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Natural estrogens enhance the engraftment of human hematopoietic stem and progenitor cells in immunodeficient mice

by Sara Fañanas-Baquero, Israel Orman, Federico Becerra Aparicio, Silvia Bermudez de Miguel, Jordi Garcia Merino, Rosa Yañez, Yolanda Fernandez Sainz, Rebeca Sánchez, Mercedes Dessy-Rodríguez, Omaira Alberquilla, David Alfaro, Agustin Zapata, Juan A. Bueren, Jose Carlos Segovia, and Oscar Quintana-Bustamante

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Title

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Short title

E4 enhances human HSPC engraftment in NSG mice

Author list and affiliations:

Sara Fañanas-Baquero[1,2], Israel Orman[1,2], Federico Becerra Aparicio[1,2], Silvia Bermudez de Miguel[1,2], Jordi Garcia Merino[1,2], Rosa Yañez[1,2], Yolanda Fernandez Sainz[1,2], Rebeca Sánchez[1,2], Mercedes Dessy-Rodríguez[1,2], Omaira Alberquilla[1,2], David Alfaro[3], Agustin Zapata[3], Juan A. Bueren[1,2], Jose Carlos Segovia[1,2], Oscar Quintana-Bustamante[1,2]

1 Hematopoietic Innovative Therapies Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain. 2 Unidad Mixta de Terapias Avanzadas. Instituto de Investigación Sanitaria Fundación Jiménez. 3 Department of Cell Biology, Faculty of Biology, Complutense University of Madrid, Madrid, Spain.

* Correspondence: oscar.quintana@ciemat.es ; jc.segovia@ciemat.es; Phone: +34-91-496-2524, +34-91-346-6268; Fax: +34-91-346-6484

Key points:

- Natural estrogens modulate human hematopoietic stem and progenitor cells.
- E2 and E4 increase human hematopoietic reconstitution in NSG mice.

Abstract:

Hematopoietic Stem and Progenitor Cells are crucial in the maintenance of lifelong production of all blood cells. These Stem Cells are highly regulated to maintain homeostasis through a delicate balance between quiescence, self-renewal and differentiation. However, this balance is altered during the hematopoietic recovery after Hematopoietic Stem and Progenitor Cell Transplantation. Transplantation efficacy can be limited by inadequate Hematopoietic Stem Cells number, poor homing, low level of engraftment, or limited self-renewal.

As recent evidences indicate that estrogens are involved in regulating the hematopoiesis, we sought to examine whether natural estrogens (estrone or E1, estradiol or E2, estriol or E3 and estetrol or E4) modulate human Hematopoietic Stem and Progenitor Cells. Our results show that human Hematopoietic Stem and Progenitor Cell subsets express estrogen receptors, and whose signaling is activated by E2 and E4 on these cells. Additionally, these natural estrogens cause different effects on human Progenitors in vitro. We found that both E2 and E4 expand human Hematopoietic Stem and Progenitor Cells. However, E4 was the best tolerated estrogen and promoted cell cycle of human Hematopoietic Progenitors. Furthermore, we identified that E2 and, more significantly, E4 doubled human hematopoietic engraftment in immunodeficient mice without altering other Hematopoietic Stem and Progenitor Cells properties. Finally, the impact of E4 on promoting human hematopoietic engraftment in immunodeficient mice might be mediated through the regulation of mesenchymal stromal cells in the bone marrow niche. Together, our data demonstrate that E4 is well tolerated and enhances human reconstitution in immunodeficient mice, directly by modulating human Hematopoietic Progenitor properties and indirectly by interacting with the bone marrow niche. This application might have particular relevance to ameliorate the hematopoietic recovery

after myeloablative conditioning, especially when limiting numbers of Hematopoietic Stem and Progenitor Cells are available.

Introduction

Hematopoietic Stem Cells (HSCs) are a rare cell population resident in the bone marrow of adult mammals and sit atop a hierarchy of progenitors that become progressively restricted to several or a single blood lineage. HSCs are capable of self-renewal and multipotent differentiation to all blood cell lineages[1], becoming crucial for the maintenance of lifelong production of all blood cells. They are homeostatically regulated through a delicate balance between quiescence, self-renewal and differentiation. Although HSCs divide infrequently, they are activated to proliferate in response to bone marrow injury to re-establish homeostasis[2]. On the other hand, Hematopoietic Stem and Progenitor Cell Transplantation (HSPCT) is routinely used to reconstitute hematopoiesis after myeloablation, as a result of leukemia or hematopoietic genetic disease treatments. However, HSPCT efficacy can be limited by inadequate number, poor homing, low engraftment, or differentiation stress of Hematopoietic Stem and Progenitor Cells (HSPCs). Different approaches have been attempted to solve these problems, such as using different sources of HSPCs[3-5], ex vivo expansion of HSPCs[6-10] or stimulating them by using accessory molecules[11, 12] or cells[13]. However, these approaches require extensive knowledge regarding HSPC regulation and how their properties can be boosted to maximize their efficacy to reconstitute the patient's blood system after HSPCT[1, 14].

Estrogen is the primary female sex hormone and, apart from its known role in the reproductive system, it is responsible for controlling many cellular and molecular processes including growth and differentiation. Estrogens act through genomic or nuclear signaling and non-genomic or membrane-initiated steroid signaling (MISS), modulating intracellular second messengers[15]. The four major naturally-occurring estrogens in women are estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4). E1 is the predominant estrogen in postmenopausal women. E2 is considered the active estrogen during the estrous cycle. E3 and E4 are synthesized during pregnancy by the placenta and fetal liver respectively, but their physiological roles are essentially unknown[16].

Recent evidence indicates that E2 is involved in regulating the proliferation and lineage commitment of HSCs[17]. But the studies are few and sometimes their results are contradictory. But despite E2 treatment was able to specifically increase the number of vascular HSCs, long term repopulating capacity of those HSCs was limited[18]. Additionally, this estrogen was shown to promote the cell cycle of HSCs and multipotent progenitors (MPP) and increase erythroid differentiation in females[19], also during pregnancy[19]. Furthermore, E2 favors hematopoietic regeneration through the activation of the telomerase activity[20-22] or the stimulation of the unfolded protein response (UPR) on mouse HSCs, which sustain protein homeostasis to favor hematopoietic regeneration[23]. On the contrary, tamoxifen, whose active metabolite (4-hydroxytamoxifen) acts as an estrogen receptor antagonist, reduces the number of MPPs and short-term HSCs but activates the proliferation of long-term HSCs[24]. In addition, E2 might modulate HSCs indirectly through activating bone marrow mesenchymal stromal cells (BM-MSCs). E2 treatment has been described to activate MSC osteogenic differentiation and also promotes the secretion of GM-CSF and IL6, which improved the number of HSCs by modulating their niche[25]. Therefore, the estrogen-mediated regulation of HSPCs can also occur indirectly to change the HSC bone marrow niche. For that reason, fully understanding the role of estrogens in HSC regulation is essential for being able to further develop the clinical potential of these hormones.

In this work, we have examined the impact of natural estrogens on human HSPCs. E2 and E4 treatment *ex vivo* expanded human HSPCs, and more importantly, the administration of E4 to immunodeficient mice previously transplanted with human HSPCs enhanced the engraftment level of human hematopoietic cells.

Methods

Human Cord Blood-CD34$^+$samples and Bone Marrow Mesenchymal Stromal Cells

Umbilical cord blood samples (CB) from healthy donors were provided by "Centro de Transfusión de la Comunidad de Madrid". All samples were collected under written consent and Centro de Transfusión de la Comunidad de Madrid's institutional review board agreement (number PKDEFIN [SAF2017-84248-P]). Mononuclear cells were obtained by fractionation in Ficoll-hypaque according to manufacturer's recommendations (GE Healthcare). Purified CB-CD34$^+$ cells were obtained using a MACS CD34 Micro-Bead kit (Miltenyi Biotec). Cells were viably frozen in 10% dimethyl sulfoxide solution and stored at liquid nitrogen until their use.

Mononuclear cells from human Bone Marrow (BM) were obtained by Ficoll-Paque Plus density gradient from heparinized BM samples obtained from healthy donors after informed consent. All the procedures were in accordance with the Helsinki Declaration of 1975, revised in 2000. Samples were then cultured at 1.6×10^5cells/cm^2 in MesenCult medium plus supplements for human cells (Stemcell Technologies). After 24h, non-adherent cells were discarded. Fresh medium was added and replaced twice a week. At 80% confluence, adherent cells were trypsinized, washed, and seeded at 4×10^3cells/cm^2. In all the experiments, BM-MSCs were used at 5–8 passages.

Hematopoietic transplant protocol in immunodeficient mice

All the mice were kept under standard pathogen-free conditions in the animal facility of CIEMAT. All animal experiments were performed in compliance with European and Spanish legislations and institutional guidelines. The protocol was approved by "Consejeria de Medio Ambiente y Ordenación del Territorio" (Protocol number PROEX 078/15).

CB-CD34$^+$ cells were administered through tail vein of female or male NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ (NSG) mice sub-lethally irradiated the day before transplant with 1.5Gy. Three days later, the animals were treated with vehicle (olive oil) or with daily

doses of either E2 or E4 (2µg of estrogen per day) intraperitoneally for four days. Four months post transplantation (mpt), mice were sacrificed and BM from long bones of these animals was collected. Additionally, when hematopoietic niche analysis was involved, the long bones were flushed, cut in small pieces and crushed before being digested with 200U/mL Collagenase IV / 2µg/mL DNaseI in HBSS at 37°C for 45 minutes. The human engraftment was analyzed by flow cytometry (LSR Fortessa; BD). The cells were stained with hCD45-APCCy7 and hCD3-APC (BioLegend), hCD45-FITC, hCD33-PE, hCD19-FITC and hCD235a-FITC (Beckman Coulter), hCD34-Pecy5 (Immunotech), hCD38-PE, hCD90-APC, mCD45.1-PE, mCD45.1-Biotin and Ter119-Biotin (BD), mCD140a-APC (Pdgfra-APC, eBiosience) and mCD144-PE (VE-Cadherin-PE, eBiosience). DAPI-positive cells were excluded from the analysis. Analysis was performed using FlowJo software.

Additionally, $hCD45^+$ population from primary mice was sorted in an Influx Cell Sorter (BD), and $1x10^6$ $hCD45^+$ cells were transplanted into sub-lethally irradiated female secondary NSG recipients. 4mpt, the animals were sacrificed and analyzed as previously described.

Results

Engraftment of human CB-CD34$^+$ is favored in female immunodeficient mice

It has been previously described that the engraftment of highly purified human HSCs (Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$) is improved when these cells are transplanted into immunodeficient female recipients, as compared to male recipients[26]. To investigate if this enhanced engrafting potential in female recipients was also present in CB-CD34$^+$ cells, we transplanted different amounts of HSPCs into sub-lethally irradiated animals. As occurred when highly purified HSCs were transplanted, we observed higher engraftment of human HSPCs in female NSG animals than in their male counterparts (Figure 1A). Human engraftment in mouse bone marrow (BM) 4mpt was 61.06±26.07% (mean±SD) in female mice and 18.94±13.93% in male mice when 5x10^4 CB-CD34$^+$ cells had been transplanted. Interestingly, this impairment in the engrafting potential was even higher when only 5x10^3 CB-CD34$^+$ cells were transplanted (38.74±30.42% BM cells were of human origin in female animals versus only 0.19±0.27% in male animals) (Figure 1B). Therefore, females exhibited 3.2- or more than a 200-fold increase in human engraftment in comparison with male recipients when 5x10^4 or 5x10^3 CB-CD34$^+$ cells were transplanted, respectively. Additionally, there was no difference in the percentage of myeloid, B, T cells or HSPCs (hCD34$^+$, hCD34$^+$hCD38$^-$ and hCD34$^+$hCD38$^-$hCD90$^+$) within the human engraftment (supplemental Figure 1). These data pointed out the importance of the gender of the NSG mouse recipients to facilitate the engraftment of human HSPCs.

Human HSPC subsets expressed both ESR1 and ESR2

To understand the potential role of sex hormones in the observed differences of human hematopoietic engraftment between male and female recipient mice, we analyzed the expression of the two main estrogen receptors, ESR1 and ESR2, in CB-CD34$^+$ cells. As shown in the immunostaining analysis of Figure 2A and 2B, most CD34$^+$ cells were positive for ESR1 staining, while ESR2 staining was dimmer in CD34$^+$ cells (Figure 2A-B; supplemental Figure 2A). Additionally, to investigate the differential expression of these receptors in the hematopoietic progenitors, different populations of HSPCs, such as

HSC/MPP ($CD34^+CD38^-CD45RA^-$), Multilymphoid Progenitors (MLP, $CD34^+CD38^-CD45RA^+$) and committed hematopoietic progenitors (Hem Prog, $CD34^+CD38^+$), were sorted out (supplemental Figure 2B) and the expression of both estrogen receptors was determined by qRT-PCR. Both *ESR1* and *ESR2* were expressed in HSCs, MLPs and in more committed hematopoietic progenitors (Figure 2C-D; supplemental Figure 2C). *ESR1* expression tends to be up-regulated between HSC/MPP and MLP compartments to again fall down in the most committed hematopoietic progenitors (Figure 2C). On the contrary, *ESR2* expression seems to follow an opposite pattern with high values in both HSC/MPP and committed hematopoietic progenitors but reduced levels in the MLP cell population (Figure 2D). In both cases, although some tendencies could be observed, no statistical significance was observed. On the other hand, the new identified estrogen receptor, *GPER1*, as in the case of *ESR1* and *ESR2*, was also detected in CB-$CD34^+$ from different donors by RT-PCR (supplementary Figure 2D). Consequently, human HSPCs might respond to natural estrogens through any of the estrogen receptors.

Natural estrogens modified human HSPCs in vitro

Once demonstrated that both estrogen receptors were expressed in HSPCs, we wanted to investigate a potential direct effect of estrogens on human HSPCs. We cultured CB-$CD34^+$ for four days with a range of concentrations, from 10nM to 500µM, of the four natural estrogens (E1, E2, E3 and E4). As shown in Figure 3A, E1 and E3 reduced the expansion of the cells in culture practically at any used concentration. On the other hand, the lowest concentrations of E2 or E4 promoted the expansion of these cells, but they impaired the cell growth at high doses. A similar behavior was detected when different subpopulations of $hCD34^+$ cells were analyzed (Figure 3B and supplemental Figure 3A-E). E1 prevented the expansion of $hCD34^+hCD38^-$ cells (supplemental Figure 3C), MLPs (supplemental Figure 3D), Multipotent Progenitors (MPPs, $hCD34^+hCD38^-hCD90^-hCD45RA^-$, supplemental Figure 3E) and most primitive HSCs ($hCD34^+hCD38^-hCD90^+hCD45RA^-$, Figure 3B). The data from the rest of tested estrogens showed an apparent amplification of these primitive populations at low concentrations, but they were toxic at the highest concentrations

(Figure 3B; supplemental Figure 3C-E). It is important to highlight that the best tolerated estrogen was E4. Concentrations up to 10μM of E4 seemed not to be detrimental to any of these HSPC subsets, including primitive HSCs. On the contrary, E2 induced apoptosis in HSPCs at high doses (supplemental Figure 3F-G), as previously described for this estrogen and tamoxifen[24, 27]. However, only human HSPCs cultured in presence of the highest concentration of E4 showed some induction of apoptosis. Furthermore, we analyzed the cell cycle of CB-CD34^{+} cells after 4-day culture in presence of 100nM E2 or E4. Estrogens, mainly E4, induced an increment of cells in G2/M phase (Figure 3C and supplemental Figure 3H), which might explain the tendency of these two estrogens to expand human hematopoietic progenitors.

Previously, E2 was described to have a positive role in enhancing both CB-CD34^{+} proliferation and in vitro hematopoietic progenitor potential after more than week in vitro treatment[28]. Hence, we cultured human HSPCs in presence of the lowest and best tolerated doses of E2 or E4 for eight days. We could detect a significant expansion of human progenitors with E4 with all the tested concentrations (Figure 3D). In addition, a similar effect was identified with 100nM E2. Furthermore, the better tolerance of E4 over E2 was also confirmed, since all the tested concentrations of E4 were non-toxic for CB-CD34^{+} cells (Figure 3D). Additionally, the *in vitro* functionality of the estrogen-treated HSPCs was assessed with Colony Forming Unit (CFU) assays. We did not observe any differences among the groups in CFU numbers or CFU types (supplemental Figure 3I).

In order to assess which estrogen receptor was involved in the role of these molecules in human HSPCs, cell cycle of CB-CD34^{+} was determined in presence of these two estrogens together with either ESR1 antagonist (MPP), ESR2 antagonist (PHTPP) or GPER1 antagonist (G-15). The treatment with E2 or E4 alone tended to increase the percentage of cells in S/G2/M-phase as previously described; however, the addition of ESR2 antagonist seemed to block the increase of cells in S/G2/M-phase induced by E4 (Supplemental Figure 3J); less clearly, ESR1 and GPER1 antagonists seemed to reduce the number of cells in

S/G2/M-phase in E2-treated HSPCs. Moreover, expression of ESR1 and ESR2 were assessed in human HSPCs cultured with estrogens for four days by immunofluorescence analysis (supplemental Figure 3K-L). While ESR1 fluorescence intensity increased slightly but significantly with 100nM of E2 or E4 (supplemental Figure 3K and supplemental Figure 3M), ESR2 expression was significantly increased in presence of both E2 and E4 (supplemental Figure 3L and supplemental Figure 3N). Moreover, estrogen treatment extended the percentage of human HSPCs showing a polarized localization of ESR1 at the membrane (supplemental Figure 3K and supplemental Figure 3O). Furthermore, the treatment with estrogens enhanced the percentage of human $CD34^+$ with cytoplasmic localization of ESR2 (supplemental Figure 3L and supplemental Figure 3P).

Altogether, the data indicate natural estrogens regulate human HSPCs through the signaling of estrogen receptors.

E2 and E4 increased the number of human HSPCs in an in vitro model of human hematopoietic niche

Subsequently, we investigated the indirect effect of E2 and E4 on HSPCs in an *in vitro* model of human hematopoietic niche. CB-$CD34^+$ cells were co-cultured on an irradiated human BM-MSC layer in the presence of 100nM or 1μM of E2 and E4 (supplemental Figure 4A). We analyzed the expansion of the hematopoietic cells in two ways: i) after a week of co-culture (Figure 4), or ii) after four weeks of co-culture with the estrogen present only during the first week (supplemental Figure 4C-D). From 10nM to 1μM E4 and the lowest concentration of E2 increased the hematopoietic cells in the culture in the first week of co-culture (Figure 4A). Likewise, the number of $hCD34^+$ cells in the co-culture was significantly higher in E4 and 10nM E2 treated conditions than in the control group (Figure 4B; supplemental Figure 4B). However, we could not detect significant differences in the functionality of those $hCD34^+$ cells in CFU assays (Figure 4C). Additionally, the effect of these two estrogens on the expansion of human hematopoietic cells or $hCD34^+$ cells seemed not to be enhanced after four weeks in co-culture with an initial single dose

(supplemental Figure 4C-D). Consequently, the positive effect of E2 and E4 on HSPCs also occurs in an *in vitro* model of human hematopoietic niche.

E2 and E4 boosted human hematopoietic engraftment in immunodeficient mice

To better evaluate the impact of E2 and E4 on the properties of HSPCs, we transplanted $5x10^4$ human CB-$CD34^+$ cells into sub-lethally irradiated male NSG mice, in order to avoid any additional effects of endogenous estrogens of female recipient mice, and three days later the animals were treated with vehicle or with daily low doses of either E2 or E4 (2μg of estrogen per day) for four days (Figure 5A). Human hematopoietic engraftment was evaluated in the mouse BM by FACS analysis four month post-transplant (mpt, supplemental Figure 5A). Surprisingly, the human hematopoietic contribution was significantly higher in the estrogen-treated animals than in vehicle-treated ones (Figure 5B; supplemental Figure 5A). Additionally, none of the estrogens altered the normal distribution of human hematopoietic lineages within the $hCD45^+$ population (supplemental Figure 5B-5D). More importantly, E4 administration significantly enhanced human $CD34^+$ population in male NSG mice (Figure 5C). No increase in the presence of the more primitive compartment, $hCD34^+hCD38^-$, was observed (Figure 5D). To explore the impact of the estrogen treatment in the long-term HSC (LT-HSC), secondary transplants were performed. One million $hCD45^+$ cells, purified from the bone marrow of the primary recipients, were transplanted into sub-lethally irradiated female NSG mice. As shown in Figure 5E, the estrogen-treated human hematopoietic cells maintained their long-term engraftment potential without any observable problem in the human hematopoietic reconstitution or any abnormal proliferation. This led us to conclude that these two estrogens, mainly E4, enhance *in vivo* human hematopoietic engraftment in male immunodeficient mice.

To study this finding more in-depth, we transplanted limited numbers of human HSPCs ($5x10^3$ CB-$CD34^+$ cells/mouse), into male NSG mice, which were treated subsequently with vehicle, E2 or E4 as previously described. The percentage of mice positive for human

engraftment, defined as percentage of $hCD45^+$ cells greater than 0.1% of the cells in the mouse BM 4mpt, tended to increase after estrogen treatment (supplemental Figure 5E). Even more, the human hematopoietic chimerism of the positive animals seemed to be higher in the group treated with E4 than in the vehicle group (supplemental Figure 5F). So, E2 and E4 might be able to improve the engraftment of human HSPCs even when a very limited number of cells were transplanted.

To explore if the engraftment enhancement mediated by estrogens occurred in female recipients as well, we repeated the transplant of this very low number of CB-$CD34^+$ into sub-lethally irradiated female NSG nice. As shown in supplemental Figure 5G, human engraftment 4mpt tended to increase in the female animals treated with either of the two estrogens, although there was no significant difference among the groups. The percentage of hematopoietic progenitors within the human population did not show larger differences between vehicle- and estrogen-treated animals (supplemental Figure 5H). Consequently, there is no clear effect of E2 or E4 in the engraftment of human HSPCs into female animals.

E4 impacts on MSCs within the mouse hematopoietic niche

To provide a further insight into the positive impact of estrogens on promoting human hematopoietic engraftment, we assessed whether estrogens act *in vivo* on human HSPCs to promote hematopoietic engraftment directly or indirectly through niche cells. Thus, $5x10^4$ CB-$CD34^+$ cells were cultured with 100nM of E2 or E4 for 4 days and the resulting cells after the culture were transplanted in NGS mice. As it is shown in Figure 6A, human engraftment of *in vitro* estrogen-treated HSPCs was lower than vehicle-treated cells, which might indicate an indirect mechanism of estrogens to enhance hematopoietic engraftment in NSG mice. Additionally, there was no difference in the percentage of lymphoid, myeloid or HSPC subpopulations among mice in the different groups (supplemental Figure 6A). To understand in depth the difference in engraftment between *in vitro* estrogen-treated HSPCs and in vivo effect of estrogens after HSCT, $5x10^4$ CB-$CD34^+$

cells were co-cultured with human irradiated BM-MSCs in presence of 100nM of E2 or E4 for a week, then the resulting cells were transplanted into sublethally irradiated NSG mice. Human engraftment and lineage distribution were similar among mice of the different groups (Figure 6B, supplemental Figure 6B), which indicated the loss of engraftment ability due to the *in vitro* estrogen-mediated expansion which might be offset by the BM-MSCs. Next, we examined the contribution of the hematopoietic niche to the engraftment of human HSPCs after in vivo estrogen-treatment. Therefore, we analyzed the mesenchymal and vascular endothelial compartments of the mouse BM niche four months after being transplanted and treated with E2 or E4. The percentage of mouse MSCs (mCD140a$^+$, also called Pdgfra$^+$) and vascular endothelial cells (mCD144$^+$, also called VE-Cadherin$^+$) was analyzed in the non-hematopoietic compartment (supplemental Figure 6C). Surprisingly, compartment of mCD140a$^+$ cells, but not mCD144$^+$ cells, was increased in the mice treated with E4 in comparison with vehicle-treated animals (Figure 6C-D). To deepen on this point, mice were sublethally irradiated and treated with estrogens without human HSPCs transplantation. Surprisingly, there were more nucleated cells in the BM of mice treated with E4 (supplementary Figure 6F). Those mouse BM cells were cultured to study their ability to form fibroblast colony-forming units (CFU-Fs). We identified a tendency to have more CFU-Fs in the BM from estrogen-treated mice than vehicle-treated mice (Figure 6E and supplemental Figure 6G), which might indicate the beneficial role of estrogens to ameliorate BM niche after irradiation.

Then, we evaluated whether human mesenchymal stromal cells might interact with these estrogens. So, the expression of *ESR1* and *ESR2* was also analyzed in the human BM-MSCs compartment by RT-PCR (Figure 6F) and immunofluorescence (Figure 6G; supplemental Figure 6H). Both estrogen receptors were present in human BM-MSCs, indicating that the presence of estrogens could influence the behavior of these human stromal cells and affect indirectly the biology and/or the engraftment of humans HSPCs. To investigate the effect of estrogens on human BM-MSCs, a limiting number of human BM-MSCs were seeded and treated with estrogens and their CFU-F potential was assessed. As it is shown

in supplemental Figure 6I, the estrogens had no effect on human BM-MSC; nevertheless, CFU-F numbers dropped when those human BM-MSCs had been irradiated previously. However, we could observe an increase in the number of CFU-Fs when the BM-MSCs were treated with estrogens after irradiation (supplemental Figure 6I). Altogether, estrogens, and more importantly E4, might facilitate and favor the hematopoietic engraftment of human progenitors through enhancing mesenchymal compartment of the hematopoietic niche, in addition to its direct effect on HSPCs.

Discussion

The present study describes the potential use of estrogens to modify human HSPCs engraftment in BM upon transplantation. On the basis of the differences in the level of human hematopoietic engraftment between female and male recipient mice (Figure 1 and supplemental Figure 1), and the expression of estrogen receptors in different subsets of human HSPCs (Figure 2 and supplemental Figure 2), we have explored the impact of estrogen treatment on hematopoietic cells engraftment. E2 and E4 showed a positive effect on the expansion of these cells *in vitro* by activating cell cycle (Figure 3 and supplemental Figure 3). Additionally, E4 was better tolerated than E2 (Figure 3 and supplemental Figure 3). Despite the modest role of these estrogens in modulating human progenitor activity *in vitro*, we found E2, and even more E4, able to boost human hematopoietic engraftment in immunodeficient mice (Figure 5 and supplemental Figure 5). This better performance of human HSPCs in estrogen-treated animals might reflect the gender differences observed. Furthermore, an apparent expansion of the mouse mesenchymal stromal compartment was identified in the animals treated with E4, which may suggest an additional indirect regulation of the estrogens enhancing human hematopoietic engraftment through niche regulation (Figure 6 and supplemental Figure 6). Thus, estrogens could act directly on HSPCs as well as indirectly, throughout the modification of the BM stroma, or BM niche, to enhance $CD34^+$ cell engraftment. These results might be clinically relevant, since the use of E4 could facilitate HSPC transplantation when only a limited number of cells can be infused.

We have shown that estrogens improve human engraftment in immunodeficient mice, which reinforces the role of sex hormones in HSPC regulation and might explain the superior performance of female mice as recipients of a hematopoietic transplant[26] (Figure 1). The importance of estrogens in regulating HSPC functions has been explored for a long time, without any clear conclusion. E3 has been found to trap mouse hematopoietic progenitors in the liver[29]. E2 has been described as promoting the cell proliferation of very primitive mouse HSCs. The increase of estrogen levels during pregnancy has also been

associated to a greater HSC division, higher HSC frequency and an increase in erythropoiesis[19, 27]. E2 has also been reported to expand human CB-CD34$^+$ *in vitro*[28]. These data contrasts with those described by Illing et al in mice, previously, where a long-term treatment with E2 stimulated mouse HSPCs in the vascular but not in the endosteal niche, impairing long term reconstitution potential[18]. On the other hand, high doses of E2 suppressed hematopoiesis in mouse bone marrow[30]. This negative effect caused by estrogens has also been pointed out in tamoxifen treatment, which increased mouse HSC proliferation, but not self-renewal, and induced apoptosis in ST-HSCs and MPPs[24]. Here, we describe human HSPCs as having different sensitivities to any of the four natural estrogens. E1, and to a lesser extent E3 and E2 were toxic for human HSPCs (Figure 3; supplemental Figure 3). In contrast, E4 was better tolerated and was able to promote some degree of expansion of the human HSPCs by activating their cell cycle and a lower induction of apoptosis (Figures 3-4; supplemental Figure 3-4). These observations might explain the apparently divergent effects described previously for the different estrogens investigated, since different doses and different estrogens have been used in the above-mentioned reports.

Additionally, we observed that E2 or E4 treatment *in vivo* enhanced human hematopoietic engraftment in male mice transplanted with $5x10^4$ human HSPCs (Figure 5B and supplemental Figure 5), but only to a minor degree in animals transplanted with very limited number of CB-CD34$^+$ (supplemental Figure 5E-G). The apparent lack of effectiveness in female mice might be due to the presence of endogenous estrogens in these female animals. Future experiments should be done with ovariectomized mice or taking the estrous cycle of female recipients into account to identify the real effect on HSPCs in female recipients. Furthermore, E4 treatment enlarged hCD34$^+$ population in the already boosted human hematopoietic engraftment, but its impact on the hCD34$^+$hCD38$^-$ and on secondary transplant was lower (Figure 5D-E). Nevertheless, although the percentage of hCD34$^+$hCD38$^-$ population was unmodified by the estrogen treatment, the

total cell number of this primitive population was enlarged since the human engraftment was higher in estrogen-treated mice (Figure 5B and 5D).

We have described a positive effect on human engraftment after E2 or E4 treatment, being the impact of E4 the most significant. It is important to note that E4 is synthesized exclusively by the human liver during pregnancy. It is detected at 9 weeks of pregnancy, reaches high levels in the second trimester, with steadily rising concentrations of E4 toward the end of pregnancy[31]. On the other hand, fetal liver is a hematopoietic organ during the last half of the gestation period. During the hematopoietic stage of the fetal liver, different signaling pathways are coordinated to promote both a massive expansion of HSCs through the activation of the HSC cell cycle and a massive production of erythroid cells. After birth, the HSCs migrate from the liver to the adult bone marrow, where the most primitive HSCs are largely quiescent[1, 32]. The concurrence of E4 synthesis in the fetal liver, when it is a hematopoietic organ, may suggest an indirect link between this estrogen and the expansion of human HSPCs during pregnancy. The association between estrogens and hematopoietic development has previously been described during zebrafish development[33], in mouse[19, 27] and in the hematopoietic differentiation of human iPSCs[28]. So estrogens have a clear impact on HSC emergence. Similarly, Oguro et at. demonstrated the coordination between E2 and 27-hydroxycholesterol to regulate hematopoiesis during pregnancy[27]. Consequently, we can hypothesize a likely role of E4 in modulating early human hematopoiesis during embryo development.

All previously reported observations could be attributed to an intricate regulation mediated by the estrogens[17]. The complexity of the estrogen signaling pathways starts with the existence of several estrogen receptors. Three of these receptors (ESR1, ESR2 and GPER[34]) are expressed in hematopoietic cells, but only ESR1 has been described to play a role in the regulation of HSCs[18, 19, 24]. A second level of complexity is that the expression of these estrogen receptors tends to differ among hematopoietic subpopulations[24] (Figure 2). Moreover, different estrogens vary in their binding affinity to different estrogen

receptors; for example, E2 shows 7 times higher affinity for ESR1 (inhibition constant, Ki=0.21nM) than for ESR2 (Ki=0.015nM), and E4 has 400 times higher affinity for ESR1 (Ki=4.9nM) than ESR2 (Ki=19nM)[35]. Even more, once the estrogen and receptor are bound, specific cell responses will be triggered by two different mechanisms: i) gene expression programs can be initiated through the estrogen nuclear signaling, or ii) the estrogens can act through membrane-initiated steroid signaling (MISS), which is a rapid extra-nuclear cellular response to the estrogen signal[15]. These two types of estrogen signaling also can explain the differences we have observed between E4 and E2, from their toxicity and expansion *in vitro* (Figure 3; supplemental Figure 3) to their *in vivo* effect (Figures 6). E4 uncouples the nuclear activation and MISS in contrast to E2 which does not[15]. For example, E4 works as an estrogen antagonist on breast cancer cells[31, 36]. Moreover, the lower affinity of E4 to estrogen receptors in comparison with E2 might suggest a very limited effect of E4 on HSPCs; however, E4 doses, whose effects on HSPCs were observed (Figures 3-4), were the same doses used by Abbot et al. in which ERE transcriptional activity could be detected[15]. What's more, since E4 lacks of MISS activity, it is likely that the impact of E2 and E4 on human HSPCs might be due to their nuclear signaling, with similar transcriptional output, but this point will have to be analyzed in depth. Additionally, the presence of E2 or E4 increased the levels of both ESR1 and ESR2 and their cellular localization. Furthermore, the increment of cells in S/G2/M-phase mediated by estrogens could be partially blocked by ESR1 and GPER antagonist in the case of E2, and by ESR2 antagonist in the case of E4 (supplemental Figure 3J). Consequently, different estrogen receptors in human HSPCs might be involved in the signaling triggered by E2 or E4; however, this point will require more in-depth study. More interestingly, E2 might activate estrogen receptor-mediated MISS, since a clear polarized location of the estrogen receptors in the cytoplasm membrane was found (supplemental Figure 30-P). On the other hand, E4 might activate nuclear estrogen signaling, since E4 is unable to induce MISS[15] and a clear increment of ESR2 in the cytoplasm was detected (supplemental Figure 3N). The consequences of ESR1 and ESR2 upregulation and localization should be explored in future experiments. Additionally, Oguro et al. [27] described two different ESR1 ligands,

such as E2 and 27-Hydroxycholesterol, which regulated HSPCs differentially during pregnancy. Both ESR1 ligands collaborated to induce HSC proliferation, mobilization and extramedullary hematopoiesis. In a similar way, E2 and E4 might collaborate together to impact differentially on human HSPCs.

We identified that the underlying mechanism mediated by estrogens is through the activation of cell cycle *in vitro*, as previously described, which promote the expansion of hematopoietic progenitors[19, 24]. However, estrogens might also cause different effects, such as the activation of telomerase activity to facilitate the expansion of HSPCs[20-22], or increase of unfolded protein response (UPR) to promote hematopoietic regeneration after a proteotoxic stress, such as irradiation[23, 37]. In our *in vivo* model, E2 or E4 might activate the gene signaling involved in the cell cycle[19, 24], telomerase[20-22] or UPR[23]; but these estrogens could also activate apoptosis[24, 27] when high doses are used (Figure 3 and supplemental Figure 3G-H). Surprisingly, this estrogen-mediated expansion observed *in vitro* was not enough to explain the improvement in human hematopoietic engraftment. Indeed, the in vitro proliferation of human HSPCs induced by the estrogens was counterproductive to the enhancement of hematopoietic engraftment (Figure 6A). This might be due to the reduction of long-term engraftment ability of cycling HSPCs [2, 38], and the decoupling of HSPC expansion and stem cell properties *in vitro*[39]. As HSC quiescence, self-renewal and differentiation is controlled through intrinsic HSC signaling and extrinsic niche signaling, we could observe that the co-culture of HSPCs with human BM-MSCs was able to expand hematopoietic cells (Figure 4) and maintain engraftment potential (Figure 6B), which indicates that *in vitro* expansion of HSPCs might be compensated by niche signaling. In accordance with this, estrogens could also modulate hematopoiesis by affecting MSC capacity to promote osteogenesis[30, 40]. Furthermore, this osteogenic differentiation might favor the proliferation of HSPCs[25]. The beneficial effect of E2 to expand both HSPCs and MSCs has been seen by Kitajima et al. previously[41]. As shown in Figure 6, an increase in MSCs was also detected in our *in vivo* model after E4 treatment. Besides, estrogen presence might favor the recovery of MSCs after irradiation (Figure 6E

and supplemental Figure 6E-G), as previously published for HSPCs[23, 37]. Consequently, the impact of estrogens on promoting human hematopoietic engraftment in immunodeficient mice might be mediated through the regeneration of the mesenchymal stromal cell compartment of the BM niche after irradiation, or a combined effect on human HSPCs (Figure 2-3) and niche cells (Figure 6). Consequently, we can hypothesize estrogens might coordinate HSPC proliferation and recovery of BM niche in a HSCT context

Based on the reported results, some significant clinical implications could be suggested. E4 has a safer therapeutic window than E2, which facilitates its clinical use[31]. Additionally, there are several clinical trials where E4 has been tested and its safety and efficacy have been assessed for different conditions, such as contraception[42], menopause[43], osteoporosis[44] or breast cancer[45]. Therefore, the clinical application of E4 to modulate HSPCs would be considered for improving HSPC transplantation in the near future. The potential clinical use of E4 might facilitate the transplantation of single CB units, the autologous transplant of gene therapy corrected HSPCs to treat inherited hematopoietic diseases or in any situation in which a reduced amount of HSPCs needs to be infused. The administration of a clinically approved estrogen, such as E4, after HSPCT could lead to an improvement in the overall hematopoietic engraftment of the transplanted patients.

Contribution

JCS, OQB conceived and designed the experiments. SFB, IO, FBA, SBM, JGM, MDR, RS, OA and OQB conducted the experiments. RY, YFS, DA, AZ provided reagents. AZ and JAB contributed with ideas. SFB, JCS and OQB wrote the manuscript.

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Conflict of interest

JAB and JCS are consultants for Rocket Pharmaceuticals (NY). The rest of the authors declare no competing interests.

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Figure Legends

Figure 1.

Human Hematopoietic Stem Progenitor Cells show a superior hematopoietic engraftment in female than in male NSG mice. (A) Representative flow cytometry analyses of human engraftment of $5x10^4$ umbilical cord blood $CD34^+$ ($CB\text{-}CD34^+$) cells into sub-lethally irradiated female (left panel) and male (right panel) NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice 4 months post transplantation. (B) Percentage of human hematopoietic cells, $hCD45^+$, in the bone marrow of female (F) or male (M) animals transplanted with $5x10^3$ or $5x10^4$ $CB\text{-}CD34^+$cells. Data are obtained from 6 independent biological replicates. Data are presented by dots and box-plots that represent the interquartile range (p75, upper edge; p25, lower edge; p50, midline; p95, line above the box; and p5, line below the box). Significance was analyzed by Mann–Whitney U test and represented by **P <0.01 and ****P<0.001.

Figure 2.

Human Hematopoietic Stem Progenitor Cells express estrogen receptors. (A) Representative immunofluorescent image of umbilical cord blood $CD34^+$ ($CB\text{-}CD34^+$) cells stained with anti-ESR1 (green), anti-hCD34 (red) and DAPI (blue). Insert, showing $ESR1^+$ $CD34^+$ cells (marked with arrows), is an enlargement of the white boxed area. (B) Representative immunofluorescent image of $CB\text{-}CD34^+$ cells stained with anti-ESR2 (green), anti-hCD34 (red) and DAPI (blue). Insert, showing $ESR2^+$ $CD34^+$ cells (marked with arrows), is an enlargement of the white boxed area. (C) qRT-PCR analysis of *ESR1* expression of sorted Hematopoietic Stem Cell/Multipotent Progenitors (HSC/MPPs, $hCD34^+hCD38^-hCD45RA^-$), Multilymphoid Progenitors (MLPs, $hCD34^+hCD38^-hCD45RA^+$) and committed Hematopoietic Progenitors ($hCD34^+hCD38^+$). (D) qRT-PCR analysis of *ESR2* expression of sorted HSC/MPPs, MLPs and Hematopoietic Progenitors ($hCD34^+hCD38^+$). Data are obtained from 3 biological replicates. Data are presented by mean±SD. Significance was analyzed by one-way Anova with Fisher's LSD test, no significant differences were found.

Figure 3.

Natural estrogens affect human Hematopoietic Stem Progenitor Cells differently. (A) Total cell number of the estrogen-treated Hematopoietic Stem Progenitor Cells (HSPCs) after 4 days in culture. Different concentrations (10nM, 100nM, 1μM, 10μM, 100μM and 500μM) of the natural estrogens (E1, E2, E3 and E4) were used. (B) Total number of the Hematopoietic Stem Cell (HSCs, $hCD34^{+}hCD38^{-}hCD90^{+}hCD45RA^{-}$) after 4 days in culture. Different concentrations (10nM, 100nM, 1μM, 10μM, 100μM and 500μM) of the natural estrogens (E1, E2, E3 and E4) were used. (C) Cell cycle analysis of HSPCs treated with 100nM E2 or E4. G0/G1-phase (left panel), S-phase (middle panel) and G2/M-phase (right panel). (D) Total cell number of the estrogen-treated HSPCs after 8 days in culture. Data are obtained from 3-5 biological replicates. Data are presented by mean±SD. Significance was analyzed by one-way Anova with Fisher's LSD test and represented by $^{*}P<0.05$ and $^{**}P <0.01$.

Figure 4.

E2 and E4 impact on Hematopoietic Stem Progenitor Cells in an *in vitro* model of human hematopoietic niche. (A) Total hematopoietic cells after 1 week of co-culture with human bone marrow mesenchymal stromal cells (BM-MSCs) in presence of estrogens. (B) Total $hCD34^{+}$cells after 1 week of co-culture with human BM-MSCs in presence of estrogens. (C) CFUs derived from Hematopoietic Stem Progenitor Cells (HSPCs) after 1 week of co-culture with human BM-MSCs in presence of estrogens. Data are obtained from 3-6 biological replicates. Data are presented by mean±SD. Significance was analyzed by one-way Anova with Fisher's LSD test and represented by $^{*}P<0.05$ and $^{**}P <0.01$.

Figure 5.

E2 and E4 enhance human engraftment in immunodeficient male mice. (A) Experimental scheme of human Hematopoietic Stem Progenitor Cells (HSPCs) into immunodeficient mice. Sub-lethally irradiated NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were transplanted

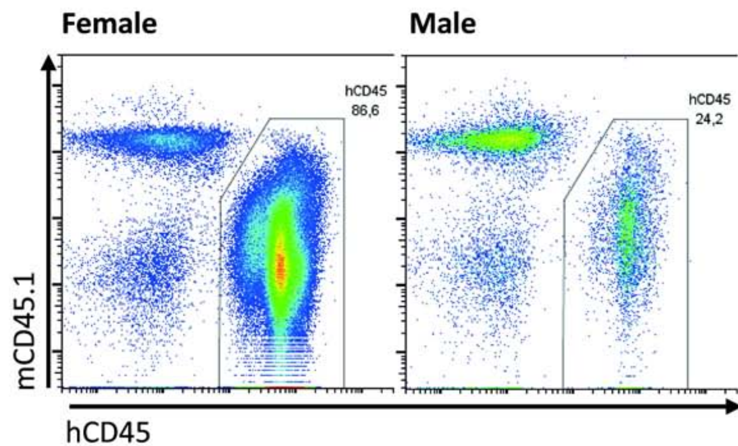
with human umbilical cord blood $CD34^+$ (CB-$CD34^+$) cells, and three days later the animals were treated with vehicle or with daily low doses of either E2 or E4 (2μg of estrogen per day) for four days. Four mpt the human hematopoietic engraftment was evaluated in the mouse bone marrow. (B) Percentage of $hCD45^+$ cells in the bone marrow (BM) of male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells 4 months post transplantation. (C) Percentage of $hCD34^+$ within the human population in the BM of male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells 4 months post transplantation. (D) Percentage of $hCD34^+hCD38^-$ within the human population in the BM of the transplanted male mice with $5x10^4$ hCB-$CD34^+$ cells. (E) Percentage of human engraftment ($hCD45^+$) in the BM of secondary NSG mice transplanted with $1x10^6$ sorted $hCD45^+$ cells from the primary recipients and analyzed 4 months post transplantation. Data were obtained from 4 biological replicates. Data are presented by dots and box-plots that represent the interquartile range (p75, upper edge; p25, lower edge; p50, midline; p95, line above the box; and p5, line below the box). Significance was analyzed by Mann–Whitney U test and represented by **P <0.01 and ****P<0.001.

Figure 6.

Estrogens modulate hematopoietic niche. (A) Human engraftment in bone marrow (BM) of male mice transplanted with the expanded cells from initial $5x10^4$ hCB-$CD34^+$ cells after 4 days in culture in presence of 100nM of E2 or E4. The human engraftment was analyzed 2 months post transplantation. (B) Human engraftment of in BM of male mice transplanted with the expanded cells from initial $5x10^4$ hCB-$CD34^+$ cells after 1 week in co-culture with irradiated human BM-MSCs in presence of 100nM of E2 or E4. The human engraftment was analyzed 3 months post transplantation. (C) Relative percentage of mouse mesenchymal stromal cells (MSCs, $mCD45^-Ter119^-hCD45^-hCD235a^-mCD140a^+$) in the BM of male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells analyzed 4mpt. (D) Relative mouse vascular endothelial cells ($mCD45^-Ter119^-hCD45^-hCD235a^-mCD144^+$) in the BM of the male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells analyzed 4mpt. (E) Number of fibroblast colony-forming units (CFU-Fs) derived from BM of vehicle- or estrogen-treated

mice after sublethal irradiation. (F) Representative agarose gel showing the PCR products of RT-PCR analysis of *ESR1* (top panel), *ESR2* (middle panel) and *HPRT1* (bottom panel) in human BM-MSCs. (G) Representative immunofluorescence image of human BM-MSCs stained with anti-ESR1 (green, left panel), anti-ESR2 (green, middle panel) or secondary antibody (green, right panel) and DAPI (blue). Data were obtained from 3 biological replicates. Data are presented by dots and box-plots that represent the interquartile range (p75, upper edge; p25, lower edge; p50, midline; p95, line above the box; and p5, line below the box). Significance was analyzed by Mann–Whitney U test and represented by **$P < 0.01$.

A



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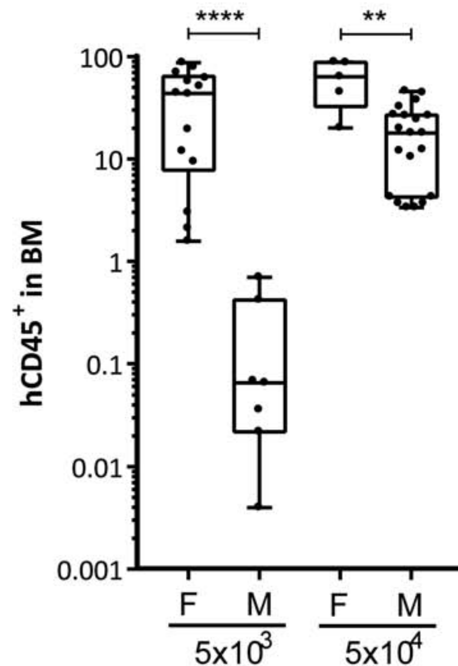

Figure 1
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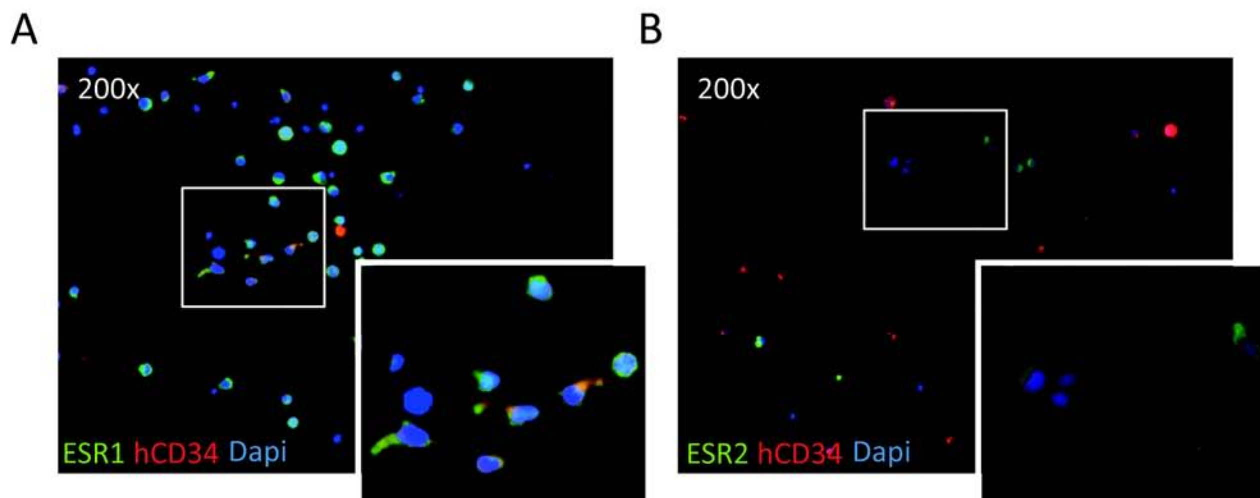

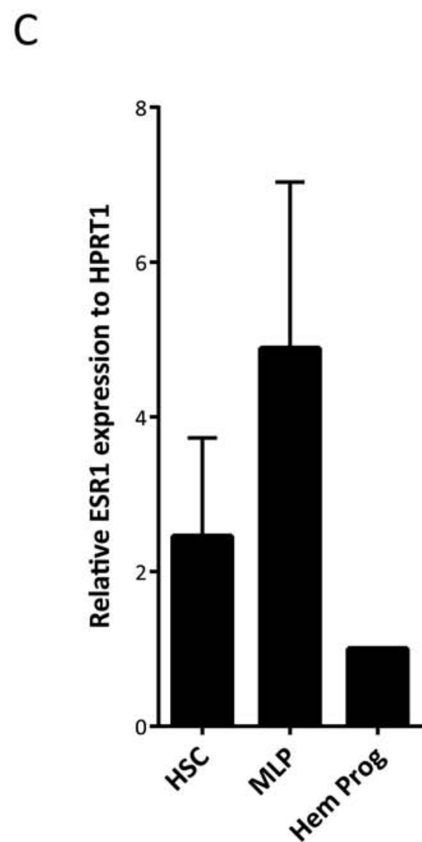

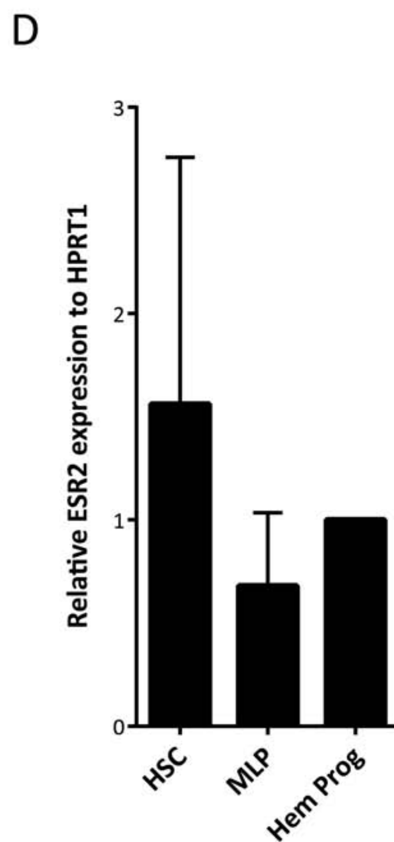

Figure 2
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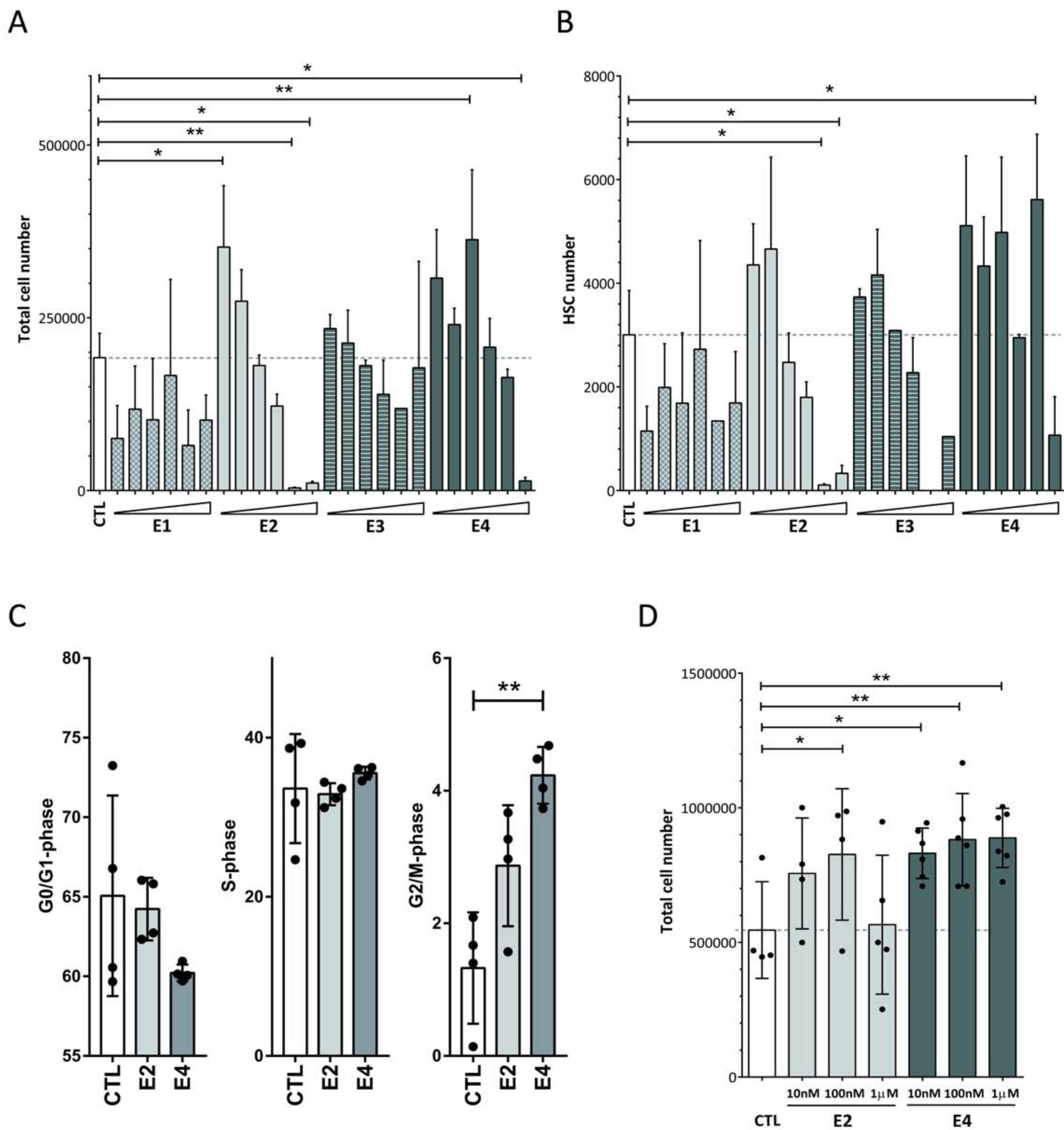

Figure 3

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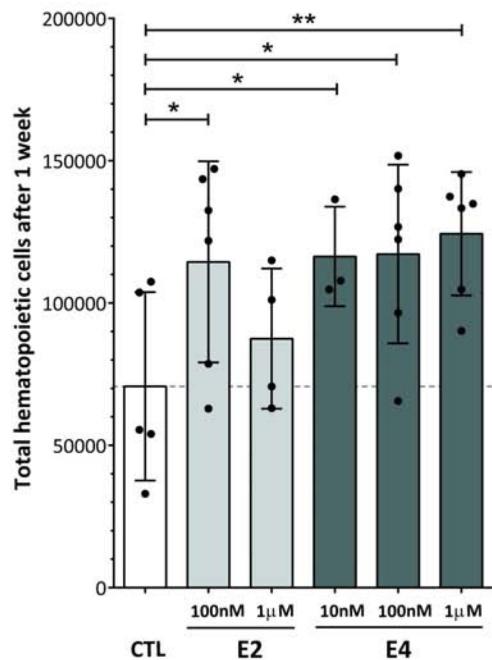

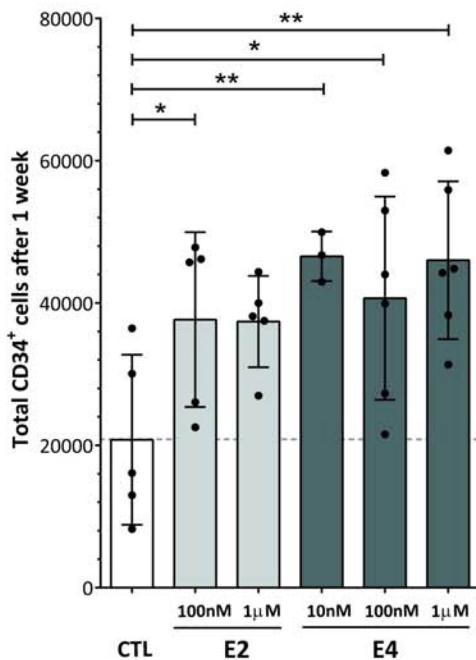

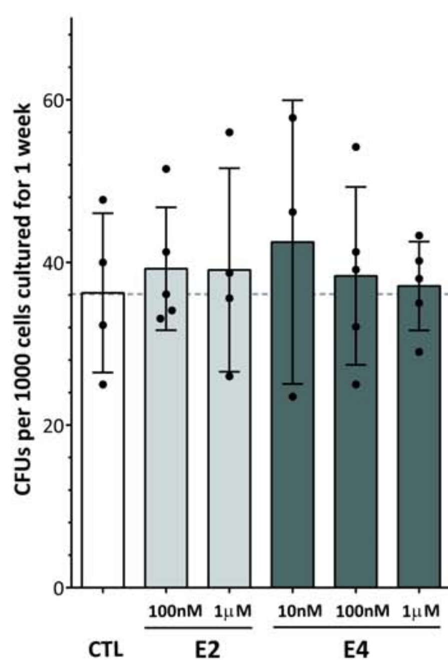

Figure 4
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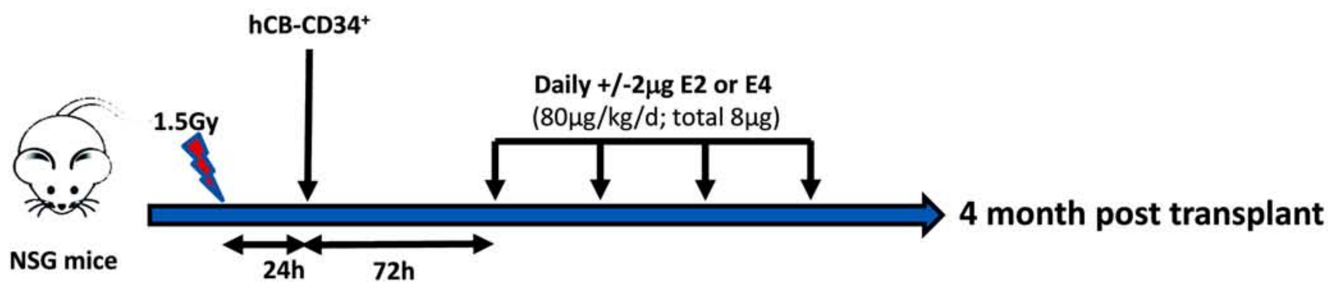

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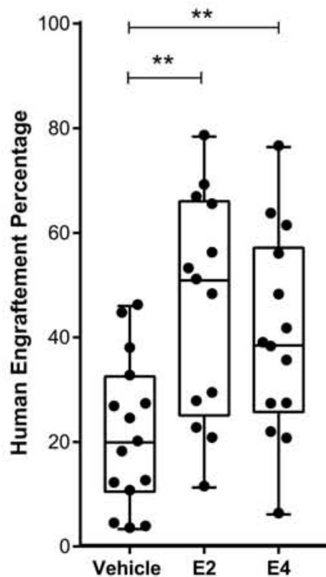

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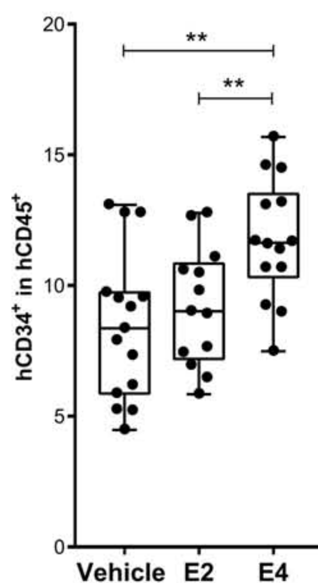

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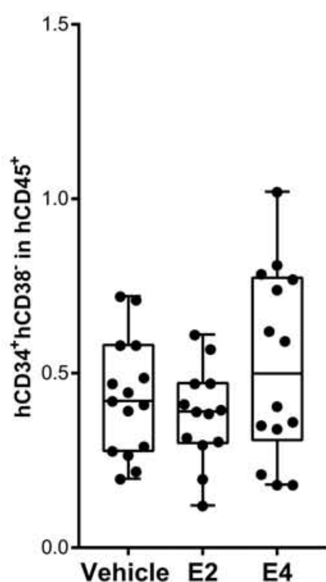


E

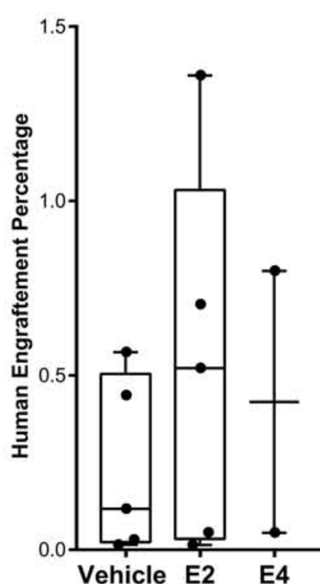


Figure 5

Fañanas-Baquero et al.

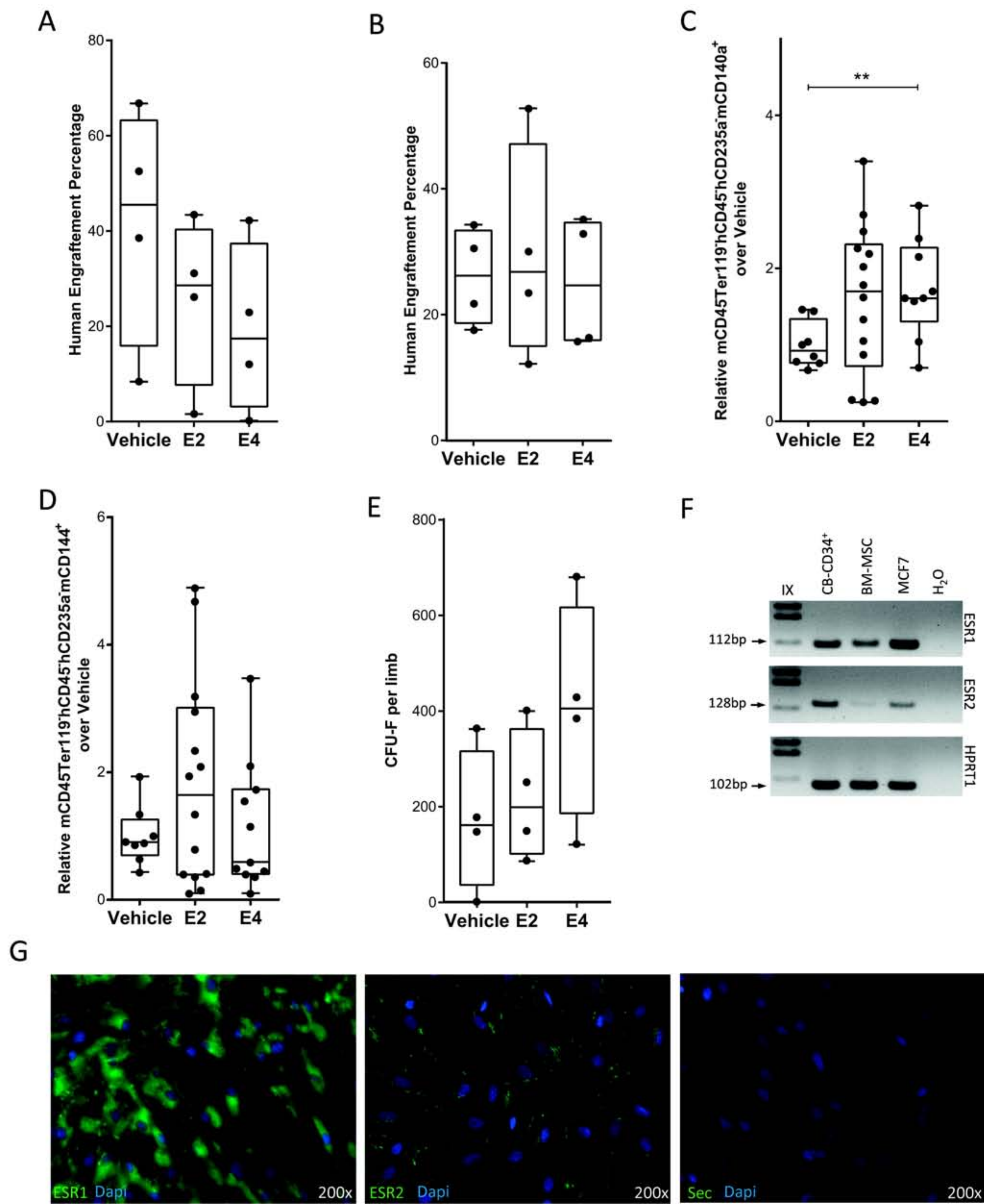


Figure 6
Fañanas-Baquero et al.

SUPPLEMENTAL METHODS

RT and qRT-PCR analysis

mRNA from whole or sorted subpopulations of CB-CD34$^+$ cells and human BM MSCs was purified by TRIzol™ reagent (ThermoFisher) protocol and retro-transcribed by SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher) to obtain cDNAs. qRT-PCR was carried out using Fast SYBR Green Master Mix (ThermoFisher). The specific primers to analyses: *ESR1* were as Forward (ATCCACCTGATGGCCAAG) and as Reverse (GCTCCATGCCTTTGTTACTCA), *ESR2* were as Forward (GATGCTTTGGTTTGGGTGAT) and as Reverse (AGTGTTTGAGAGGCCTTTTCTG) and *HPRT1* were as Forward (ATGATGGGGCTGATGTGG) and as Reverse (TTCTACGCATTTCCCCTCA). The relative expression of each estrogen receptor to *HPRT1* was calculated according Pfaffl's method[1]. Additionally, the specific size of the PCR products was verified in 2% agarose gel. *GPER1* expression was analyzed through RT-PCR using specific primers, as Forward (AAAACAAATTTGCCGGCCCT) and as Reverse (TGAACCTCACATCCGACTGC), and visualized in an agarose gel.

Immunofluorescence analysis

CB-CD34$^+$ cells and human BM-MSCs were cultured in Retronectin-treated chambers for one day. Then, they were fixed with 4% PFA for 10 minutes, blocked and permeabilized for 1 hour with PBS/1% BSA/10% FBS/0.3M Glycine/0.1% Tween20. Finally, the cells were stained by either rabbit anti-ESR1 (Abcam) or anti-ESR2 (Abcam) together with mouse anti-hCD34-PE (Becton Dickinson Pharmingen, BD) and then washed and stained by a secondary anti Rabbit-Alexa488 (Molecular Probes) and counterstained by 4',6-Diamidino-2-phenylindole (DAPI; Roche) to visualize cell nuclei. All the images were visualized in Axioplan 2 imaging (Zeiss) fluorescent microscope.

ESR1 and ESR2 fluorescence intensity and cellular localization were analyzed in immunofluorescence images acquired with the same exposure settings of CB-CD34$^+$ cells cultured for 4 days with CTL, 100nM E2, 1µM E2, 100nM E4 or 1µM E4. From 20 to 55

different HSPCs were analyzed per condition to calculate fluorescence intensity and cellular localization: 1) cells with a reduced cellular presence of ESR1 or ESR2, 2) cells with polarized localization of ESR1 or ESR2 at the membrane, 3) cells with a localization of ESR1 or ESR2 at the membrane and 4) cells with a cytoplasmic localization of ESR1 or ESR2. Immofluorescence analysis was performed with ImageJ 1.45 software (National Institutes of Health).

Estrogens

E1, E2, E3 and E4 (Sigma-Aldrich) were dissolved in ethanol for in vitro experiments or in olive oil for in vivo administration. Estrogen receptors antagonists, such as MPP (ESR1 antagonist), PHTPP (ESR2 antagonist) or G-15 (GPER1 antagonist), all of them from Tocris, were suspended in DMSO.

Human CB-CD34$^+$ culture

Purified CB-CD34$^+$ cells were cultured in serum-free X-Vivo 20 media (BioScience-Lonza) without phenol red and supplemented with 100ng/mL rSCF (EuroBioSciences), 100ng/mL FLT3L (EuroBioSciences), 100ng/mL TPO (Bio-Techne) and 0.5% Penicillin-Streptomycin (Thermofisher) at 5x10^5 cells/mL. The indicated estrogens concentrations were added in each condition. Viable cells were determined with Trypan blue. The human hematopoietic phenotype was analyzed by flow cytometry (LSR Fortessa; BD). The cells were stained with hCD34-PECy7 (BioLegend), hCD45RA-FITC (Beckman Coulter), hCD38-PE and hCD90-APC (BD). DAPI-positive cells were excluded from the analysis. Analysis was performed using FlowJo software. Colony-forming Unit (CFU) assay was performed in HSC-CFU media (StemCell Technologies), and 14 days after, the number and type of hematopoietic CFUs were analyzed. In some experiments, 4-day cultured cells were transplanted into sub-lethally irradiated NSG mice.

For Cell Cycle analyses, HSPCs were fixed and permeabilized with BD Cytofix/Cytoperm kit (Thermofisher) and the DNA was stained with DAPI. Cell cycle analyses were done using

Moffitt software. To investigate the effect of the different estrogen receptors antagonist, CB-CD34$^+$ cells were cultured as previously indicated adding MPP (1µM), PHTPP (1µM) or G-15 (3µM) for 4 days. Additionally, apoptosis of HSPCs was analyzed after 4 days in culture with different concentrations of E2 or E4, and the apoptotic cells were determined through flow cytometry with FITC Annexin V (BD Bioscience) and DAPI staining.

Additionally, co-culture experiments were carried out by adding 5x10^4 CB-CD34$^+$ cells on 30Gy irradiated BM-MSC layer. The cells were maintained in no phenol red MEM alpha with 15% Horse serum, 15% Hyclone^{TM} Fetal Bovine Serum and 0.5% Penicillin-Streptomycin (all from Thermofisher). Half of the media was refreshed every week. Different concentrations of estrogens were added at the beginning of the culture. After 1 or 4 weeks, the culture was tripsinized and cell number, human hematopoietic phenotype and CFU assay were performed as previously described. In some experiments, cultured cells were transplanted into sub-lethally irradiated NSG mice.

Fibroblast colony-forming unit assay

Male NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ (NSG) mice sub-lethally irradiated with 1.5 Gy. Three days later, the animals were treated with vehicle (olive oil) or with daily doses of either E2 or E4 (2µg of estrogen per day) intraperitoneally for four days. Two weeks after irradiation, mice were sacrificed and BM from long bones of these animals was collected. The long bones were flushed, cut in small pieces and crushed before being digested with 250µg/mL LiberaseDL (Roche) / 200U/mL DNaseI in HBSS at 37°C for 20 minutes. BM-MNCs were counted and 3x10^5 cells were seeded in Mesencult media (StemCell Technologies) for 1 month. The Colony-Forming Cells (CFCs) were stained with hematoxylin and quantified under a stereo microscope.

Human CFCs were generated after seeding 1000 human BM-MSCs from different healthy donors. One day after, the human BM-MSCs were irradiated with 10Gy or 20Gy or non-irradiated (0Gy) and cultured in no phenol red MEM alpha (Thermofisher) supplemented

with 5% platelet lysate (Cook medical), 1% penicillin/streptomycin (Thermofisher). After two weeks, CFCs were stained and quantified as previously mentioned.

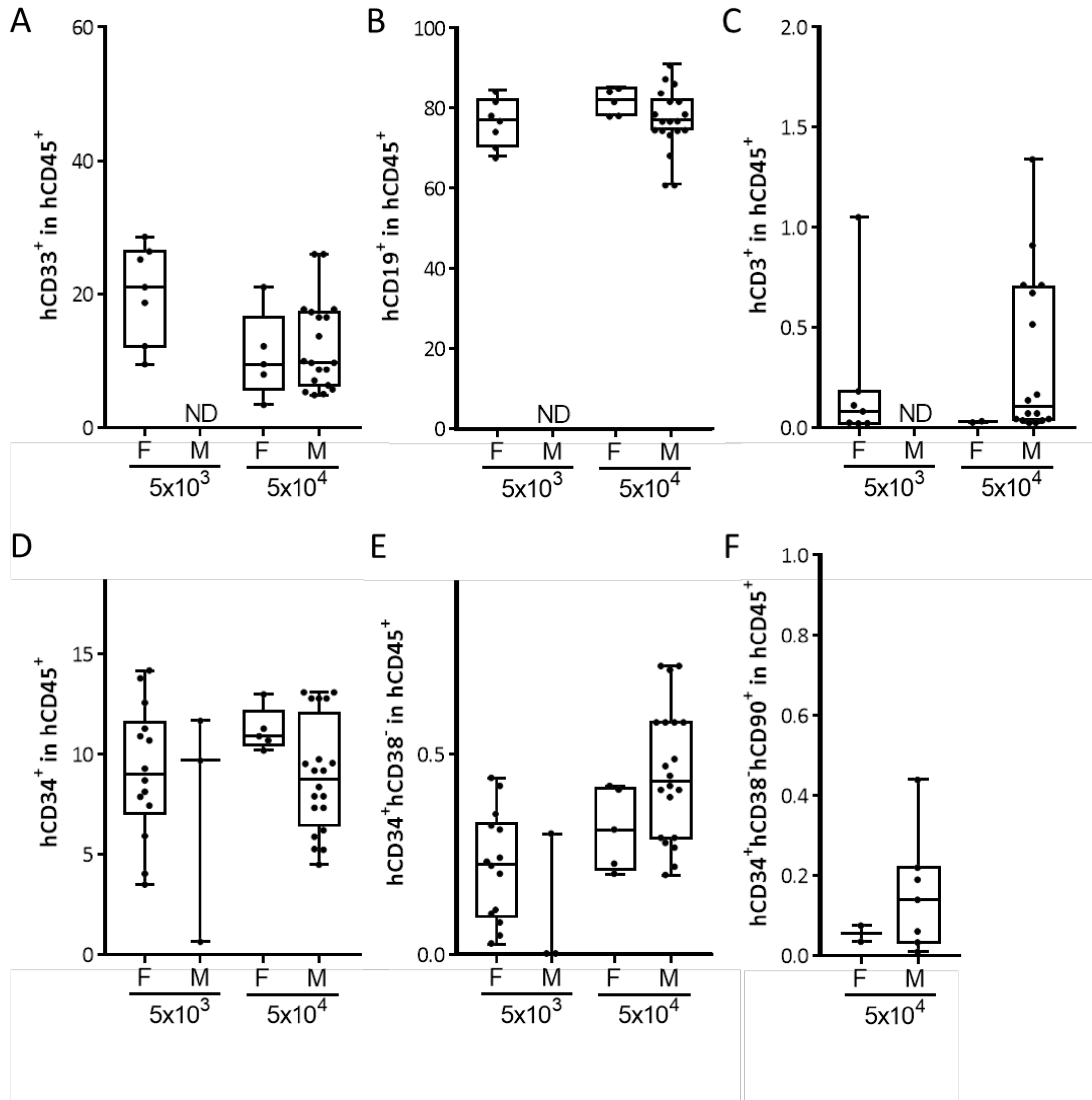
Statistical analysis

Statistical significance was determined using 1-way Anova with Fisher's LSD test, Fisher's exact test or nonparametric Mann–Whitney U test or with GraphPad Prism 7. The mean±SD or dots and box-plots, which represent the interquartile range (p75, upper edge; p25, lower edge; p50, midline; p95, line above the box; and p5, line below the box), are shown in each graph. Additionally, the significance was represented by P-values: *P<0.05, **P<0.01, ***P<0.005 and ****P<0.001.

SUPPLEMENTAL REFERENCES

1. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45.

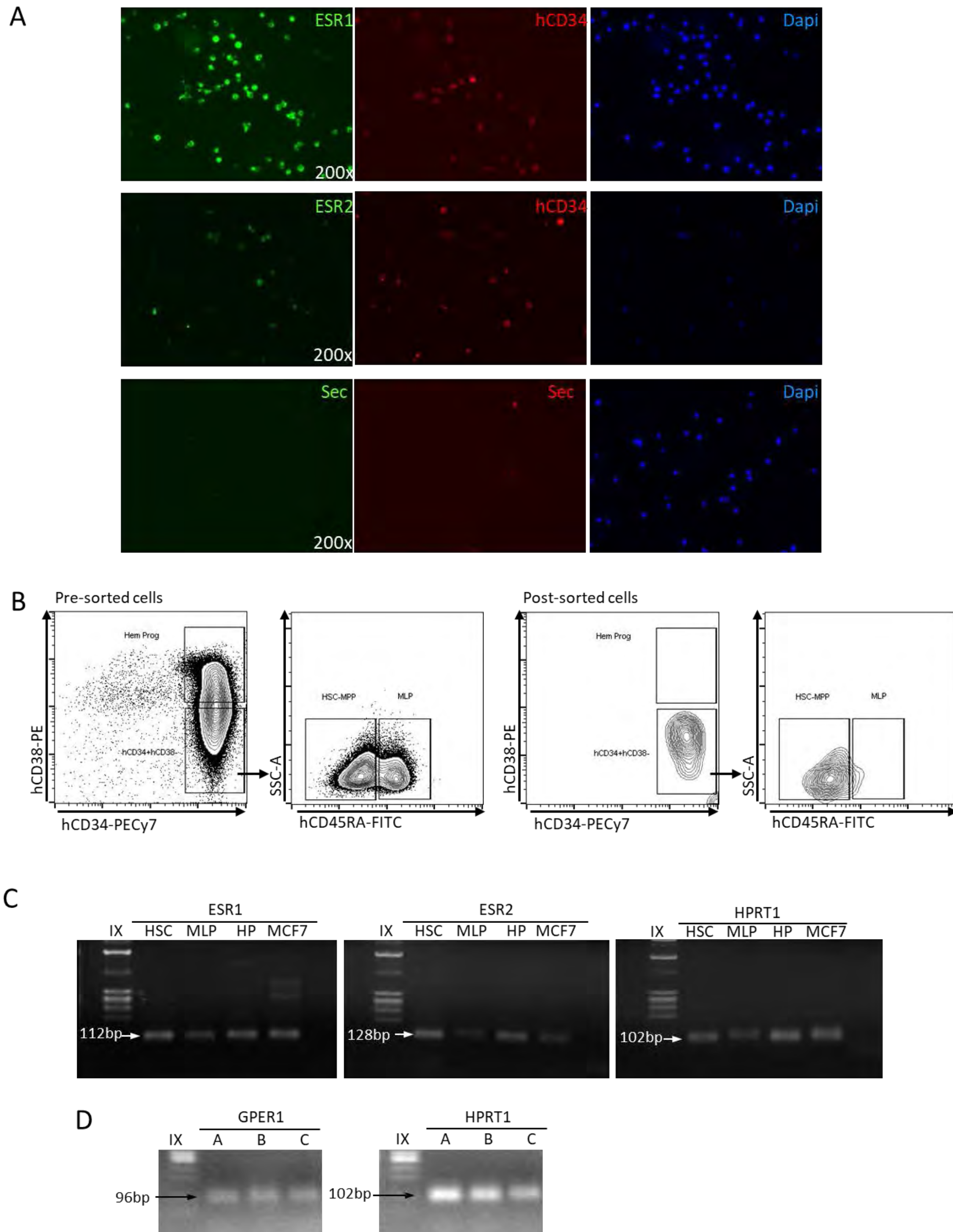
SUPPLEMENTAL FIGURES



Supplemental Figure 1.

Human hematopoietic cell lineages distribution is maintained in engrafted female or male mice. (A) Percentage of myeloid cells ($hCD33^+$) within human hematopoietic population engrafted in female (F) or male (M) NSG mice transplanted with $5x10^3$ or $5x10^4$ CB-$CD34^+$ cells. (B) Percentage of B-cells ($hCD19^+$) within human hematopoietic population. (C) Percentage of T-cells ($hCD3^+$) within human hematopoietic population. (D) Percentage of hematopoietic progenitors ($hCD34^+$) within human hematopoietic population. (E) Percentage of primitive hematopoietic progenitors ($hCD34^+hCD38^-$) within

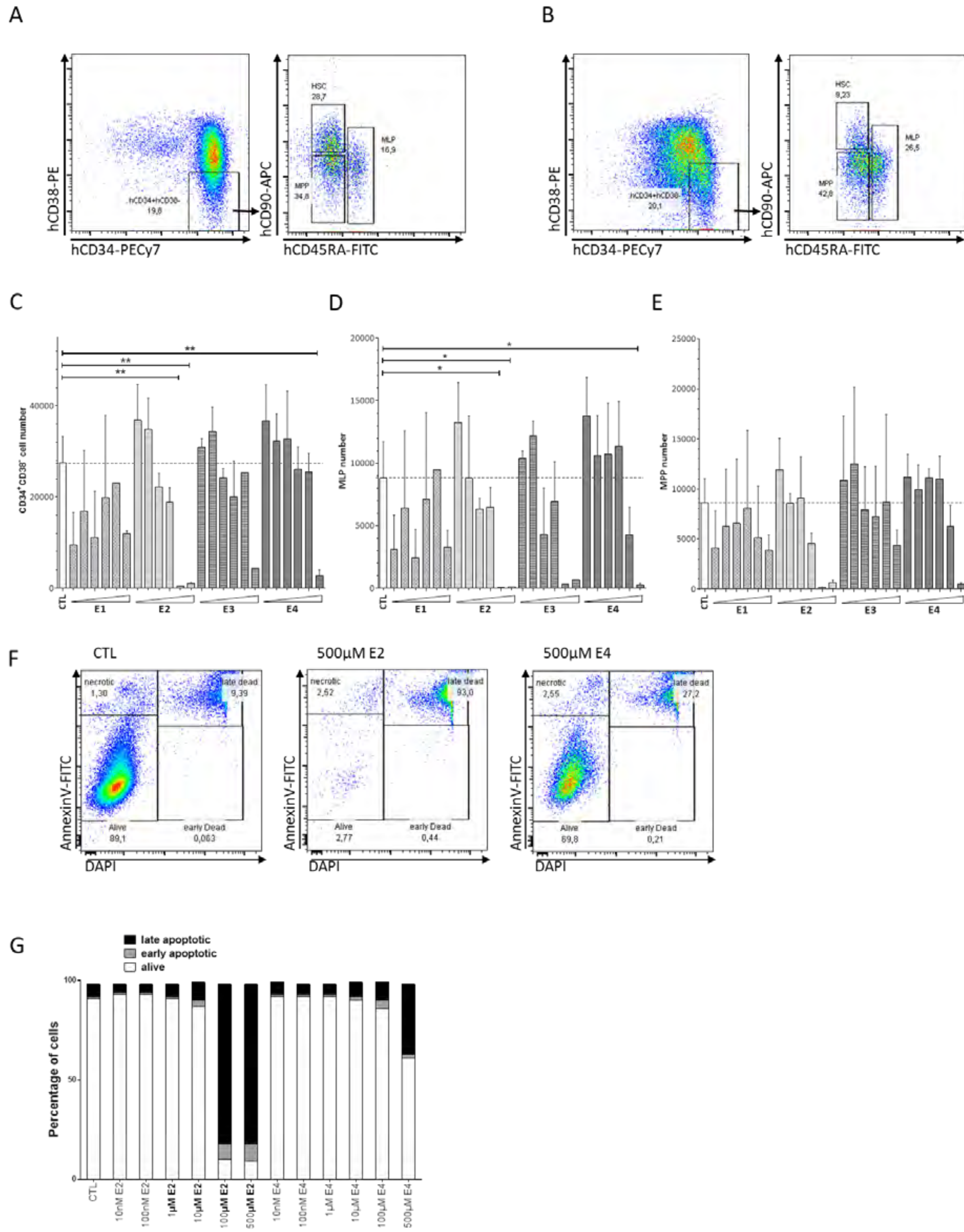
human hematopoietic population. (F) Percentage of HSCs (hCD34$^+$hCD38$^-$hCD90$^+$) within human hematopoietic population. ND no detected. Data are obtained from 6 independent biological replicates. Data are presented by dots and box-plots that represent the interquartile range (p75, upper edge; p25, lower edge; p50, midline; p95, line above the box; and p5, line below the box). Significance was analyzed by Mann–Whitney U test, no significant differences were found.



Supplemental Figure 2.

Human HSPCs express estrogen receptors. (A) Single channels of the immonufluorescent images shown in Figure 2A-B. CB-CD34^{+} cells were stained with anti-ESR1 (top row, green)

or anti-ESR2 (middle row, green) or only secondary antibody (bottom row, green), and anti-hCD34 (top and middle row, red) or with the specific IgG (bottom row, red), and DAPI (blue). (B) Representative cell sorting strategy to purify the different subsets of CB-CD34^{+} cells: HSC/MPPs (hCD34^{+}hCD38^{-}hCD45RA^{-}), MLPs (hCD34^{+}hCD38^{-}hCD45RA^{+}) and committed Hematopoietic Progenitors (hCD34^{+}hCD38^{+}). Pre-sorted cells and post-sorted HSC-MPP cells are shown at left and right panels respectively. (C) Representative agarose gel showing the PCR products of qRT-PCR analysis of the Figure 2C-D, *ESR1* (left panel), *ESR2* (middle panel) and *HPRT1* (right panel). Human breast cancer line MCF7 was used as control. (D) Representative agarose gel showing the PCR products of RT-PCR analysis of *GPER1* (left panel) and *HPRT1* (right panel) of three different CB-CD34^{+} samples. Significance was analyzed by one-way Anova with Fisher's LSD test. Data are obtained from 3 biological replicates (A-B-C).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HSC
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MLP
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E2
E3
E4
F
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early Dead
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G
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early apoptotic
alive
Percentage of cells
CTL
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100nM E2
1µM E2
10µM E2
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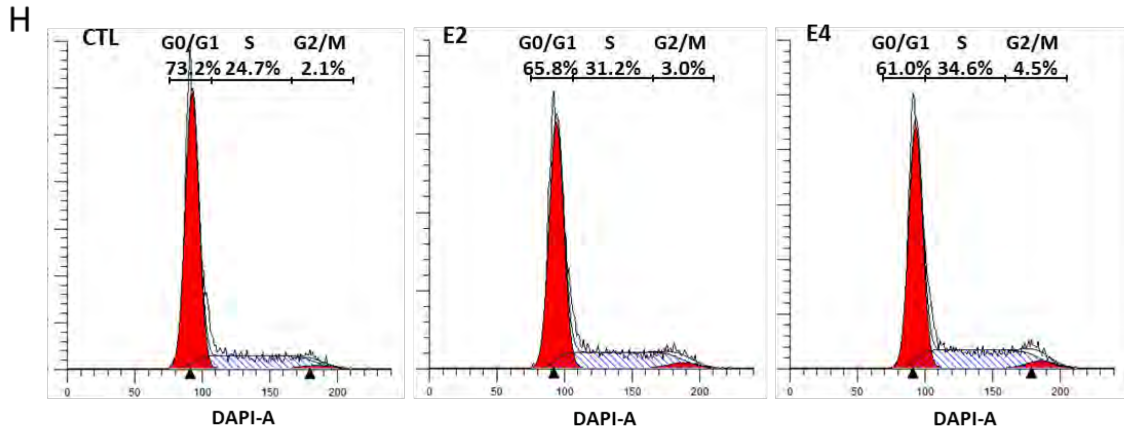

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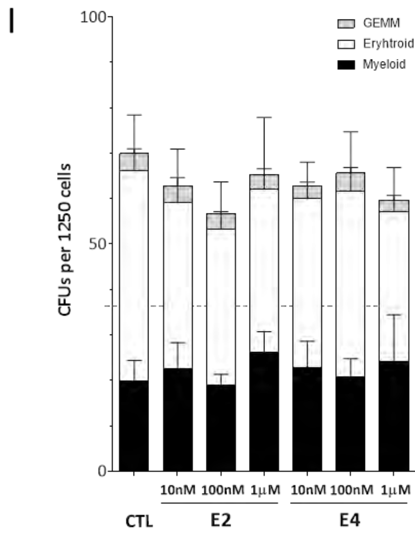

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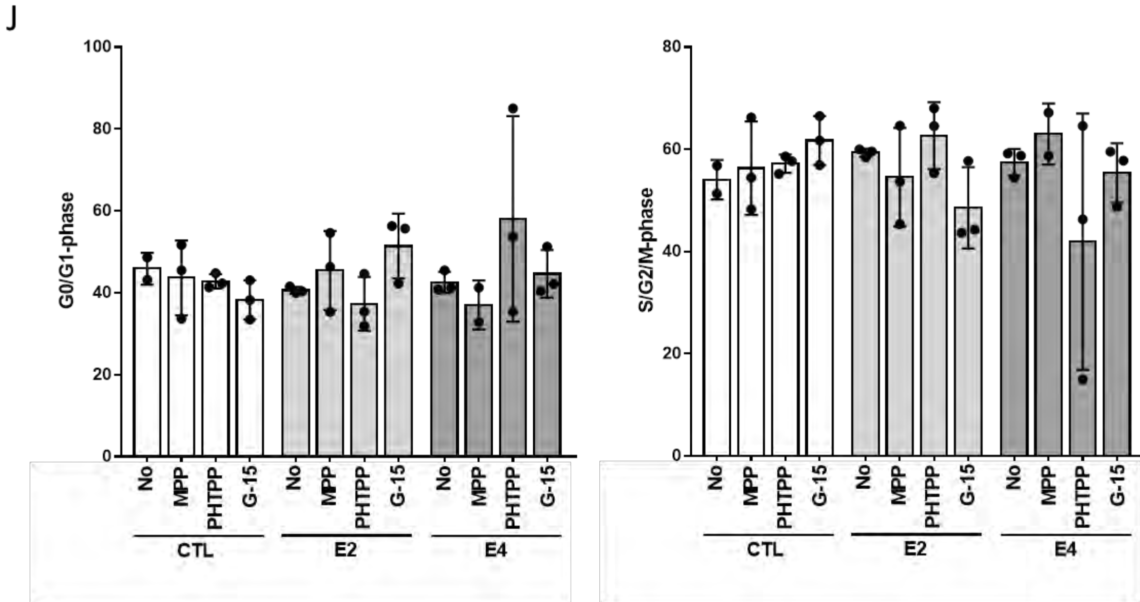

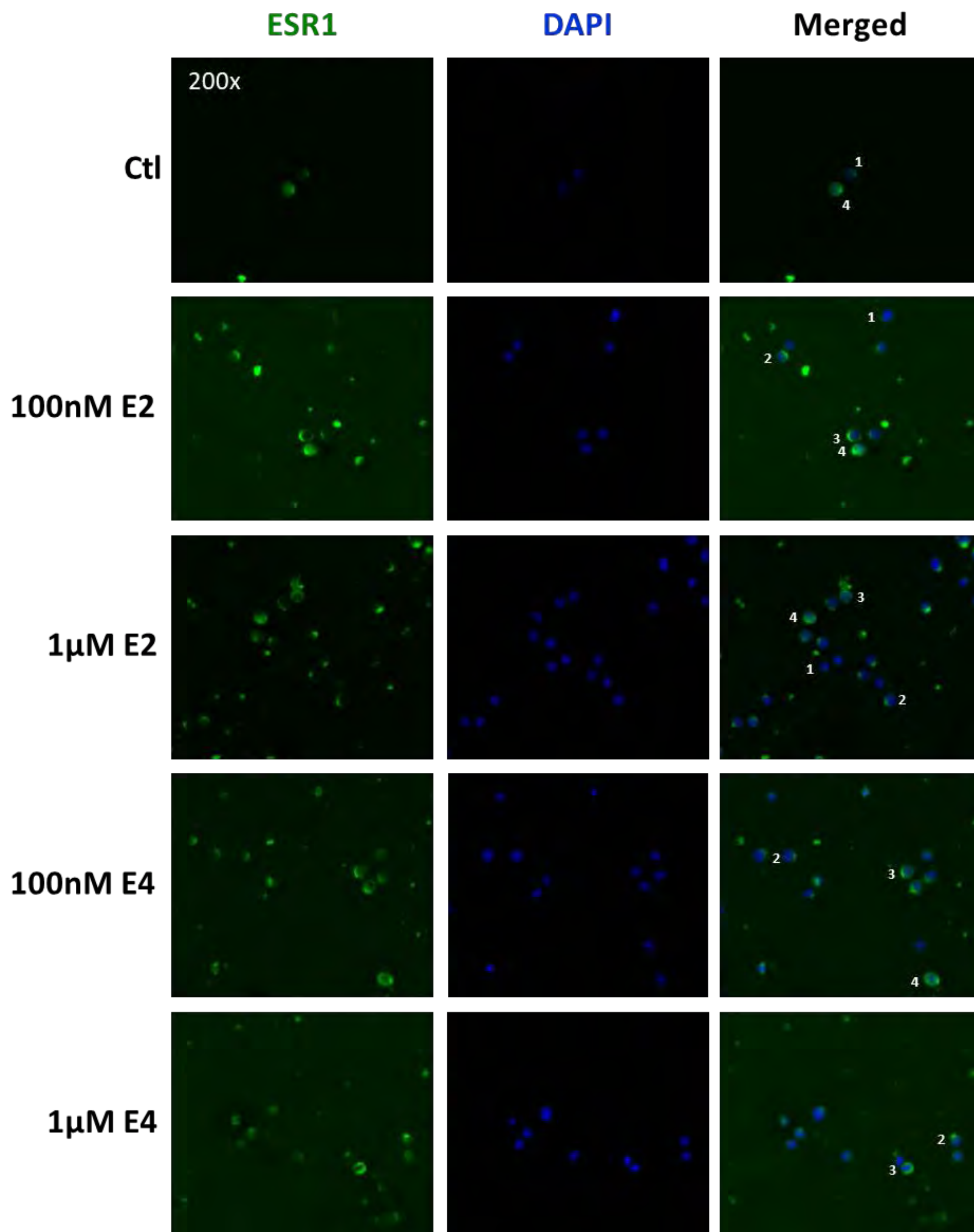
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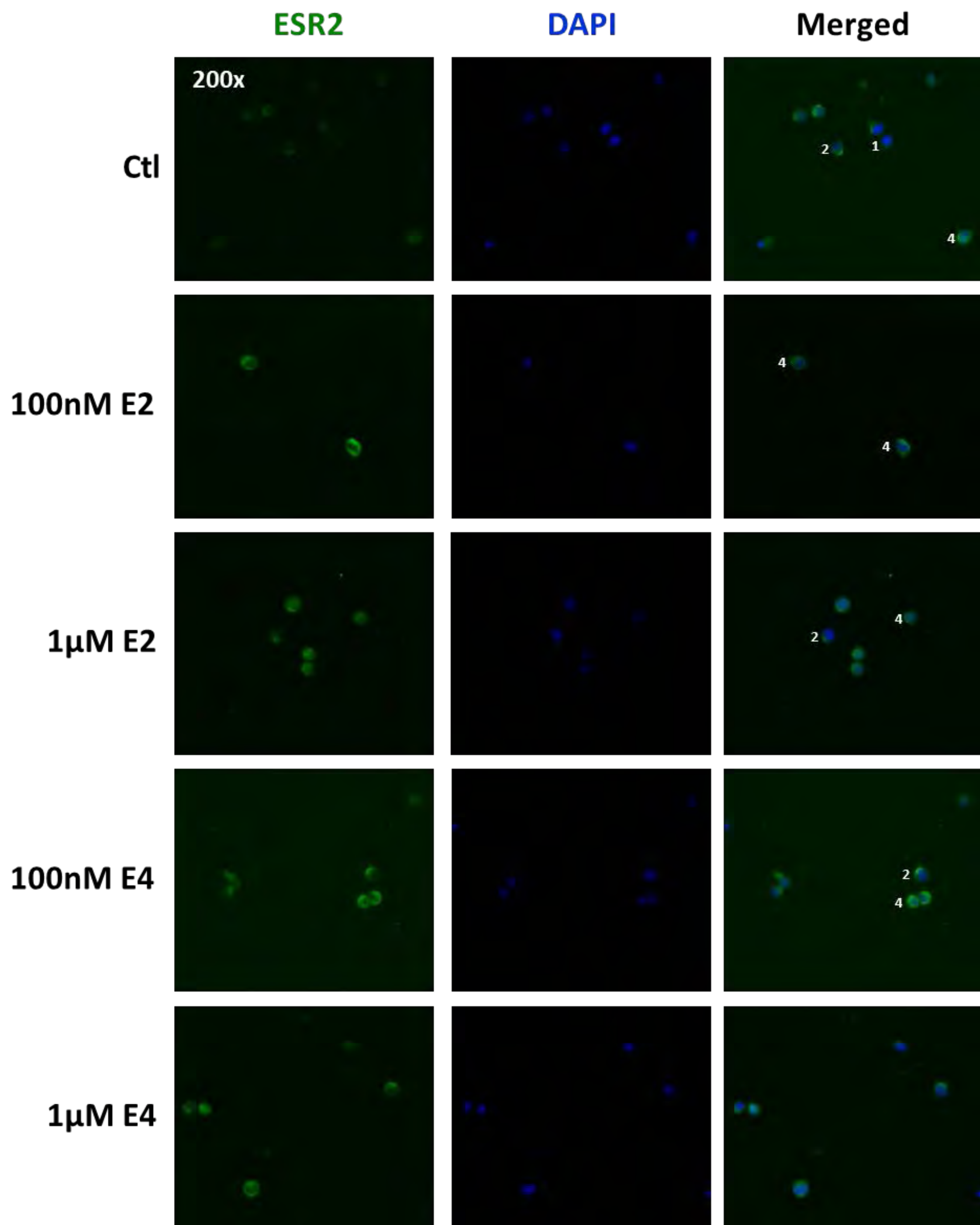
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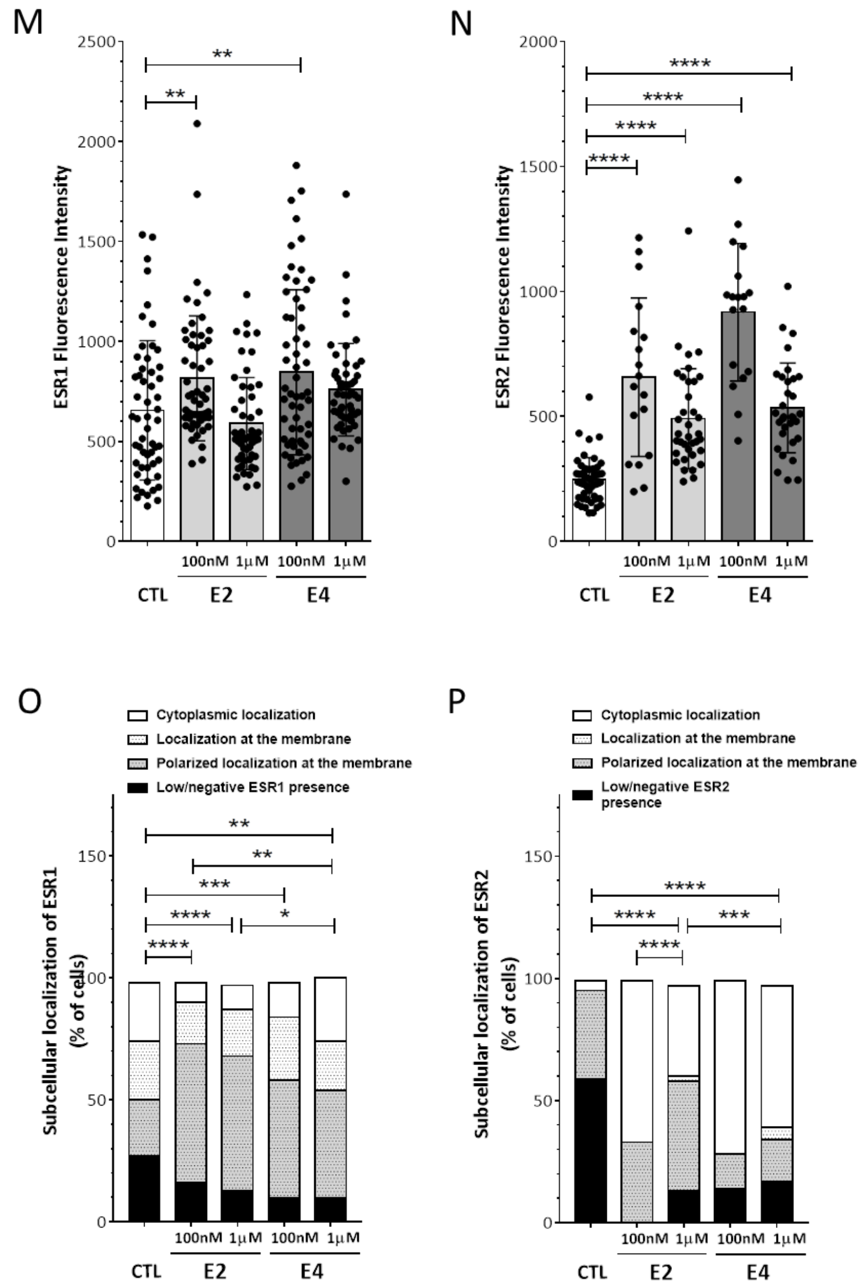
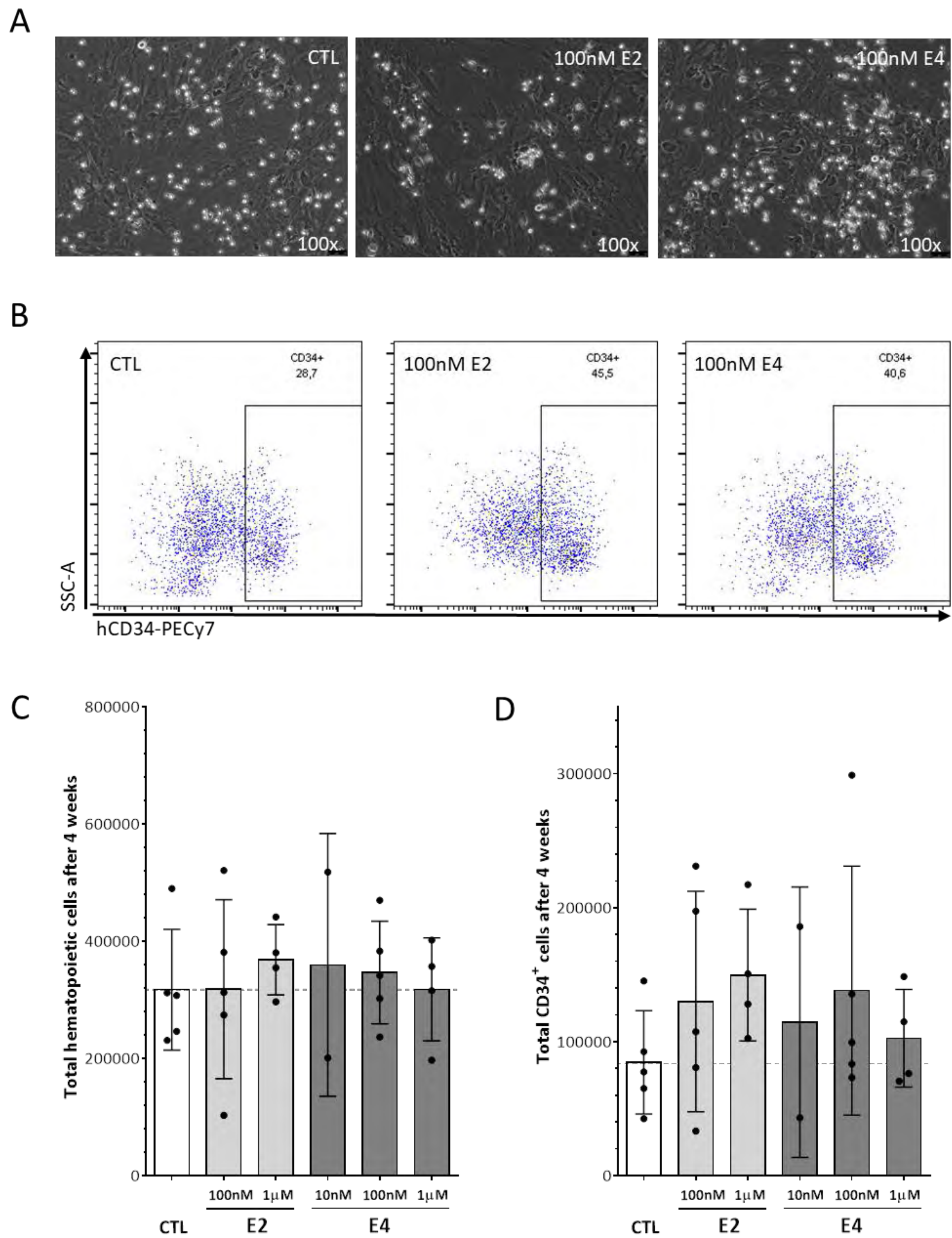


Supplemental Figure 3.

Natural estrogens affect different human HSPCs. (A) Representative dot-plots showing the flow cytometry analysis of different HSPC subsets, such as $hCD34^{+}hCD38^{-}$, MLP ($hCD34^{+}hCD38^{-}hCD90^{-}hCD45RA^{+}$), MPP ($hCD34^{+}hCD38^{-}hCD90^{-}hCD45RA^{-}$) and HSC ($hCD34^{+}hCD38^{-}hCD90^{+}hCD45RA^{-}$). (B) Representative dot-plots showing cytometry analysis of E2-treated HSPCs for four days. (C) Number of $hCD34^{+}hCD38^{-}$ cells within of estrogen-treated HSPCs after 4 days in culture. Different concentrations (10nM, 100nM,

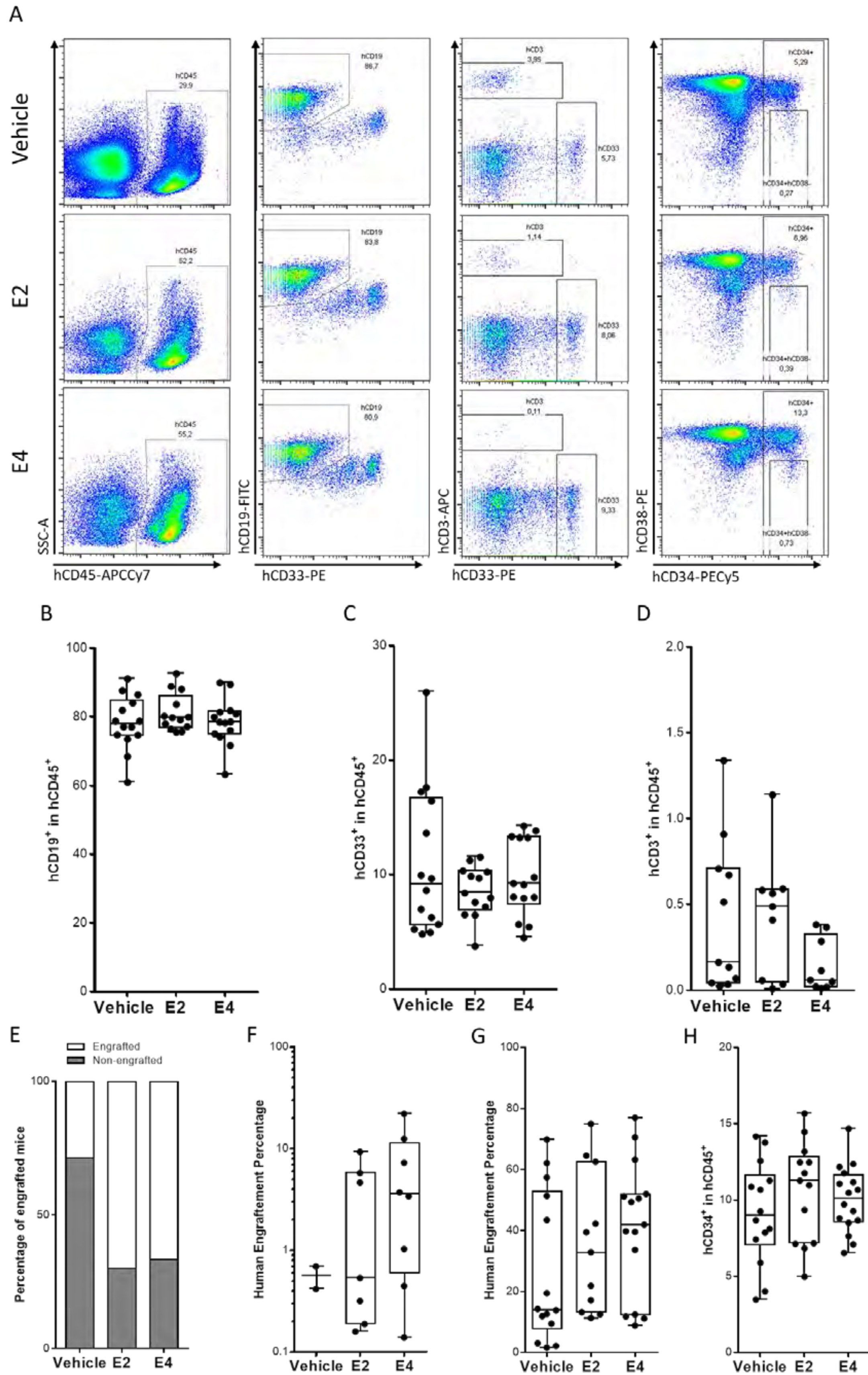
1μM, 10μM, 100μM and 500μM) of the natural estrogens (E1, E2, E3 and E4) were used. (D) Number of the MLPs within of estrogen-treated HSPCs after 4 days in culture. Different concentrations (10nM, 100nM, 1μM, 10μM, 100μM and 500μM) of the natural estrogens (E1, E2, E3 and E4) were used. (E) Number of the MPPs within of estrogen-treated HSPCs after 4 days in culture. Different concentrations (10nM, 100nM, 1μM, 10μM, 100μM and 500μM) of the natural estrogens (E1, E2, E3 and E4) were used. (F) Representative dot-plots showing Annexin V analysis of E2- and E4-treated HSPCs for four days. (G) Percentage of alive, early apoptotic and late apoptotic HSPCs after estrogen treatment for four days. Different concentrations (10nM, 100nM, 1μM, 10μM, 100μM and 500μM) of E2 and E4 were used. (H) Representative histogram showing Cell Cycle analyses of control (CTL) or HSPC treated with 100nM E2 or E4 for 4 days in culture. (I) Colony-Forming Unit (CFU) analysis from HSPCs cultured for 8 days in presence or absence of E2 or E4 at 10nM, 100nM or 1μM. (J) Cell cycle analysis of HSPCs culture with 100nM of E2 or E4 in presence of different antagonists of estrogen receptors, such as MPP (ESR1 antagonist, 1μM), PHTPP (ESR2 antagonist, 1μM) or G-15 (GPER1 antagonist, 3μM). Data are obtained from 3-5 biological replicates. (K) Representative ESR1 immunofluorescent images of CB-CD34^{+} cells cultured for 4 days with CTL, 100nM E2, 1μM E2, 100nM E4 or 1μM E4. ESR1 (green, left panels), DAPI (blue, middle panels) and DAPI (blue). (L) Representative ESR2 immunofluorescent images of CB-CD34^{+} cells cultured for 4 days with CTL, 100nM E2, 1μM E2, 100nM E4 or 1μM E4. ESR2 (green, left panels), DAPI (blue, middle panels) and DAPI (blue). (M) Analysis of ESR1 fluorescence intensity in CB-CD34^{+} cells cultured for 4 days with CTL, 100nM E2, 1μM E2, 100nM E4 or 1μM E4. (N) Analysis of ESR2 fluorescence intensity in CB-CD34^{+} cells cultured for 4 days with CTL, 100nM E2, 1μM E2, 100nM E4 or 1μM E4. (O) Classification of CB-CD34^{+} cells cultured for 4 days with CTL, 100nM E2, 1μM E2, 100nM E4 or 1μM E4 according ESR1 expression and subcellular. Several examples of human CD34^{+} with different ESR1 cellular localization are indicated as: 1. Low/negative ESR1 presence, 2. Polarized localization at the membrane, 3. Localization at the membrane and 4. Cytoplasmic localization. (P) Classification of CB-CD34^{+} cells cultured for 4 days with CTL, 100nM E2, 1μM E2, 100nM E4 or 1μM E4 according ESR2 expression and

subcellular. Several examples of human CD34^{+} with different ESR2 cellular localization are indicated as: 1. Low/negative ESR2 presence, 2. Polarized localization at the membrane, 3. Localization at the membrane and 4. Cytoplasmic localization. From 20 to 55 different HSPCs were analyzed. Data are presented by mean±SD. Significance was analyzed by by one-way Anova with Fisher's LSD test or Chi-square test and represented by *P <0.05, **P <0.01, ***P <0.005 and ****P <0.001.



Supplemental Figure 4.

Impact of either E2 or E4 impact on HSPCs co-cultured with human BM-MSCs. (A) Representative bright-field microscopy images of control HSPCs (left panel), E2-treated HSPCs (middle panel) and E4-treated HSPCs (right panel) co-cultured with human BM-MSCs for 1 week. (B) Representative dot-plots of control HSPCs (left panel), E2-treated HSPCs (middle panel) and E4-treated HSPCs (right panel) co-cultured with human BM-MSCs for 1 week. Samples were stained with hCD34-PECy7. (C) Total number of control and estrogen-treated HSPCs after 4 weeks of co-culture with human BM-MSCs. (D) Percentage of the $hCD34^+$ population within HSPCs co-cultured with BM-MSCs for 4 weeks. Data are obtained from 2-6 biological replicates. Data are presented by mean±SD. Significance was analyzed by by one-way Anova with Fisher's LSD test.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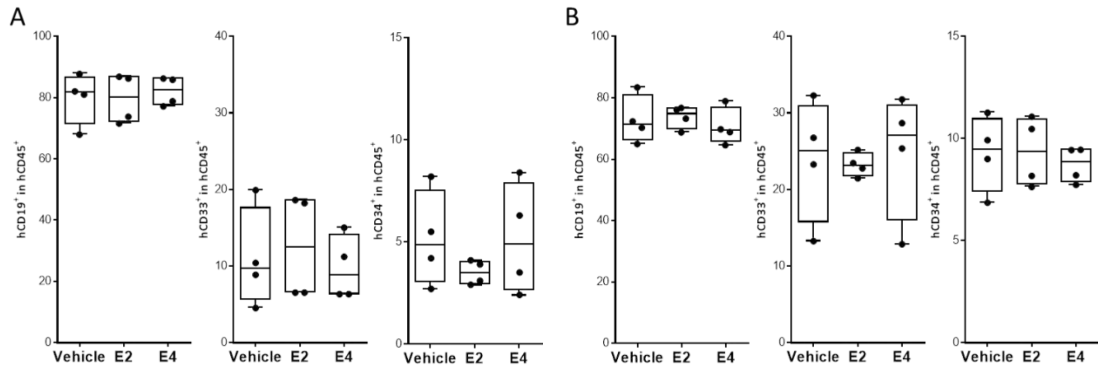
B
hCD19+ in hCD45+
Vehicle
E2
E4
C
hCD33+ in hCD45+
D
hCD3+ in hCD45+
E
Engrafted
Non-engrafted
Percentage of engrafted mice
F
Human Engraftement Percentage
G
Human Engraftement Percentage
H
hCD34+ in hCD45+

Supplemental Figure 5.

E2 and E4 enhance human engraftment in immunodeficient mice (A) Representative dot-plots of the flow cytometry analysis of NSG male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells 4mpt. Human engraftment, $hCD45^+$ cells (far left panels), human myeloid cells ($hCD33^+$) and human B-cells ($hCD19^+$) in the human population (center-left panels), human T-cells ($hCD3^+$) and human B-cells ($hCD19^+$) in the human population (center-right panels) and HSPC subsets ($hCD34^+$ and $hCD34^+hCD38^-$) in the human population (far right panels) of vehicle-(top row) or E2- (middle row) or E4-treated mice. (B) Percentage of $hCD19^+$ cells within the human population in the BM of male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells 4mpt. (C) Percentage of $hCD33^+$ cells within the human population in the BM of male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells 4mpt. (D) Percentage of $hCD3^+$ cells within the human population in the BM of male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells 4mpt. (E) Percentage of engrafted male NSG mice (percentage of $hCD45^+$ cells within mouse BM higher than 0.1%) and non-engrafted ones 4mpt after transplanting $5x10^3$ hCB-$CD34^+$ cells. Significance was analyzed by Fisher´s exact test, no significant differences were found. (F) Percentage of $hCD45^+$ cells in the BM of engrafted male mice transplanted with $5x10^3$ hCB-$CD34^+$ cells 4mpt. (G) Percentage of $hCD45^+$ cells in the BM of female mice transplanted with $5x10^3$ hCB-$CD34^+$ cells 4mpt. (H) Percentage of $hCD34^+$ cells within the human population in the BM of female mice transplanted with $5x10^3$ hCB-$CD34^+$ cells 4mpt. Data are obtained from 3-4 biological replicates. Data are presented by dots and box-plots that represent the interquartile range (p75, upper edge; p25, lower edge; p50, midline; p95, line above the box; and p5, line below the box). Significance was analyzed by Mann–Whitney U test and represented by **P <0.01.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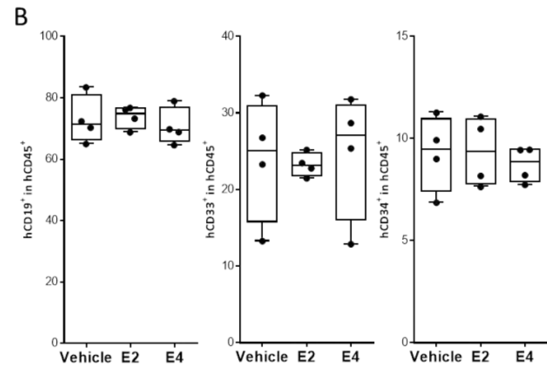
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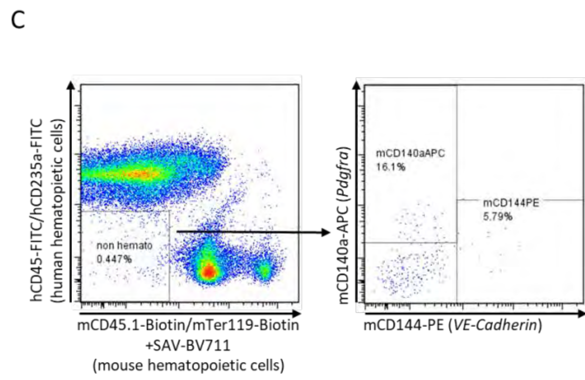
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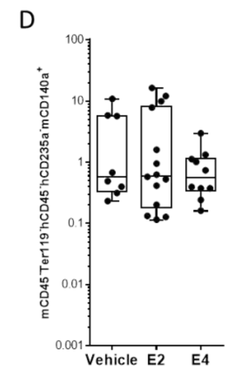
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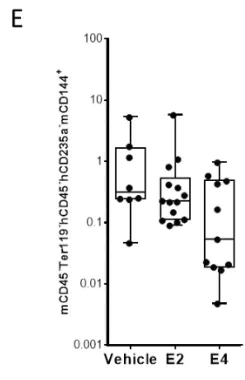
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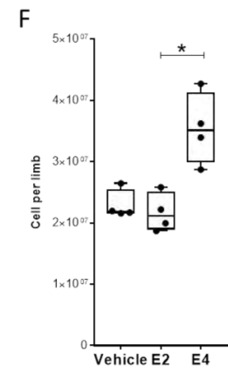
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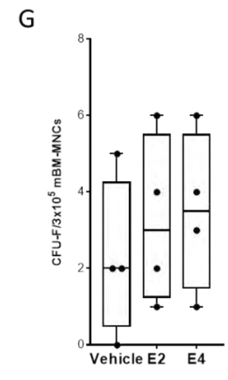
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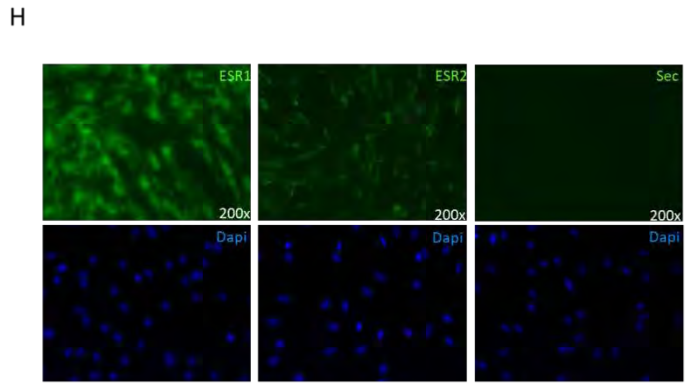
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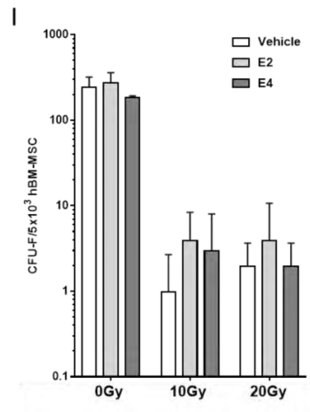
I
Vehicle
E2
E4
0Gy
10Gy
20Gy

Supplemental Figure 6.

Estrogens modulate hematopoietic the niche. (A) Percentage of $hCD19^+$ cells (left panel) $hCD33^+$ (center panel) and $hCD34^+$ cells (right panel) within the human population in BM of male mice transplanted with the expanded cells from initial $5x10^4$ $hCB-CD34^+$ cells after 4 days in culture in presence of 100nM of E2 or E4. The human engraftment was analyzed 2mpt. (B) Percentage of $hCD19^+$ cells (left panel) $hCD33^+$ (center panel) and $hCD34^+$ cells (right panel) within the human population in BM of male mice transplanted with the expanded cells from initial $5x10^4$ $hCB-CD34^+$ cells after 1 week in co-culture with irradiated human BM-MSCs in presence of 100nM of E2 or E4. The human engraftment was analyzed 3mpt. (C) Representative dot-plots of the flow cytometry analysis of mouse MSCs ($mCD140a^+$) and mouse vascular endothelial cells ($mCD144^+$) within the non-hematopoietic population ($hCD45^-hCD235a^-mCD45.1^-mTer119^-$) in the BM of NSG male mice transplanted with $5x10^4$ $hCB-CD34^+$ cells 4mpt. (D) Percentage of mouse MSCs ($mCD140a^+$) within the non-hematopoietic population ($mCD45^-Ter119^-hCD45^-hCD235a^-$) in the BM of male mice transplanted with $5x10^4$ $hCB-CD34^+$ cells 4mpt. (E) Percentage of mouse vascular endothelial cells ($mCD144^+$) within the non-hematopoietic population ($mCD45^-Ter119^-hCD45^-hCD235a^-$) in the BM of male mice transplanted with $5x10^4$ hCB-$CD34^+$ cells 4mpt. (F) Total number of BM-MNCs per limb of mice two weeks after being sublethally irradiated and treated with vehicle or estrogens. (G) Number of Colony-Forming Cells (CFCs) per $3x10^5$ mouse BM-MNCs seeded in mesenchymal media. (H) Single channels of the immonufluorescent images shown in Figure 6C. Human BM-MSCs cells were stained with anti-ESR1 (left panel, green) or anti-ESR2 (middle panel, green) or only secondary antibody (right panel, green) and DAPI (blue). (I) Number of CFCs derived from human BM-MSCs of three different healthy donors after being irradiated with 10Gy or 20Gy or without irradiation, and treated with vehicle, E2 or E4. Data are obtained from 3 biological replicates. Data are presented by mean±SD or dots and box-plots that represent the interquartile range (p75, upper edge; p25, lower edge; p50, midline; p95, line above the box; and p5, line below the box). Significance was analyzed by Mann–Whitney U test or one-way Anova with Fisher's LSD test and represented by *P <0.05.