Estrogen Receptor α Expression in Both Endothelium and Hematopoietic Cells Is Required for the Accelerative Effect of Estradiol on Reendothelialization

Céline E. Toutain, Cédric Filipe, Audrey Billon, Coralie Fontaine, Laurent Brouchet, Jean-Charles Guéry, Pierre Gourdy, Jean-François Arnal, Françoise Lenfant

Objectives—E2 accelerates reendothelialization through estrogen receptor α (ERα), and we now aimed at defining the precise local and systemic cellular actors of this process.

Methods and Results—The respective roles of endothelial and hematopoietic targets of E2 were investigated in a mouse carotid injury model, using confocal microscopy, to follow endothelium repair. Grafting ERα−/− mice with ERα+/+ bone marrow (BM) was not sufficient to restore the accelerative effect of E2 on reendothelialization, demonstrating the necessary role of extrahematopoietic ERα. Using an endothelial-specific inactivation of ERα (Cre-Lox system), we showed that endothelial ERα plays a pivotal role in this E2 action. Conversely, in ERα+/+ grafted with ERα−/− BM, the E2 regenerative effect was abolished, demonstrating that ERα-expressing hematopoietic cells are also needed. As eNOS expression in BM was required for this action, both endothelial progenitor cells and platelets could be the hematopoietic targets that participate to this beneficial E2 effect.

Conclusions—We demonstrate that endothelial ERα plays a pivotal role in E2-mediated reendothelialization. However, endothelial targeting alone is not sufficient because the concomitant stimulation of a subpopulation of BM ERα is necessary. This cooperation should be taken into account in strategies aimed at optimizing in-stent reendothelialization. (Arterioscler Thromb Vasc Biol. 2009;29:1543-1550.)

Key Words: estradiol • reendothelialization • carotid injury model • endothelium • confocal microscopy

Angioplasty followed by stent implantation is a commonly used procedure to treat coronary or peripheral artery stenosis. The major complication of bare metal stent implantation is intransient stenosis because of neointimal hyperplasia. Drug-eluting stents have emerged as a potential solution against restenosis, but they also inhibit proliferation of endothelial cells. Late thrombosis has become a major concern, because delayed arterial healing, characterized by incomplete reendothelialization, constitutes an important underlying substrate for coagulation. Endothelium constitutes an antithrombogenic layer and limits neointima formation, and optimization of endothelial healing should be considered of primary importance.

Various treatment strategies to promote endothelial regrowth after arterial injury have been proposed. Administration of angiogenic growth factors such as vascular endothelial growth factorα and fibroblast growth factor-2 (FGF-2) increased endothelial healing in animal models. Endogenous mobilization or injection of ex vivo expanded endothelial progenitor cells (EPCs) was associated with enhanced reendothelialization. Pharmacological agents including angiotensin-converting enzyme inhibition, statins, and estrogens promote endothelial healing.

17β-estradiol (E2), the main endogenous estrogen, exerts many vascular protective effects by increasing basal production of endothelial nitric oxide (NO), accelerating reendothelialization and inhibiting neointima formation. We previously demonstrated that E2 accelerates reendothelialization in a mouse model of perivascular carotid injury, through estrogen receptor (ER) α but not ERβ. E2 increases both migration and proliferation of cultured endothelial cells in vitro, and these direct actions contribute to accelerate reendothelialization in vivo. In addition, E2 exerts major effects on hematopoietic cells, as mobilization of EPCs, actions on inflammatory immune cells and platelets, that could contribute to endothelial repair. However, the
respective contributions of E2 on endothelium and BM cells in this enhancing effect on the healing process have not been directly addressed. In the present work, we sought to determine whether endothelial ERα, BM ERα, or both participate to this beneficial effect of E2 on reendothelialization.

Methods
Animal Studies
Female mice were housed in groups of 6, kept in a temperature-controlled facility, on a 12-hour light–dark cycle, and fed normal laboratory chow diet. All procedures were performed in accordance with the guidelines established by the National Institute of Medical Research. In carotid injury experiments, mice were ovariectomized at 4 weeks of age and implanted or not with a pellet releasing E2 (17β-estradiol, 0.1 mg, 60-days release (ie, 80 μg · kg⁻¹ · d⁻¹, Innovative Research of America). E2 treatment was initiated 2 weeks before carotid injury and continued until the sacrifice (Figure 1A). ERα⁻/⁻ mice and eNOS⁻/⁻ mice were maintained in our animal facility. We systematically checked that ovariectomized and ERα-deficient mice had an atrophied uterus (<20 mg) whereas those implanted with an E2- pellet had a significant increase in uterine weight, except for the ERα⁻/⁻ mice (Table).

Generation of Tie2Cre and LysMCre ERα KO Mice
Endothelium and macrophage/granulocyte-selective deletion of ERα was achieved by the loxP-Cre recombination system. Mice homozygous for floxed ERα in exon 2 (ERαfloxfloxfloxfloxfloxfloxflox) were crossed with transgenic mice expressing Cre recombinase under the control of the Tie2 or LysM promoter/enhancer, obtained from the Jackson Laboratory (Bar Harbor, Me). We obtained Tie2Cre⁻/⁻ ERαfloxfloxfloxfloxfloxfloxflox mice which lack ERα in the endothelium (named Tie2Cre hereafter) and Tie2Cre⁻/⁻ ERαfloxfloxfloxfloxfloxfloxflox mice with intact ERα in the endothelium (named Tie2Cre hereafter, used as control littermates). LysMCre⁻/⁻ ERαfloxfloxfloxfloxfloxfloxflox mice which lack ERα in macrophages/granulocytes (named LysMCre hereafter) and LysMCre⁻/⁻ ERαfloxfloxfloxfloxfloxfloxflox mice with intact ERα (named LysMCreCre hereafter, used as controlled littermates) have been specifically phenotyped, showing a specific inactivation of ERα in macrophages (unpublished data, Calippe B, Guery J.C.G., Gourdy P., 2009). Genotyping of the mice was performed for the Cre transgene and ERα by genomic PCR.

Mouse Carotid Injury
Mice were anesthetized by injection of ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹) by intraperitoneal route. The perivascular carotid electric injury was performed as previously described. Briefly, surgery was carried out with a stereomicroscope (Nikon...
We previously demonstrated that ERα mediates the accelerative effect of E2 on reendothelialization.11 Several studies strongly suggested that BM-derived cells as EPCs are involved in endothelial healing.20–22 To clarify whether BM cells are sufficient to mediate the entire effect of E2 on endothelial healing, we generated hematopoietic chimeric mice by grafting ERα+/+ BM to ERα−/− mice. We verified by PCR the restoration of a normal expression of ERα in the hematopoietic compartment (data not shown).

We then measured the reendothelialized area by "en face" confocal microscopy. Whereas the accelerative effect of E2 was still observed in control irradiated mice as expected, it was completely abolished in the chimeric ERα+/+ → ERα−/− mice (P=0.45; Figure 2), demonstrating that ERα-expressing hematopoietic cells are not sufficient to mediate this effect.

**Results**

**ERα Expression in the Hematopoietic Compartment Alone Is Not Sufficient to Mediate E2 Reendothelialization**

We recently demonstrated that ERα mediates the accelerative effect of E2 on reendothelialization.11 Several studies strongly suggested that BM-derived cells as EPCs are involved in endothelial healing.20–22 To clarify whether BM cells are sufficient to mediate the entire effect of E2 on endothelial healing, we generated hematopoietic chimeric mice by grafting ERα+/+ BM to ERα−/− mice. We verified by PCR the restoration of a normal expression of ERα in the hematopoietic compartment (data not shown).

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**Endothelial Expression of ERα Is Necessary for the Accelerative Effect of E2 on Reendothelialization**

Three days after injury, the lengths of the reendothelialized areas were 424 ± 14 μm and 448 ± 30 μm in untreated and E2-treated Tie2Cre+ animals, respectively. In contrast, in control littermates, the lengths of the reendothelialized areas were 455 ± 40 μm and 681 ± 52 μm in untreated and E2-treated Tie2Cre− animals, respectively (Figure 4A and 4B). Thus, the beneficial effect of E2 on reendothelialization was strongly suggested that BM-derived cells as EPCs are important candidates. Endothelium-specific inactivation of ERα appeared to be natural candidates. Endothelium-specific inactivation of ERα was achieved by breeding floxed ERα mice with Tie2Cre transgenic mice, as Tie2 is expressed in endothelial cell-lineage.23 We showed that the floxed and deleted ERα gene were still detected in isolated aorta from Tie2Cre+ mice while scraping endothelium strongly abrogated expression of the deleted allele, demonstrating that the Tie2Cre transgene leads to specific inactivation of ERα in endothelial cells (Figure 3B).

However, Tie2Cre+ mice also present an inactivation of ERα in hematopoietic cells.24 Indeed, quantitative RT-PCR showed that expression of ERα in BM, spleen, and blood was dramatically reduced in Tie2Cre+ mice (Figure 3C), confirming deletion of ERα in hematopoietic cell-lineage. To restrict the ablation of ERα in endothelial cells, Tie2Cre+ mice were irradiated and then grafted with BM from wild-type mice. As expected, quantitative RT-PCR analysis showed that ERα expression in BM, spleen, and blood from these chimeric mice was restored by 100% (Figure 3C).

**Table. Effect of E2 Treatment on Uterine Weights**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uterine Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα+/+ → ERα−/+</td>
<td></td>
</tr>
<tr>
<td>0 VX</td>
<td>7.9 ± 0.9</td>
</tr>
<tr>
<td>0 VX + E2</td>
<td>73.1 ± 4.2</td>
</tr>
<tr>
<td>ERα+/+ → ERα+/−</td>
<td></td>
</tr>
<tr>
<td>0 VX</td>
<td>10.1 ± 1.5</td>
</tr>
<tr>
<td>0 VX + E2</td>
<td>10.0 ± 1.7</td>
</tr>
<tr>
<td>ERα−/+ → ERα+/+</td>
<td></td>
</tr>
<tr>
<td>0 VX</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>0 VX + E2</td>
<td>78.5 ± 5.2</td>
</tr>
</tbody>
</table>

| Tie2Cre+                       |                    |
| 0 VX                           | 9.5 ± 1.0          |
| 0 VX + E2                      | 80.1 ± 10.3        |

| WT → Tie2Cre−                  |                    |
| 0 VX                           | 12.8 ± 1.7         |
| 0 VX + E2                      | 92.4 ± 16.8        |

| WT → Tie2Cre+                  |                    |
| 0 VX                           | 9.3 ± 1.0          |
| 0 VX + E2                      | 48.3 ± 4.1         |

Results are expressed as means (mg) ± SEM. 0 VX indicates ovariectomized mice; 0 VX + E2, ovariectomized mice treated with E2.

**Generation of Tie2Cre+ Mice With Endothelial-Specific ERα Deletion**

Among the potential local targets of E2, endothelial cells appeared to be natural candidates. Endothelium-specific inactivation of ERα was achieved by breeding floxed ERα mice with Tie2Cre transgenic mice, as Tie2 is expressed in endothelial cell-lineage.23 We showed that the floxed and deleted ERα gene were still detected in isolated aorta from Tie2Cre+ mice while scraping endothelium strongly abrogated expression of the deleted allele, demonstrating that the Tie2Cre transgene leads to specific inactivation of ERα in endothelial cells (Figure 3B).

However, Tie2Cre+ mice also present an inactivation of ERα in hematopoietic cells.24 Indeed, quantitative RT-PCR showed that expression of ERα in BM, spleen, and blood was dramatically reduced in Tie2Cre+ mice (Figure 3C), confirming deletion of ERα in hematopoietic cell-lineage. To restrict the ablation of ERα in endothelial cells, Tie2Cre+ mice were irradiated and then grafted with BM from wild-type mice. As expected, quantitative RT-PCR analysis showed that ERα expression in BM, spleen, and blood from these chimeric mice was restored by 100% (Figure 3C).
completely abolished in Tie2Cre^+ mice, as compared with their control littermates Tie2Cre^-/^- mice. To reverse the deletion of ERα also present in hematopoietic cells from Tie2Cre^-/^- mice, we studied reendothelialization in Tie2Cre^-/^- mice grafted with wild-type BM. Tie2Cre^-/^- mice grafted with wild-type BM were used as controls. The E2 effect on reendothelialization was conserved in Tie2Cre^-/^- mice grafted with wild-type BM, whereas it was abolished in Tie2Cre^-/^- mice grafted with wild-type BM (Figure 4C). Thus, ERα expression in endothelial cells is absolutely required to mediate the accelerative effect of E2 on reendothelialization.

BM ERα Is Also Required for the E2 Effect on Reendothelialization

Although the present data clearly indicate that activation of endothelial ERα is necessary for the accelerative effect of E2, previous experimental studies suggested that BM-derived cells, especially EPCs, could be key actors in this action.15,25 To determine whether the accelerative effect of E2 could be obtained by activating only extrahematopoietic ERα (ie, the endothelial ERα) we grafted ERα^-/- BM to irradiated ERα^-/- mice (chimeric mice deficient in hematopoietic ERα). Surprisingly, the accelerative effect of E2 was abolished in these chimeric mice (P=0.46; Figure 5A). Interestingly, the E2 effect was also abolished when eNOS was absent in BM-derived cells, whereas it was still present in eNOS^-/- mice grafted with wild-type BM (Figure 5A). Altogether, the presence of both ERα and eNOS in BM cells is mandatory for the accelerative effect of E2 on reendothelialization.

To further determine the effects of these hematopoietic cells, we established a BM transplantation model in which BM cells could be identified by GFP expression (Figure 5B through 5F). Three days after carotid injury, we observed that only a few GFP-positive cells were present at the level of the endothelium (Figure 5C and 5F) with no difference in density between untreated and E2-treated mice (4.1%±0.3 and 3.7%±0.4, respectively; Figure 5G). None of these cells were observed in the intimal layer 30 days after injury (data not shown). No BM-derived GFP cells were found in the medial layer 3 days after injury (Figure 5D and 5F), whereas they were very abundant in the adventitia (Figure 5E and 5F), an infiltration that persisted at day 30 postinjury (data not shown).

Macrophages and Granulocytes Expression of ERα Is Dispensable for the Accelerative Effect of E2 on Reendothelialization

Because the GFP-positive cells in the adventitia were thought to be mainly macrophages, we sought to explore their role using cell-specific inactivation of ERα, by breeding LysMCre mice with floxed ERα (LysM is expressed by macrophages and neutrophils). The accelerative effect of E2 on reendothelialization was then investigated. Three days after injury, the lengths of the reendothelialized areas were increased by E2 in both LysMCre^+ and LysMCre^-/- mice (431±19 μm and 556±24 μm in untreated and E2-treated LysMCre^+ [means±SEM], respectively). In control littermates, the lengths of the reendothelialized areas were 456±19 μm and 579±23 μm in untreated and E2-treated LysMCre^-/- mice (means±SEM), respectively. Thus, macrophages and granulocyte ERα expression is dispensable for E2-mediated reendothelialization. These data indicated that cells from myeloid lineage do not significantly contribute to the E2 effect on reendothelialization.
Discussion

We previously reported that ERα is the key molecular target of E2-mediated reendothelialization. Despite many studies aimed at understanding the cellular targets involved in reendothelialization, the precise role of the local and systemic actors of this process has never been clearly established.

In the last decade, literature focused on the potential role of BM-derived progenitors/supporting cells not only in angiogenesis but also in reendothelialization. We first sought to investigate whether BM-derived cells were sufficient to support the accelerative effect of E2 on reendothelialization. However, E2 failed to accelerate reendothelialization in ERα−/− mice grafted with ERα+/+ BM (still deficient in extrahematopoietic ERα). This result indicates that E2 action restricted to BM derived cells is not sufficient to mediate the effect on endothelial healing.

The 2 major extra-BM candidates are resident endothelial cells and smooth muscle cells that constitute the main and almost unique cell type of the intima and media, respectively. To our knowledge, implication of smooth muscle cells in the reendothelialization process in vivo has not yet been directly investigated. However, when we recently compared the endovascular (where smooth muscle cells are preserved) and the perivascular model used in the present work (where complete decellularization of both endothelial and smooth muscle cells is achieved), we found that both basal and E2-stimulated velocity of reendothelialization were similar in the 2 models. This result strongly indicates that the underlying smooth muscle cells play a limited role, if any, in the accelerative effect of E2 on reendothelialization.

Thus, we decided to explore directly the action of E2 on endothelial ERα, using Tie2Cre mice. We found that the beneficial effect of E2 was completely abolished in this model. Furthermore, the lack of accelerative action of E2 also persisted in Tie2Cre+ grafted with the wild-type BM, despite the restoration of ERα expression in BM-derived cells. Indeed, inactivation of ERα using the Cre-Lox system under the control of the Tie2 promoter concomitantly inactivates expression of ERα in the endothelium and in the hematopoietic compartment, as previously described and as confirmed here (see Figure 3). Endothelial ERα is therefore absolutely required for the accelerative effect of E2 on reendothelialization, an observation reminiscent of the stimulatory action of E2 on the migration and proliferation of endothelial cells in vitro.

However, we found that this E2 action on endothelial ERα is not sufficient in vivo and that other cellular targets of E2 are mandatory. Indeed, inactivation of ERα in the hematopoietic compartment completely abolished the E2 effect in this model. Thus, ERα expression in endothelial cells is absolutely necessary to mediate the E2 effect on reendothelialization, but not sufficient, because it also requires involvement of BM-derived cells.

Accordingly, previous studies suggested that several BM-derived cells, such as EPCs, macrophages, and platelets, are potential candidates to mediate this action. Indeed, intravenous infusion of EPCs accelerated reendothelialization, and E2 increased the number of circulating EPCs as well as the incorporation of BM-derived cells in the reendothelialized area. However, most of these experiments showed a correlation between EPC increase and acceleration of reendothelialization rather than a causative relationship. To further characterize the relationship between BM-derived cells and the regenerating endothelial cell monolayer, we grafted GFP-BM to wild-type mice and found that only a few GFP-positive cells (3% to 4% of intima cells) were present at the level of the regenerated endothelial cell monolayer, irrespective of the E2 treatment. Noteworthy, they were no longer detectable 30 days postinjury, indicating that these cells did not incorporate into the regenerated endothelium. If EPCs are defined as cells differentiating into endothelial cells...
and incorporating into the new vascular network, the absence of GFP-positive cells in healed carotid artery (30 days post injury) suggests no direct contribution of “true” EPCs. At least some initially called EPCs appear to be BM-derived cells with monocyte characteristics. Thus, if these intimal GFP-positive cells play a role in the E2 action and although their density were not influenced by E2 at day 3, they could act essentially in a paracrine manner to promote reendothelialization as reported for angiogenesis. To make the story even more complex, true endothelial progenitors

Figure 5. Bone marrow–derived cell implication in reendothelialization. A, E2 treatment fails to accelerate reendothelialization in ERα-/- or eNOS-/- BM. B, Representation of the vessel wall. C through F, GFP-BM transplantation. 3% to 4% of BM-GFP cells were found within the endothelium 3 days after injury, irrespective of the treatment (C and G), whereas they were absent in the media (D) and very abundant in the adventitia (E). F, Carotid transversal view. H, The E2 accelerative effect on reendothelialization is maintained in LysMCre- mice. G, Quantification of BM-deprived GFP-positive cells in the endothelium 3 days after injury.
with high proliferative potential, detected in the vessel wall itself, might not be restricted to BM but could derived directly from the vessel wall.\textsuperscript{31,32}

Monocytes/macrophages are clearly direct targets for E2\textsuperscript{33} and were previously reported to modulate angiogenesis. Interestingly, they could thereby also be a candidate for endothelial regeneration, although macrophage colony-stimulating factor had no effect on reendothelialization.\textsuperscript{34} Moreover, Geissman et al demonstrated that a subset of resident monocytes patrol and are rapidly recruited in inflammatory sites where they differentiated into macrophages.\textsuperscript{35} Because this population is patrolling the surface of the endothelium through long-range crawling, it could therefore be involved in the modulation of reendothelialization. In consequence, we used LysMCre ER\textsubscript{a}\textsuperscript{lox/lox} mice to clarify the potential role of myeloid cell lineage in E2-mediated reendothelialization. In double mutant mice, harboring LysMCre and loxP-flanked target genes, Förster et al showed a deletion efficiency of 83% to 98% and near 100% in mature macrophages and granulocytes, respectively, whereas partial deletion (16%) was detected in dendritic cells.\textsuperscript{36} Our results indicate that neither macrophages nor neutrophils contribute to the accelerative effect of E2 on reendothelialization.

Platelets are also abundant in the deendothelialized area\textsuperscript{37} and release factors that can directly\textsuperscript{38} or indirectly\textsuperscript{39} contribute to the acceleration of reendothelialization. In this respect, it is noteworthy that E2 was reported to modulate platelet physiology.\textsuperscript{40,41} Finally, a yet unrecognized circulating factor secreted by E2-stimulated hematopoietic cells could have contributed to its effect on reendothelialization.

We reported here that eNOS expression in BM is important to mediate the E2 effect on reendothelialization, whereas the E2 effect persisted in chimeric eNOS\textsuperscript{−/−} grafted with wild-type BM, although lesser in magnitude. Both EPCs\textsuperscript{42} and platelets\textsuperscript{43} were reported to express eNOS. Moreover, we have previously shown that BM-derived FGF-2 was absolutely required in the accelerative effect of E2 in reendothelialization, whereas in nonhematopoietic cells FGF-2 was dispensable,\textsuperscript{29} showing that both FGF-2 and eNOS are key molecular actors of the E2 action at the level of the BM.

Thus, the identification of the specific BM-derived cell populations responsible for the reendothelialization process remains difficult because it will require the development of the cell-specific inactivation of ER\textsubscript{a} in these BM-derived populations, tools that are not yet available in our laboratory.

In summary, the present study demonstrates that expression of ER\textsubscript{a} in both BM compartment and endothelial cells is required to mediate the accelerative effect of E2 on reendothelialization. This should be taken into account in designing strategies aimed at promoting postangioplasty reendothelialization. For instance, E2-eluting stents could not be sufficient to efficiently accelerate reendothelialization. Accordingly, in the only in-man randomized trial (ETHOS I trial), E2-eluting stents did not show any benefit for treatment of coronary lesions when compared to bare metal stents.\textsuperscript{44} Recognizing the BM-derived actors that contribute to promote reendothelialization represents an important challenge to optimize in-stent vascular healing.

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Disclosures
None.

References


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Supplemental Material

**Bone marrow transplantation**

Two weeks after ovariectomy, recipient mice were irradiated (9 Gy, γ-source) and 24 h later, received a retro-orbital injection of 12·10⁶ donor BM cells suspended in 200µl PBS 1X. Donor BM cells were prepared by flushing the tibia and femur BM with PBS 1X, followed by centrifugation at 1,200 g and resuspension in PBS 1X. Following BM transplantation, mice recovered for 4 weeks and sulfamethoxazole 200mg.ml⁻¹ and trimethoprim 48mg.ml⁻¹ (Bactrim®, Roche) was added in drinking water. Mice were then implanted or not with an E2 pellet. Carotid injury was performed 2 weeks after pellet implantation, at 12 weeks of age (Figure 1B).

**Quantification of ERα mRNA expression by real-time RT-PCR**

Total RNA from BM cells, splenocytes and blood was prepared using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA (1 µg) was reverse transcribed for 10 min at 25°C and 2h at 37 °C in a 20-µl final volume with the High Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCRs were performed on ABI Prism 7900 (Applied Biosystem). Primers were validated by testing PCR efficiency using standard curves (95% ≤efficiency≤ 105%) (mERα: 5’-GATCATGGAGTCTGCCAAGGA-3’ and 3’-CGTTACTGATACGGAGACGA-5’ ; mGAPDH: 5’-AGGTCGGTGTGAACGATTTG-3’ and 3’-ACTGGAGTTGATTGTACCAGATGT-5’). Gene expression was quantified using the comparative $C_t$ (threshold cycle) method and GAPDH was used as reference.

**Southern blot analysis**

Genomic DNA from isolated aorta, before or after removal of endothelium by scraping, was isolated using proteinase K and phenol/chloroform. Ten micrograms of DNA were digested with *BamH* I (Invitrogen, Carlsbad, CA), loaded on 0.7% agarose gels and transferred to
positively-charged nylon membranes (MP Biomedicals, Illkirch, France). A previously described ERα probe (P3') was radiolabeled with (α-32P) dCTP (Perkin Elmer, Boston, MA) and hybridized overnight at 42°C. To reveal the 4.4 kb and 8.8 kb bands, representing the targeted (floxed) allele and the deleted allele, respectively (figure 3A), medical X-ray films (Agfa-Gevaert, Mortsel, Belgium) were exposed at −80°C for 1 to 2 weeks.

**En face confocal microscopy**

Mice were anesthetized and perfused by an intra cardiac injection of PBS 1X to remove intravascular blood. The left common carotid was dissected from the aortic arch to the carotid bifurcation. Carotids were fixed for 30 min in 100% methanol and then longitudinally opened. Carotids were permeabilized with HCl 2N at 37°C for 7 minutes. After 3 washes in PBS, samples were treated with a Borate solution (24.6 g/L Na2B4O7, 4.36 g/L KH2PO4) during 2x15 min. Carotids were stained with propidium iodide (1/500) for 1h at room temperature and washed with PBS 1X. All preparations were mounted with Kaiser’s glycerol gelatin (Merck).

**Reendothelialization quantification**

Microscopy imagery was performed on a ZEISS LSM 510 confocal microscope and quantification was performed with ZEISS LSM 510 software. The reendothelialized area was scanned with special emphasis on endothelial cells (z stacks of about 1.5µm). The assembly of micrographs to reconstruct the whole overview of the carotids was achieved with PhotoStich v. 3.1 (Canon, Courbevoie, France). The lengths of the reendothelialized area are means of at least 10 measures of endothelial cells that have maximal migration from the line of injury spanning the carotid (Figure 1), obtained with the software Zeiss LSM Image Browser v.3.1 (Carl Zeiss Jena GmbH, Jena, Germany).

**Statistics**

Results are expressed as means ±S.E.M. The effect of E2 treatment and the role of chimerism or genotype were tested with a 2-factor ANOVA model followed by a Bonferroni post-hoc
test using the software Prism (Prism®, GraphPad). When an interaction was observed between the two factors, the groups were compared by a one-factor ANOVA. $P<0.05$ was considered as statistically significant. *$p<0.05$, **$p<0.01$.