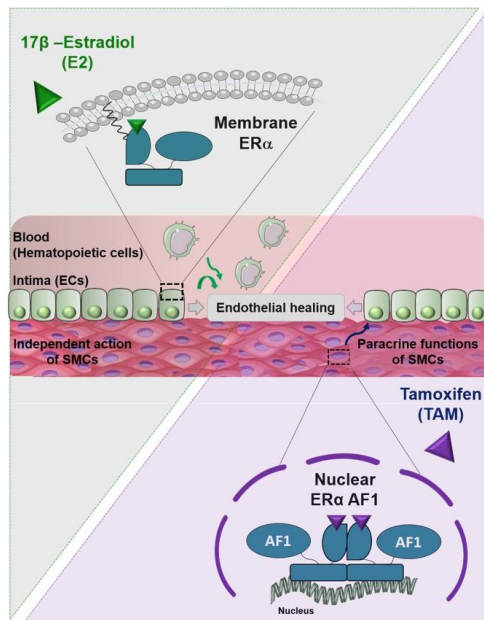
Objective: Here, we tested whether tamoxifen is able to accelerate endothelial healing and analyzed the underlying mechanisms.

Methods and Results: Using three 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 E2 and tamoxifen elicited differential gene expression profiles in the carotid artery. The use of transgenic mouse models targeting either whole ER α in a cell-specific manner or ER α sub-functions (membrane/extracellular *versus* genomic/transcriptional) demonstrated that E2-induced acceleration of endothelial healing is mediated by membrane ER α in endothelial cells, while the effect of tamoxifen is mediated by the nuclear actions of ER α in smooth muscle cells.

Conclusion: Whereas tamoxifen acts as an anti-estrogen and ER α antagonist in breast cancer, but also on the membrane ER α of endothelial cells, it accelerates endothelial healing through activation of nuclear ER α in smooth muscle cells, inviting to revisit the mechanisms of action of selective modulation of ER α .

Keywords:

Estrogen, tamoxifen, estrogen receptor (ER α), vascular endothelium, smooth muscle cell, endovascular repair.



Graphical abstract

Nonstandard Abbreviations and Acronyms:

AF1:	Activation Function-1
AF2:	Activation Function-2
AI:	Aromatase Inhibitors
CVD:	Cardiovascular Diseases
E2:	17 β -Estradiol
ECs:	Endothelial cells
ER:	Estrogen receptor
HH:	Hedgehog
MISS:	Membrane-Initiated Steroid Signaling
SERM:	Selective Estrogen Receptor Modulator
SMCs:	Smooth Muscle Cells
TAM:	Tamoxifen

INTRODUCTION

Cardiovascular diseases (CVD) 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 CVD, was recently reviewed ¹, demonstrating shared risk factors and cardiovascular toxicity effects of cancer therapy. The treatments of patients with estrogen receptor α (ER α +) breast cancer are mainly endocrine therapies such as tamoxifen (TAM) and/or aromatase inhibitors (AIs) that are both very efficient in reducing the risk of cancer recurrence in women ² but also in men ³. However, AIs appears to increase the risk of CVD through suppression of the cardiovascular protective effects of estrogens ⁴⁻⁶, whereas TAM seems to prevent acute myocardial infarction ^{5,6}. Experimental data in mice directly demonstrated several vasculoprotective effects of TAM. Indeed, TAM prevents atherosclerosis ⁷ and neointimal hyperplasia after artery injury ⁸. These beneficial vascular effects are mediated by ER α ^{8,9}.

ER α belongs to the nuclear receptor family and can regulate gene transcription through activation functions (AF)-1 and/or (AF)-2. Whereas E2 activates both ER α AFs, TAM exerts antagonist actions on ER α AF2 and agonist actions on ER α AF1 ⁸⁻¹¹. In addition to these classical, nuclear, genomic actions, a sub-population of ER α is present at/or near the plasma membrane where it can elicit rapid, non-genomic, membrane-initiated steroid signaling (MISS) effects ¹². We previously demonstrated that 17 β -estradiol (E2) accelerates endothelial healing after electric injury through ER α , but not through ER β ¹³, and more precisely through membrane ER α 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 TAM in three complementary mouse models of arterial injury, *i.e.* *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 TAM and E2. The molecular mechanisms underlying the effects of TAM were finally characterized using transgenic mice in which the ER α AF1 and the membrane ER α 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 Online Supplemental Materials.

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). *Tie2CreER $\alpha^{lox/lox}$* , *α SMACreER T2 ER $\alpha^{lox/lox}$* , *ER α AF1 0* and *ER α -C451A* mouse line were generated as described previously^{8,14,23,24}. *α SMACreER $^{T2-}$ ER $\alpha^{lox/lox}$* (control mice) and *α SMACreER $^{T2+}$ ER $\alpha^{lox/lox}$* 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 Online Supplementary Data.

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 two weeks of recovery, mice were subcutaneously implanted with pellets releasing either tamoxifen (TAM, 5 mg for 60 days, i.e. 4mg/kg/d; Innovative Research of America, Sarasota, FL), 17 β -Estradiol (E2, 0.1 mg for 60 days, i.e. 80 μ g/kg/d; Innovative Research of America, Sarasota, FL) or vehicle (Veh). These doses of E2 and TAM 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 non-detectable circulating levels of E2, whereas those implanted with E2 or TAM 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 re-endothelialization.

Endovascular injury of the carotid artery was performed on mice as described previously²⁷. 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 two times into common carotid artery (5 mm length in total) and blood flow was restored. Five-days post-injury, 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 re-endothelialization was calculated relative to the initial deendothelialized area (day 0).

Ex vivo re-endothelialization of mouse carotid artery.

Ex vivo endovascular injury of the carotid artery was performed as described previously²⁸. Briefly, carotid arteries were isolated from mice and a 2mm-portion of artery was de-endothelialized with a 0.35mm diameter flexible wire with a 0.25mm tip. The carotid arteries were then flushed with endothelial growth cell medium MV (CC-22020, Promocell) medium 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 min in 4% paraformaldehyde, opened longitudinally and were then permeabilized for 15 min with PBS containing 0.3% TritonX100. After blocking with PBS containing 3% BSA, 1% FBS and 0.1% tween-20 for 1hour, carotids arteries were immunostained with primary antibody (CD31 antibody (sc-1506, Santa Cruz, catalog # sc1506; 1:300), anti-VE-Cadherin antibody (555289, BD Pharmingen, catalog #555289; 1:300) for ECs and anti- α SMA (abcam-7817, catalog #7817; 1:300) for SMCs were performed overnight at 4°C. Then, the arteries were incubated with secondary antibodies for 2h (anti-mouse (Alexa Fluor 488, catalog#715-545-150, 1:300) or anti-rat (Alexa Fluor 594, #714-585-150, 1:300) for 1 hour at room temperature. Nuclei were stained with DAPI (5 μ g/mL). Control experiments using the appropriate secondary antibody alone was 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 (ref #20020595). Eleven cycles of 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 (2x150 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 portal²⁹ and can be accessed at GSE154268 accession number.

The reads were first trimmed for adapters and low-quality ends by the TrimGalore! 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 (mm10) by bowtie2 (-- fr, --no-mixed, --no-discordant, -- sensitive)³⁰. Reads were further processed by the featureCounts tool (-B, -C, -O) (Lio et al., 2013) to evaluate gene expression levels using the mus_musculus. GRCm38.98 Ensembl version of genes' annotations. All sequencing statistics are provided within the **(Online Table I)**.

Primary evaluation of the 18 samples expression profiles by PCA (plot within **Online Figure 1**) evidenced that one OVX and one TAM 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 (**Online Table II**), 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 (**Online Figure I**). Differential gene expression analysis was next performed using the R-interfaced

DESeq2 suite³¹ following the removal of genes with no detected expression. We used the HTSfilter³² to set the threshold values to filter the weakly expressed genes. Genes were declared as differentially regulated when their FC was > 1.5 or < 0.66 with a BH adjusted *P*-value < 0.05. Functional annotations were made using the GSEA program (v4.0.3)³³ interrogating MSigDB hallmarks³⁴ 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., Cedex, France) and total RNA from tissues was prepared using TRIzol (Invitrogen, Carlsbad, CA). 500 ng of RNA was reverse transcribed (RT) at 25°C for 10 min and then at 37°C for 2 h 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 validated by testing the PCR efficiency using standard curves (95% < efficiency < 105%). Gene expression was quantified using the comparative C_t (threshold cycle) method; tumor protein, translationally-controlled 1 (*Tpt1*) was used as reference. Details about Q-PCR conditions are described in Online Supplementary Data.

Statistical analysis.

Data are shown as means ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA; www.graphpad.com). A difference of *P*<0.05 was considered significant. Gaussian (normal) distribution was determined using Shapiro-Wilks normality test (n≥5). For normally distributed populations, student's t-test (2 groups) or 1-way ANOVA followed by Tukey's 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's post-test was subsequently performed. For data that failed normality testing (including n≤5), Mann-Whitney test (2 groups) or Kruskal-Wallis with Dunn's 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 Online Supplemental Data. 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 smooth muscle cells.

To evaluate the effect of TAM 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 TAM (Figure 1A). E2 was used as a positive control of full ERα activation. As expected, both chronic treatment with E2 or TAM led to uterine hypertrophy, confirming the agonist effect of TAM alone in this organ (Figure 1B). Indeed, TAM 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 re-endothelialized area showed 30 % of endothelial regeneration in vehicle-treated mice compared to about 70 % in both E2 and TAM treated female mice (Figures 1C and 1D). Interestingly, tamoxifen also accelerated endothelial healing in intact or castrated male mice with the same efficacy than in female mice (Online Figure II). In ovariectomized female mice, combination of E2 and TAM treatment induced a similar acceleration of endothelial healing than E2 or TAM alone (Figure 1E). This contrasts with the partial antagonist action of TAM on uterus.

Indeed, as previously described³⁶, animals treated with a combination of E2 plus TAM had significantly smaller uterine weights than the ones treated with E2 alone (64.2 mg ± 4.1 *versus* 100 mg ± 7.93) (Online Figure III). To evaluate the regenerated endothelium after complete re-endothelialization, we performed VE-cadherin staining of the arteries 10 days after injury. The VE-cadherin staining pattern of re-endothelialized area was similar in vehicle and treated mice, suggesting the efficiency of functional integrity of the endothelium after healing, regardless of the treatment (Figures 1F and 1G).

These findings are in contrast with our previous work reporting that TAM failed to accelerate endothelial healing after perivascular electric injury⁹. We first confirmed that TAM, unlike E2, does not accelerate endothelial healing after electric injury of the carotid artery (Figures 2A and 2B). We previously demonstrated that E2 effect on endothelial healing after electric injury is mediated by membrane ER α ¹⁴. The fact that TAM was not able to elicit this membrane ER α action raises two hypotheses: 1) either TAM fails to bind membrane ER α , or 2) TAM is able to bind but does not activate membrane ER α . To address this question, we co-administrated E2 and TAM and found no acceleration of endothelial healing after electric injury of the carotid from these mice compared to vehicle. Thus, TAM has an antagonist activity on membrane ER α 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 smooth muscle cells^{13,27,37,38} (Online Figures IV and V). Our result thus demonstrates, that TAM accelerates endothelial healing but, at variance to E2, its action requires smooth muscle cells integrity. Recently, two studies highlighted the importance of SMCs-ECs paracrine communication during this regenerative process^{28,39}. Depending on the context, SMCs could either promote re-endothelialization through CXCL7-mediated recruitment of ECs³⁹ or on the contrary prevent this process *via* CXCL10 secretion²⁸. However, no difference in either *Cxcl7* or *Cxcl10* mRNA levels were observed in TAM or E2 treated mice (Online Figure VI).

TAM, 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 TAM action during re-endothelialization process, we used a model of *ex vivo* endothelial healing²⁸. De-endothelialization 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 revealed that SMCs are viable and functional at the end of the procedure (Online Figure VII). Experiments performed on carotid arteries from mice treated with either vehicle, E2 or TAM indicated that *in vivo* “preconditioning” with TAM, but not with E2, was sufficient to accelerate endothelial healing *ex vivo* (Figure 2D). This *in vivo* pre-treatment of the mice with TAM was necessary since *ex vivo* treatment of the carotid artery with 4-hydroxytamoxifen (4-OHT), the main active metabolite of TAM, was unable to accelerate endothelial healing. Altogether, these results concur to demonstrate that i) the cellular actors involved in E2 and TAM endothelial healing are quite different and ii) a period of *in vivo* TAM exposure before the injury is necessary and sufficient to subsequently accelerate endothelial healing (Online Figure VIII), suggesting long-term genomic actions as explored in the next paragraph.

Chronic E2 and TAM treatment induces different gene expression profiles in the carotid artery.

We therefore reasoned that performing RNA sequencing on non-injured carotid from ovariectomized mice treated chronically with E2 or TAM may help to identify a subset of genes involved in this effect. Gene regulations by E2, TAM 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 TAM exposure, especially for up-regulated

genes. Importantly, the patterns of genes regulated by E2 and TAM (absolute fold change >1.5; P value <0.05) were substantially different (Figure 3B). Indeed, only 68 genes were commonly regulated by these two ER ligands from totals of 717 and 227 genes regulated by E2 and TAM, respectively.

Independent RT-qPCR quantification confirmed the changes indicated by RNAseq analysis, except for some genes (*Tgm3*, *Tent5b*, *Aqp5*) which were found to be regulated by TAM but not by E2 (Online Figure IX). Of note for these genes, RNAseq analysis indicated a more pronounced effect of TAM (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 ER α dependent since transcriptional regulation in response to TAM was totally abolished (Online Figure X).

Functional annotation of the gene subsets regulated by both E2 and TAM by the GSEA method³³ revealed MSigDB³⁴ 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 up-regulated by E2^{41,42} and also by TAM. Interestingly, Hedgehog (Hh) signalling was the only hallmark specifically enriched for TAM-regulated genes in non-injured carotid arteries (Figure 3C). Furthermore, expression of *Gli1/2* and *Ptch1/2*, the main readouts of Hh signalling activity was up-regulated in the carotid arteries of TAM-treated mice compared to vehicle-treated mice during the re-endothelialization process *i.e* 1, 3 or 5 days after endovascular injury (Online Figure XI A). Since Hh pathway is well described to be involved in angiogenesis, in particular through *Vegf* upregulation⁴³⁻⁴⁵, we analyzed genes linked this pathway during endothelial healing process. Interestingly, we found that *Vegfa*, *Vegfd* and *Flk1* mRNA levels were higher in the carotid from TAM-treated mice compared to those from the control group (vehicle-treated mice) (Online Figure XI B). We then took advantage of the published transcriptome single-cell analysis obtained on mouse aorta⁴⁶, 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 TAM are representative of genes expressed in SMCs and fibroblasts (Online Figure XII).

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

In contrast to E2, TAM effect on endothelial healing requires smooth muscle cells (SMCs) ER α but not endothelial/hematopoietic ER α .

Thus, we used the Cre-lox strategy (*Tie2Cre ER $\alpha^{lox/lox}$* and *α SMA CreER^{T2} ER $\alpha^{lox/lox}$* mice) to directly evaluate the role of ER α in endothelial/hematopoietic cells *versus* smooth muscle cells respectively. The efficiency of treatment with either E2 or TAM was verified by the uterine response in each mouse model (Online Figure XIII). First, we showed that E2 regenerative effect on endothelium after endovascular injury was abolished in *Tie2Cre+ER $\alpha^{lox/lox}$* mice, demonstrating the crucial role of ER α in hematopoietic/endothelial compartment in response to E2 in this model of endovascular injury, similar to that observed for perivascular electric injury¹⁵. In contrast, TAM effect on endothelial healing was fully preserved in *Tie2Cre+ER $\alpha^{lox/lox}$* 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 *ER $\alpha^{lox/lox}$* mice to evaluate whether SMCs ER α is involved in endothelial healing after mechanical injury in response to TAM. In both genotypes, vehicle-treated mice displayed a similar low percentage of re-endothelialization, close to 40 % of re-endothelialization. As expected, both E2 and TAM treatment increased endothelial healing in control mice, raising 80 % of re-endothelialization. In *α SMA CreER^{T2}+ ER $\alpha^{lox/lox}$* mice, TAM failed to have such an effect, by contrast to the fully preserved effect of E2 (50 % of re-endothelialization in TAM 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 TAM on endothelial healing.

Importantly, this result highlights that, although the accelerative action on endothelial healing by E2 and TAM are both mediated through ER α , the cellular targets involved in this process are completely different.

Whereas E2 action on endothelial healing requires ER α MISS, this effect is mediated by nuclear ER α in response to TAM.

We previously showed that membrane ER α mediates acceleration of re-endothelialization after electric injury in response to E2¹⁴ independently of nuclear ER α ⁴⁷. Using a mice model harboring a mutation of ER α cysteine 451, recognized as the key palmitoylation site required for ER α plasma membrane location (*ER α -C451A*), we confirmed the crucial role of membrane ER α in response to E2 in this process using the model of endovascular injury. Indeed, we observed no effect of E2 on endothelial healing in *ER α -C451A* mice. By contrast, the effect of TAM was fully preserved in this mouse model of loss of membrane localization of ER α (Figure 5A). Because ER α AF1 is necessary to mediate actions of TAM *in vitro*^{48,49} and *in vivo*^{50,51}, we then sought to evaluate the role of this sub-function on endothelial healing. To this aim, ovariectomized *ER α AF1^{+/+}* and *ER α AF1⁰* mice were treated with E2, TAM or a vehicle, and were submitted to mechanical injury of the carotid. E2 treatment increased endothelial healing in both *ER α AF1^{+/+}* and *ER α AF1⁰* control mice, extending the previous results obtained in the perivascular model²⁴. TAM failed to have such an effect using *ER α AF1⁰* mice, highlighting the crucial role of ER α AF1 in the vascular response to TAM (Figure 5B). Accordingly, RT-qPCR analysis performed in the aorta of these mice revealed the crucial role of ER α AF1 in the transcriptional action of TAM since the induction of the most regulated ER α target genes in response to TAM was abrogated in *ER α AF1⁰* mice (Figure 6A). Importantly, analysis of this gene regulation profile in the smooth muscle cell enriched-fraction (media) further reinforces the importance of SMCs in the transcriptional response of TAM in the vessel (Figure 6B). Altogether, our data indicate that, E2 and TAM act *via* the same receptor: ER α to induce acceleration of endothelial healing ; but these two ligands target different sub-functions of the receptor: membrane ER α for E2 and nuclear ER α AF1 in response to TAM.

DISCUSSION

Clinical studies report a protective action of TAM 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 TAM on endothelial healing and to explore the cellular and molecular targets involved. Here, we demonstrated for the first time that TAM accelerated endothelial healing after endovascular injury in both female and male mice. By contrast, in the model of perivascular electric injury, in which medial smooth muscle cells are destroyed, TAM failed to accelerate endothelial healing, suggesting an important role of this cell type in TAM action. Accordingly, we found that ER α 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 re-endothelialization through CXCL7-mediated recruitment of ECs from uninjured endothelium³⁹. Lupieri *et al.* also demonstrated that SMCs through CXCL10 prevent endothelial healing²⁸. 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 TAM treatment in both intact and injured carotids.

The major findings of this study are that TAM can mimic estrogen vasculoprotective effects through the activation of different/divergent cellular and molecular targets. Indeed, both E2 and TAM can accelerate endothelial healing but this beneficial action is dependent on the experimental model (*i.e.*

dependent on the cell type environment of the model used). Indeed, TAM but not E2 required the presence of the underlying medial SMCs (comparison of perivascular *versus* endovascular injuries). In addition, the *vivo* pre-treatment with TAM, 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 TAM action is mediated by ER α in SMCs, whereas E2 action relies on endothelial/hematopoietic ER α . Hence, transcriptomic analysis reveals differential gene expression profiles in the carotid artery from mice treated chronically with E2 or TAM. Functional annotation of the gene subsets regulated by either ER ligand indicated that inflammatory response was the major MSigDB³⁴ hallmark enriched for E2 regulated genes but not in response to TAM. The Hedgehog signaling pathway was specifically enriched for TAM-regulated genes in intact carotid arteries. The expression of several genes involved in the Hedgehog signaling pathway were also significantly higher in carotid from TAM-treated mice compared to control mice during the endothelial healing process (1, 3 or 5-day post-injury). 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 endothelial cells exerted by SMCs and/or fibroblasts^{43-45,52,53}. Interestingly, the comparison of genes selectively regulated by TAM with the single-cell data on mouse aorta⁴⁶ revealed that these genes are mainly expressed in SMCs. By contrast, genes regulated by E2 are reminiscent of the expression pattern of immune cells.

SERMs, such as TAM, 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 TAM is an antagonist, TAM induces the recruitment of co-repressors instead of co-activators to ER target promoters¹¹.

In contrast, in the uterus where TAM acts as an agonist, it recruits co-activators instead of co-repressors to ER target genes¹¹. The present results highlight, for the first time, to our knowledge, that the cell/tissue specificity of SERM may also involve different cell types of ER α , shedding a new light on the mechanisms of action of Selective Modulation of ER α . This new dimension will therefore have to be taken into account when examining the mechanisms underlying the benefit/risk profile of TAM for the treatment of breast cancer. Among other interrogations raising from our finding, since ER α regulates the immune system, it appears crucial to (re)-consider the action of TAM on immune surveillance in cancer⁵⁴. Interestingly, estrogens and TAM, but not pure antiestrogens, were also recently shown to partially protect against severe acute respiratory syndrome (SARS) in a mouse-adapted SARS-CoV model⁵⁵.

We have developed for two decades several mouse models of carotid artery injury. Thanks to the combination of i) genetically models targeting ER α or its sub-functions and ii) pharmacological tools, we were able to demonstrate that acceleration of endothelial healing in response to E2 is entirely dependent on membrane ER α ^{14,47,56}. In line with our previous work⁹, we first confirmed that TAM failed to activate membrane ER α , raising the question of whether this is due to the failure of TAM to bind to membrane ER α or the failure of membrane ER α to become activated by TAM binding. Co-administration of TAM and E2 validated the later hypothesis, as it failed to accelerate endothelial healing after electric injury, showing that TAM is not only devoid of ER α MIS, but TAM is also able to antagonize these E2 MIS effects. The role of this membrane/extra-nuclear action of TAM in another pathophysiological context, in particular, breast cancer angiogenesis⁵⁷ should be studied in future works. The inhibition of the migration and invasion of breast cancer cells induced by extra-nuclear signaling in response to estrogen would be of peculiar interest⁵⁸.

The use of mice harboring mutated specific functions of ER α revealed that TAM acts through nuclear ER α activation, and more precisely through the AF1 to accelerate endothelial healing. It is highly likely that the nuclear ER α AF1 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 TAM^{48,49}. More recently, we

showed that ER α AF1 is also necessary *in vivo* to relay the effects of TAM on: i) the proliferation of uterine epithelial cells ⁵⁰, ii) the prevention of atherosclerosis ⁹, and iii) the prevention of high-fat diet-induced metabolic disorders ⁵¹, since all these beneficial actions are abrogated using mouse model selectively deficient for ER α AF1 (*ER α AF1*⁰). Interestingly, ER α AF1 is dispensable to mediate these two latter actions in response to E2 ^{24,59}.

To conclude, the present study demonstrates that TAM and E2 both accelerate endothelial healing through an ER α -dependent effect. However, the cellular (ECs *versus* SMCs) and molecular (ER α sub-functions) target accounting for this protective action are quite dissimilar. These results should serve as a paradigm to revisit the action of estrogens and SERMs in breast cancer initiation and growth, angiogenesis and immune surveillance, but also in arterial pathophysiology. Although successfully used for 40 years in medicine, the understanding of the mechanisms of action of TAM are 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.

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DISCLOSURE

The authors have nothing to disclose

SUPPLEMENTAL MATERIALS

Online Supplemental Materials include:

- Supplemental Methods
- Online Figures I – XVIII
- Online Tables I-II
- Major Resources Table
- Statistics Dataset

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FIGURE LEGENDS

Figure 1. As E2, TAM induces acceleration of endothelial healing after endovascular injury *in vivo*.

(A) Four-week-old female mice were ovariectomized and 2 weeks after received either vehicle, E2 (80 µg/kg/day), TAM (4 mg/kg/day) or combination of both E2 and TAM. Three weeks later, mice were submitted to endovascular injury of the carotid artery. Carotid re-endothelialization was analyzed 5 days after injury. (B) Representative photography of uterus. (C) Representative Evans blue or (D) CD31+ staining of carotids (Scale Bar, 500µm) and (E) quantitative analysis of re-endothelialization, expressed as a percentage of re-endothelialized area compared to day 0. The outlined zones correspond to the de-endothelialized area. Results are expressed as means ± SEM (n=5–12 per group). Carotids were also analyzed 10 days after the injury: (F) Immunostaining of endothelial cells with anti-VE-cadherin antibody day 10 after endovascular injury (Scale Bar, 20µm) 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 were performed (**P<0.01, ***P<0.001).

Figure 2. The cellular actors involved in the acceleration of endothelial healing in response to TAM and E2 are different.

Four-week-old female mice were ovariectomized and 2 weeks after were given either vehicle, E2 (80 µg/kg/day), TAM (4 mg/kg/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 panel) and quantitative analysis of re-endothelialization expressed as a percentage of re-endothelialized area compared to day 0 (right panel). Scale bar, 500µm. (C) Three weeks later, carotid arteries were isolated and de-endothelialized *ex vivo*. Subsequently, they were placed in an endothelial cell-specific medium for 5 days. (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, 450µm. Quantification of re-endothelialization expressed as the percentage of CD31+ area compared to 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 one-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).

Figure 3. E2 and 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/day) or TAM (4 mg/kg/day) for 3 weeks and sequenced. The heatmap shown illustrates the relative expression values of all genes significantly regulated following E2 and/or TAM treatment (threshold of fold-change of expression >1.5 over control with a BH corrected p-value < 0.05). HCL clustering regroups each sample with its corresponding treatment group. (B) Venn diagram representing the overlap of genes regulated by E2 and/or TAM in carotid arteries. (C) GSEA analysis representing the different hallmarks pathways regulated by E2 and TAM. Calculated 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.

Figure 4. Estrogen receptor α (ER α) in smooth muscle cells is necessary to induce endothelial healing in response to TAM but not to E2. Schematic representation of (A) *Tie2CreER $\alpha^{lox/lox}$* and (B) *α SMACre^{ERT2}ER $\alpha^{lox/lox}$* mouse models.

Four weeks old mice were ovariectomized and received E2 or TAM treatment for 3 weeks. Mice were then submitted to endovascular injury of the carotid artery (day 0). De-endothelialized area was evaluated after blue Evans staining. Quantitative analysis of re-endothelialization was expressed as a percentage of re-endothelialized area at day 5 compared to day 0. The outlined zones correspond to the de-endothelialized area. Scale bar, 500µm. Results are expressed as

means \pm SEM (n=5–14 per group). To test the effect of E2 or TAM treatments in each genotype, Kruskal-Wallis test were performed (*P<0.05, **P<0.01, ***P<0.001).

Figure 5. Nuclear but not membrane ER α is necessary to induce endothelial healing in response to TAM.

Schematic representation of (A) *ER α C451A* and (B) *ER α AF1⁰* mouse models. Four weeks old mice were ovariectomized and received E2 or TAM treatment for 3 weeks. Mice were submitted to endovascular injury of the carotid artery (day 0). De-endothelialized area was evaluated after blue Evans staining. Quantitative analysis of re-endothelialization was expressed as a percentage of re-endothelialized area at day 5 compared to day 0. Results are expressed as means \pm SEM (n=4–8 per group). To test the effect of E2 or TAM treatments in each genotype, a 2-way ANOVA (A) or a Kruskal-Wallis test (B) were performed (*P<0.05, **P<0.01, ****P<0.0001).

Figure 6. ER α AF1 is necessary for the transcriptional response to TAM in the aorta and in the enriched SMCs fraction (media).

mRNA levels relative to *Tpt1* mRNA level from (A) aorta (B) and smooth muscle cell (SMC) enriched-fraction from *ER α AF1^{+/+}* and *ER α AF1⁰* mice treated by E2 (80 μ g/kg/day) or TAM (4 mg/kg/day) during 3 weeks were quantified by RT-qPCR. Results are expressed as means \pm SEM (n=4-9/group). To test the effect of TAM treatment in each genotype, Kruskal-Wallis test were performed (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

NOVELTY AND SIGNIFICANCE

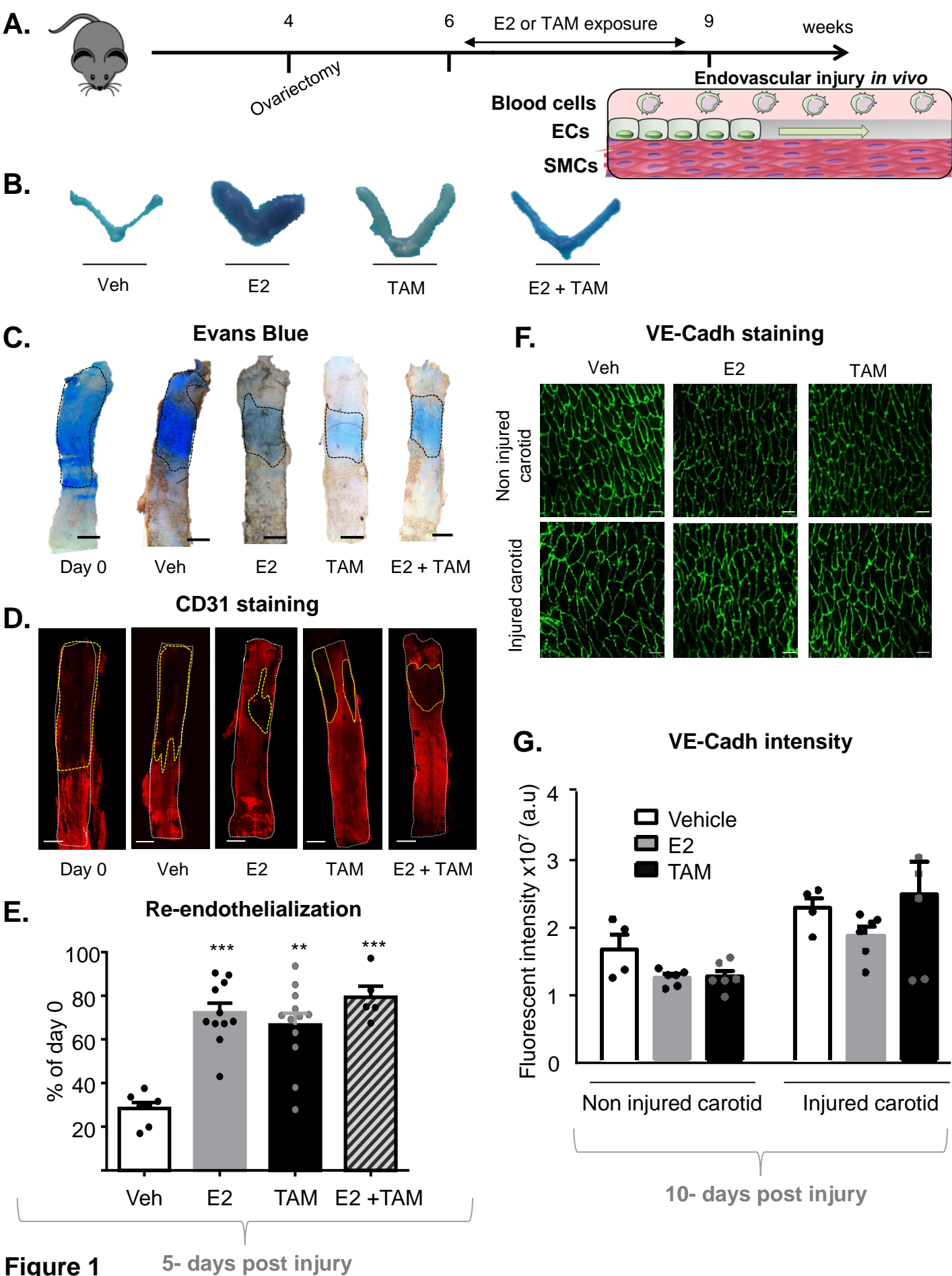
What Is Known?

- The main estrogen, 17 β -estradiol (E2) accelerates endothelial healing by targeting membrane ER α in endothelial cells.
- Tamoxifen is a selective ER α 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 ER α in smooth muscle cells.
- Tamoxifen accelerates endothelial healing by targeting nuclear ER α , 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 17 β -estradiol (E2) action depending on the tissues. We show here that as E2, Tamoxifen contributes to vascular protection through acceleration of endothelial healing. Surprisingly, even though both E2 and Tamoxifen actions on endothelial healing are mediated by ER α , activation of this receptor occurs in different cell types. In contrast to E2, which activates membrane ER α in endothelial cells, Tamoxifen accelerates endothelial healing by targeting nuclear ER α 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.



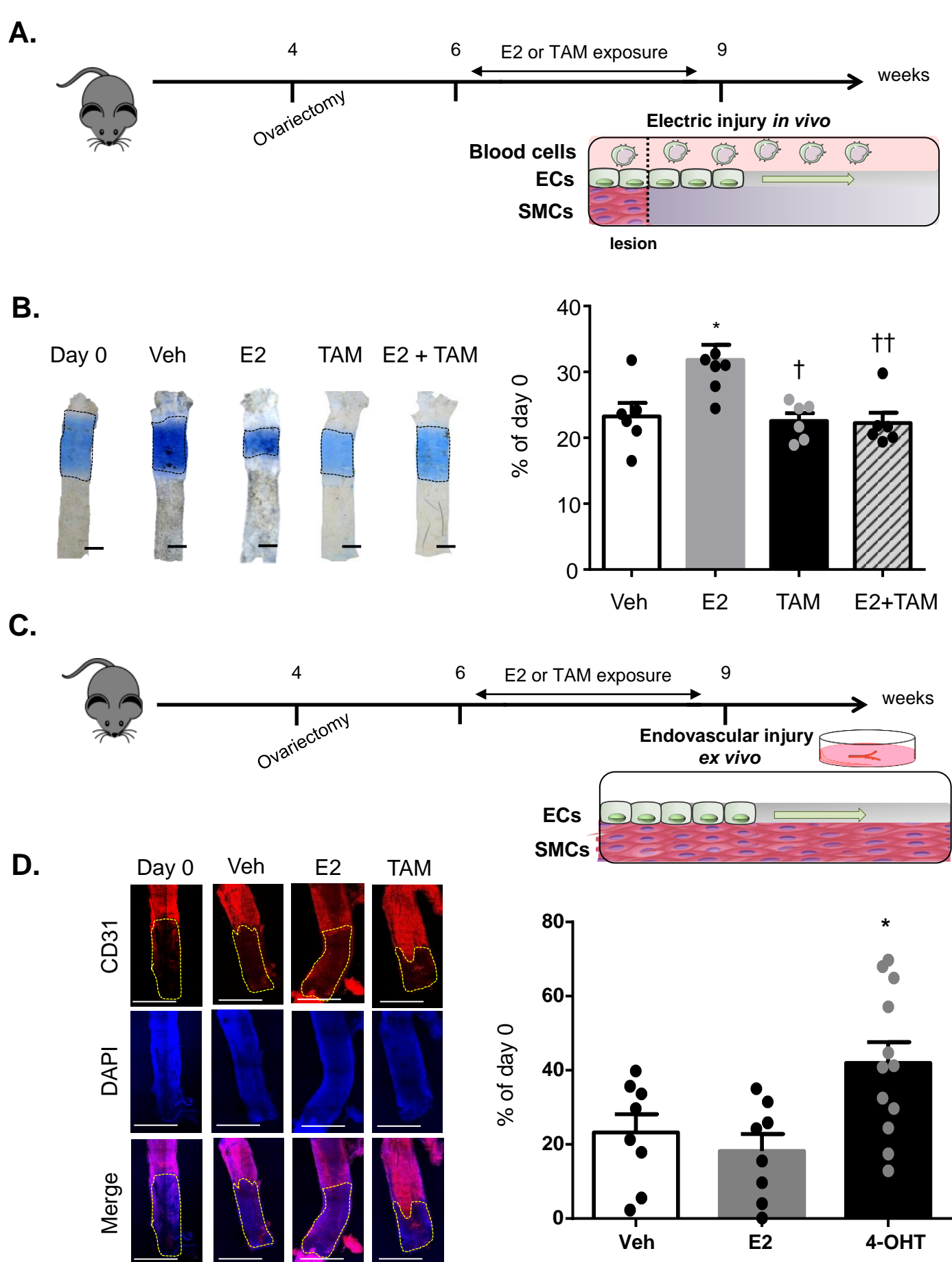
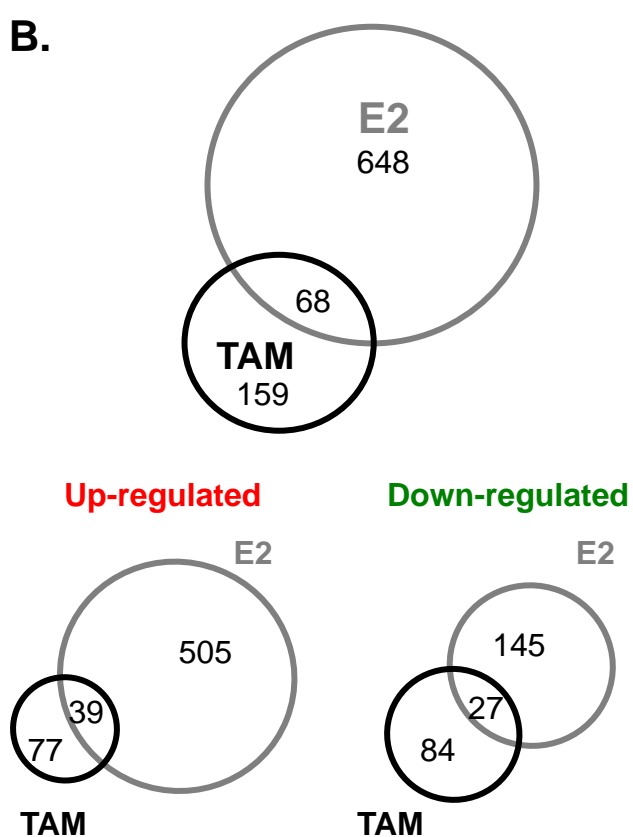
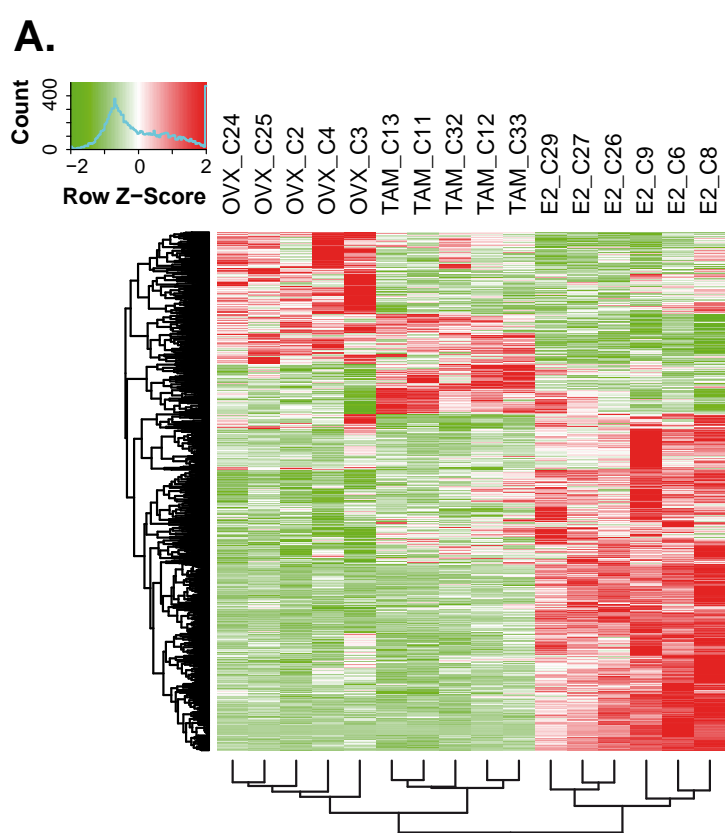


Figure 2



C.

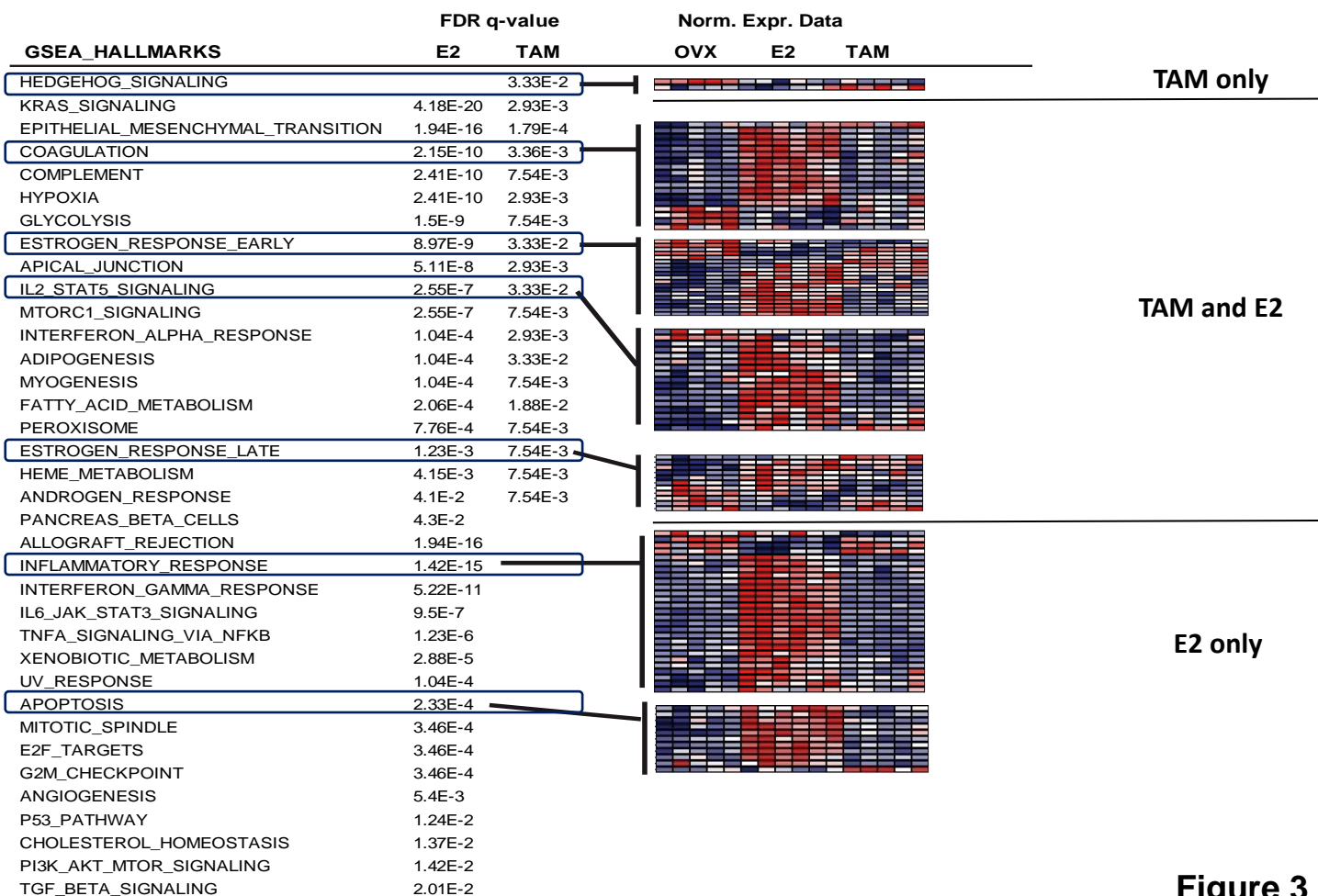
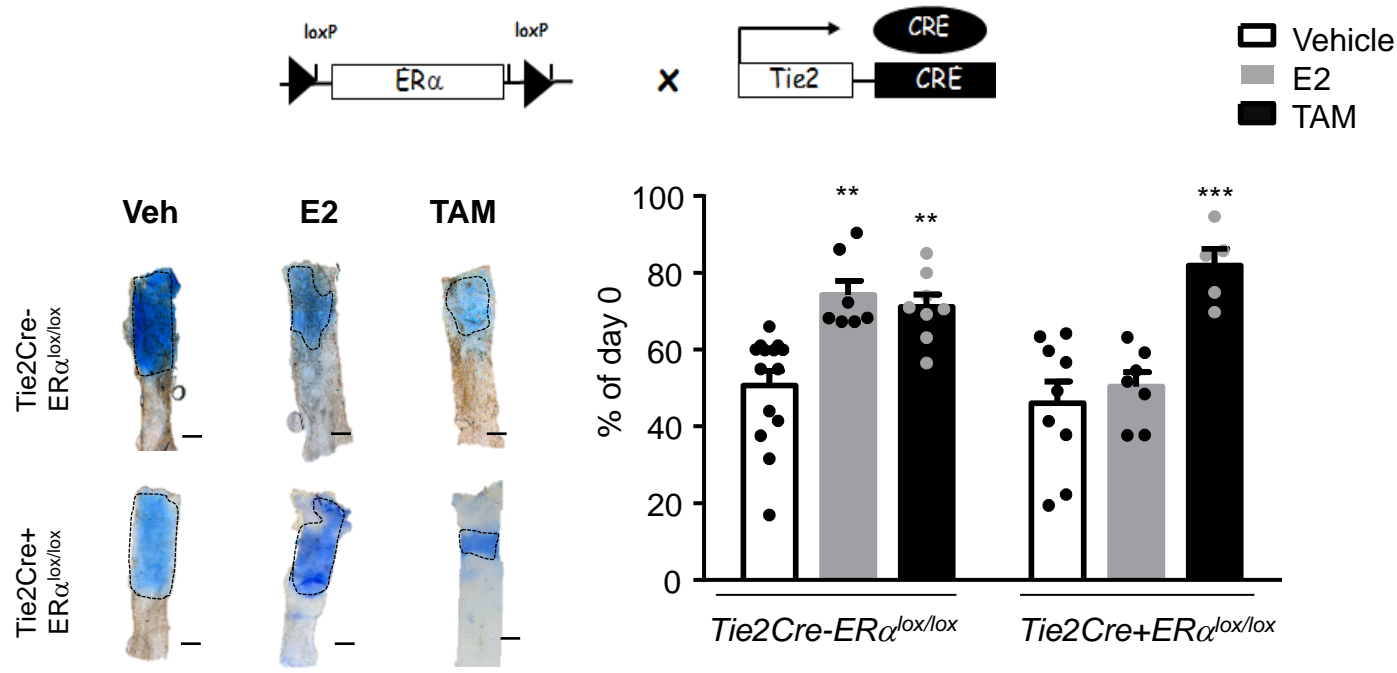


Figure 3

A. Selective endothelial /hematopoietic ERα knock-out



B. Selective Smooth Muscle Cells ERα knock-out

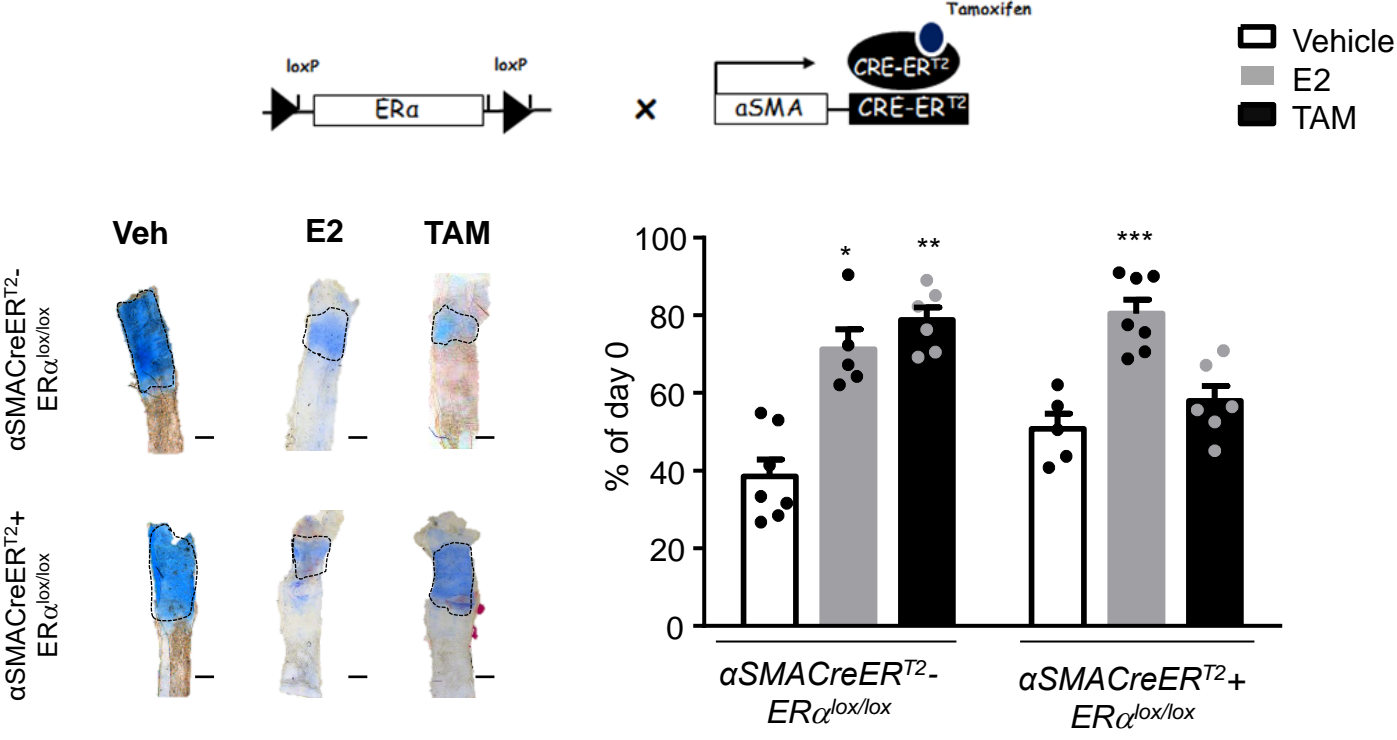
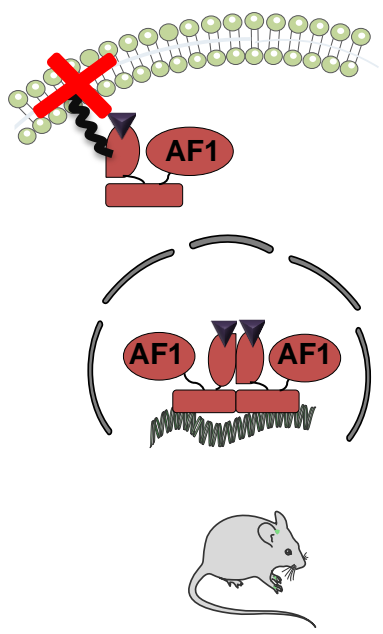
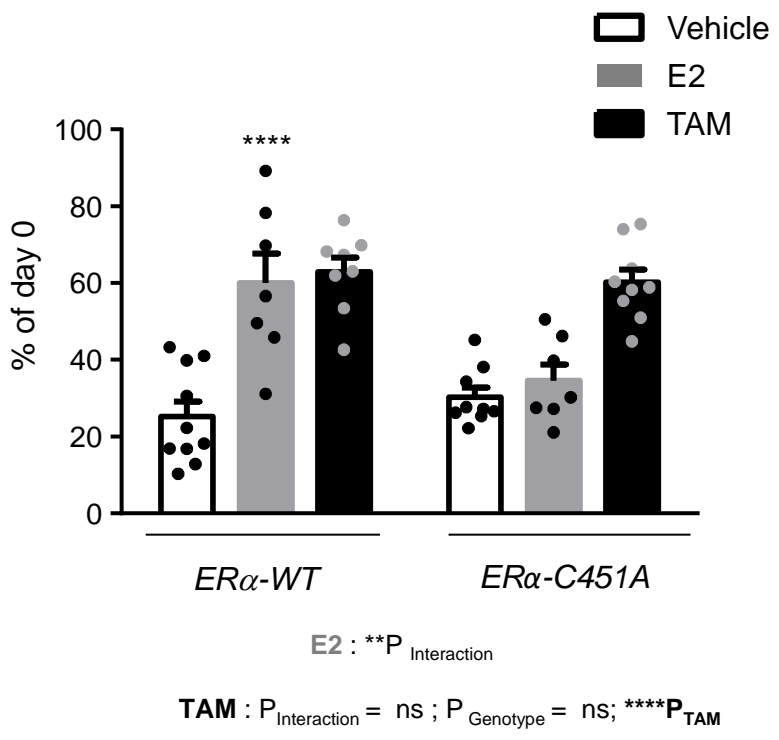


Figure 4

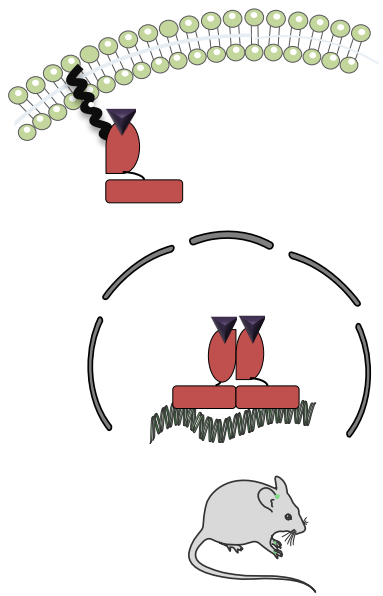
A. Abrogation of membrane ERα localization



ERα-Cys451Ala



B. Abrogation of nuclear ERαAF1



ERα-AF1⁰

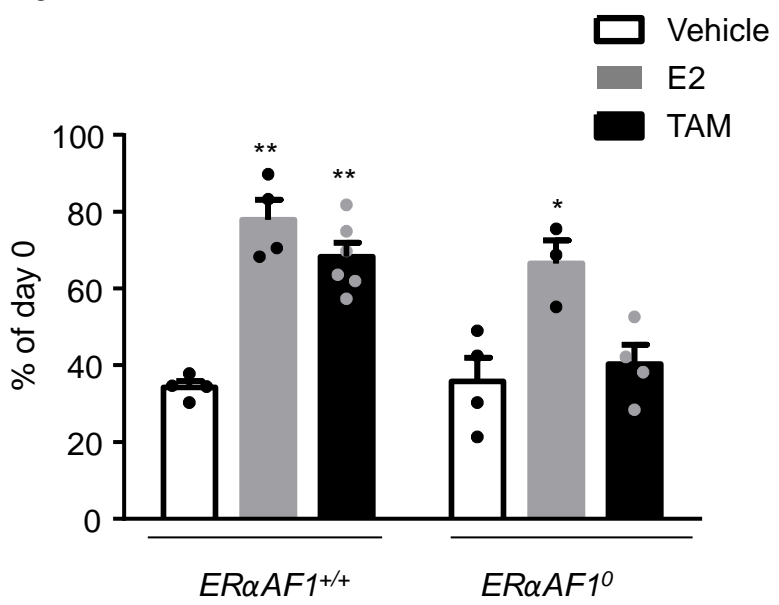
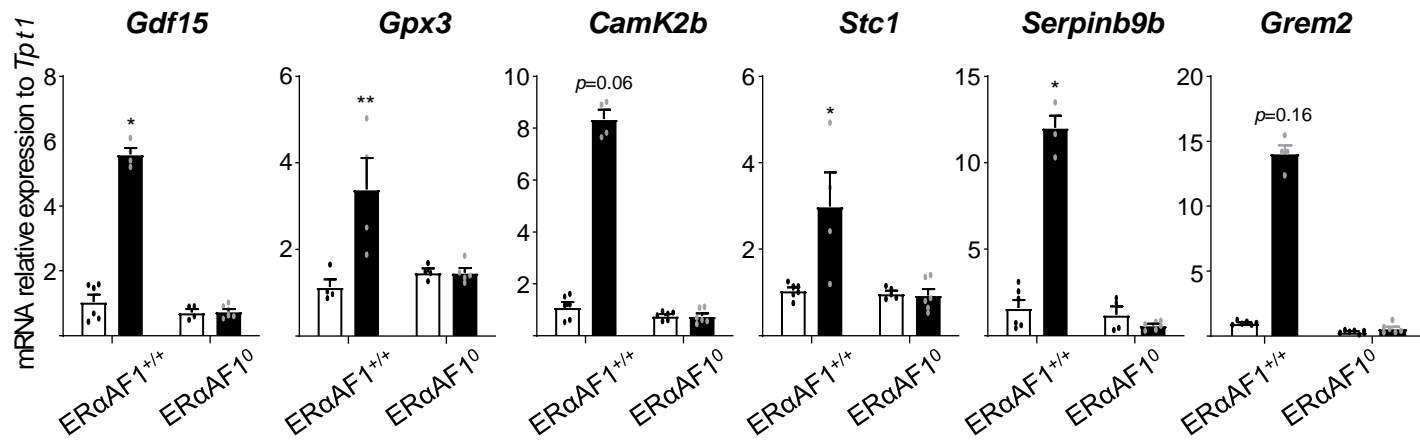
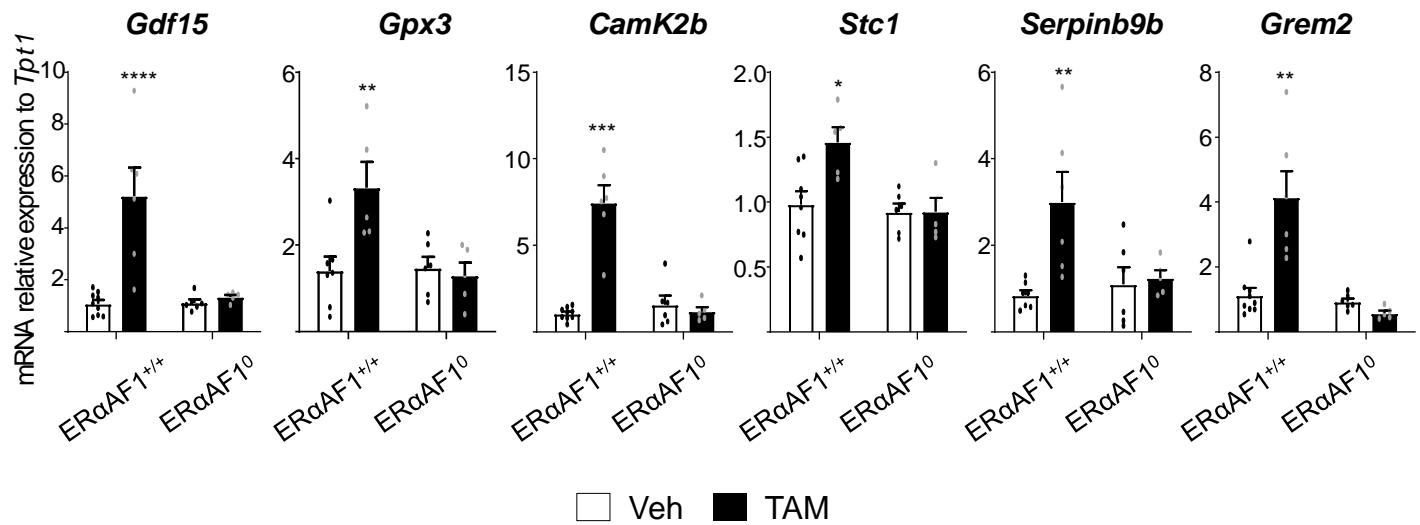


Figure 5

A.**Aorta****B.****Enriched-SMCs fraction (media)**

□ Veh ■ TAM

Figure 6