SUPPLEMENTARY APPENDIX

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

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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.

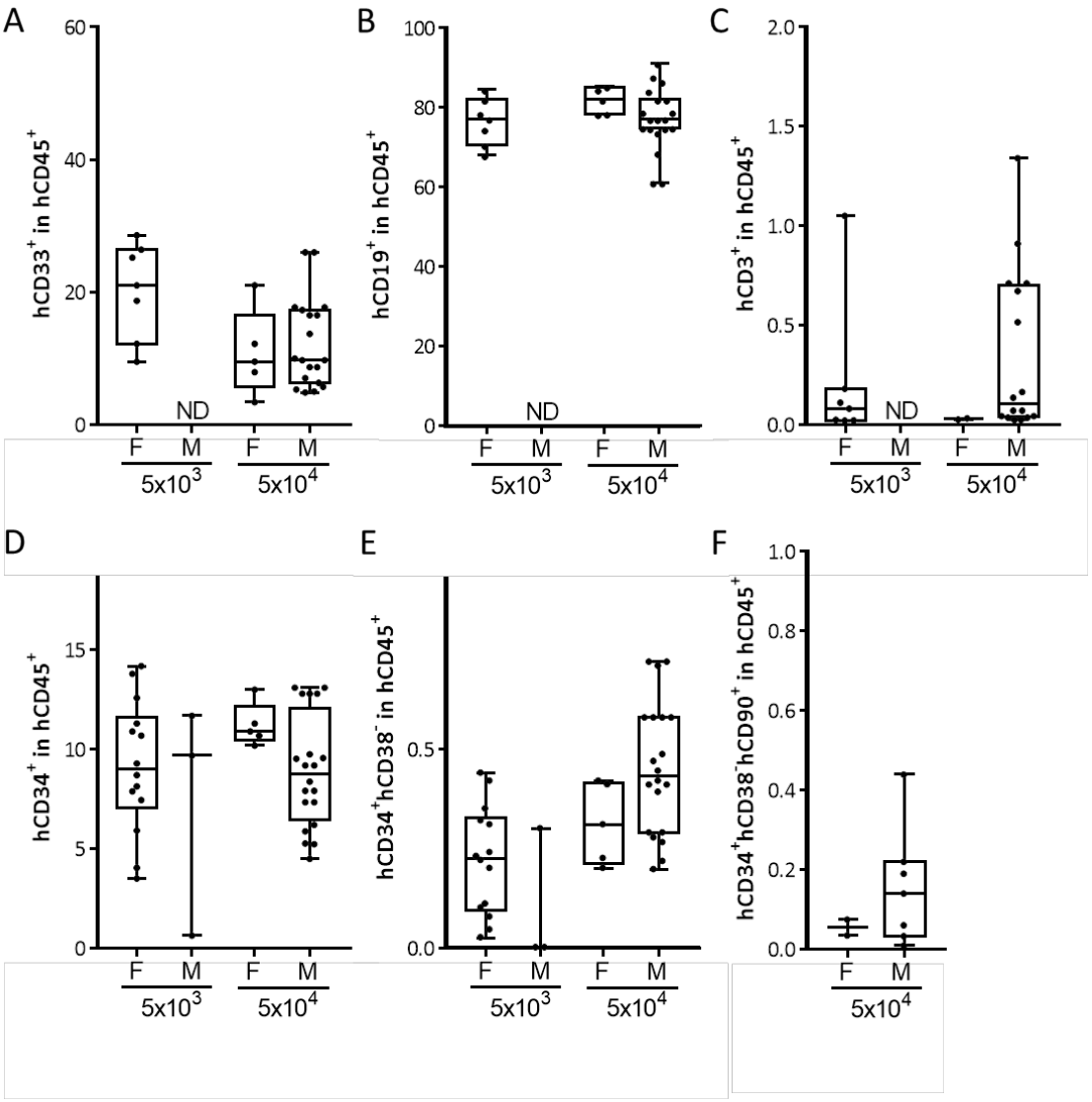
Statistical analysis

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.

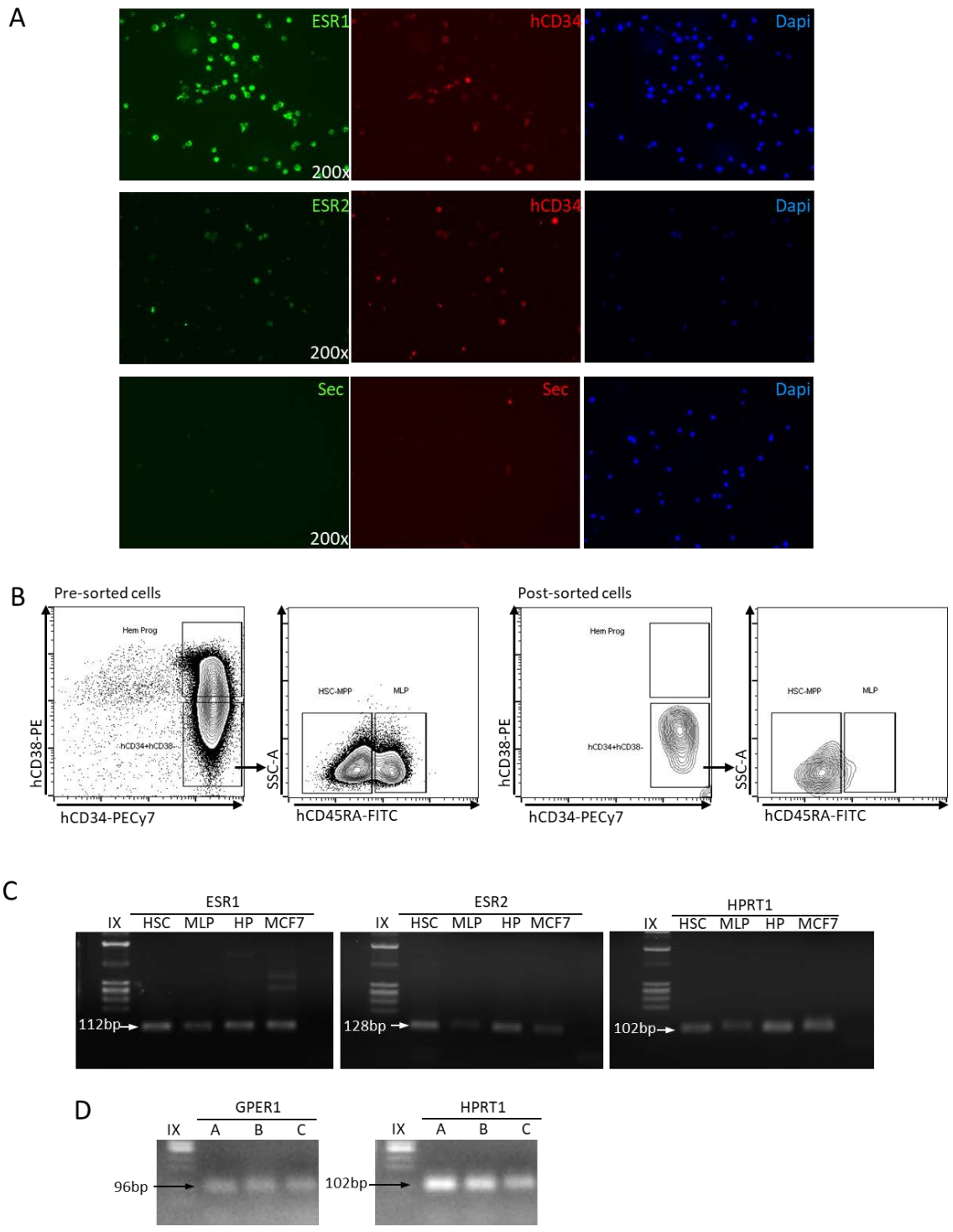
SUPPLEMENTAL FIGURES



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

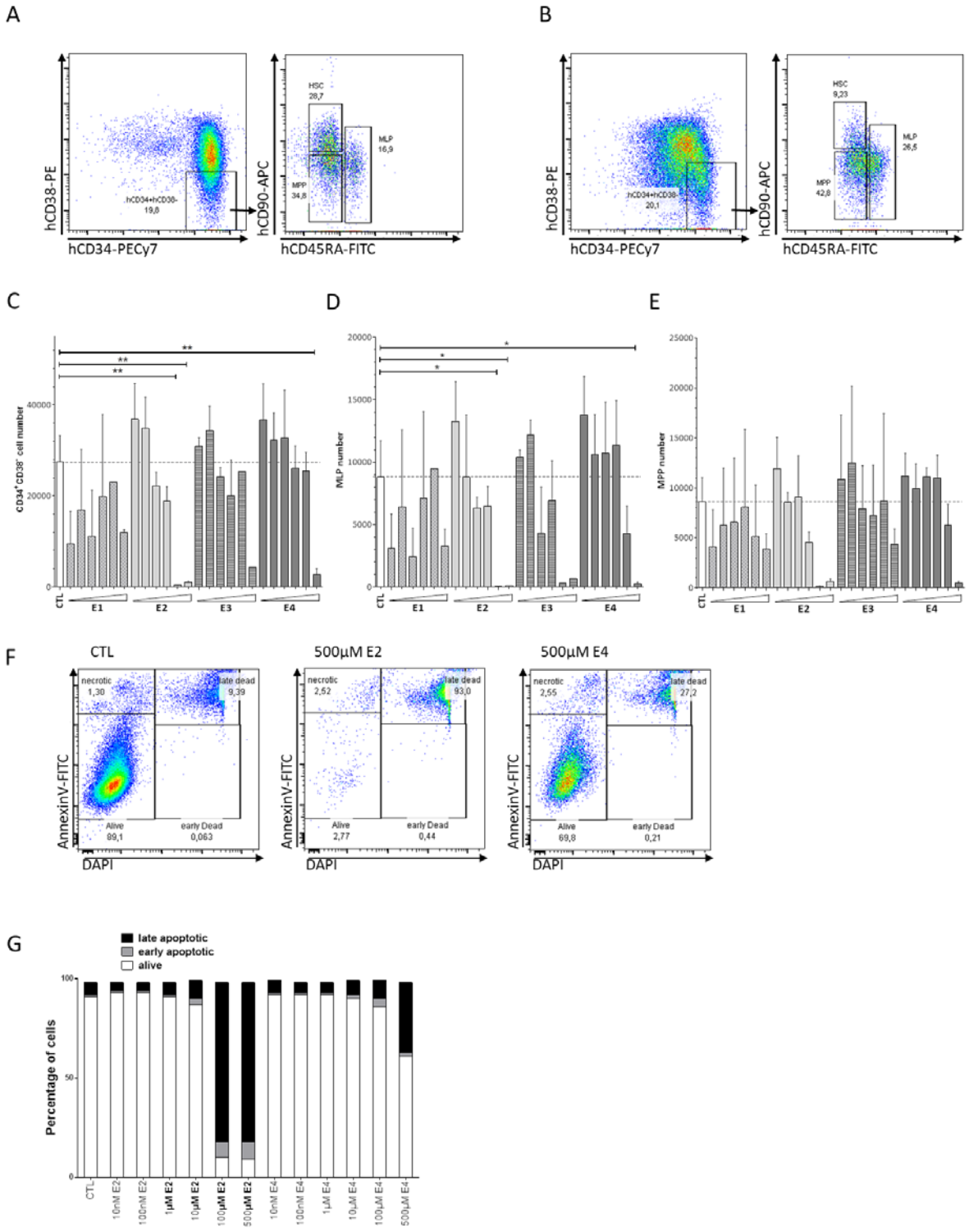
human hematopoietic population. (F) Percentage of HSCs ($hCD34^{+}hCD38^{-}hCD90^{+}$) within human hematopoietic population. ND no detected. Data are obtained from 6 independent biological replicates. Data are presented by dots and box-plots that represent the interquartile range (p75, upper edge; p25, 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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hCD34+hCD38-
19,8
hCD90-APC
hCD45RA-FITC
HSC
28,7
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16,9
MPP
34,8
B
hCD38-PE
hCD34-PECy7
hCD34+hCD38-
20,1
hCD90-APC
hCD45RA-FITC
HSC
9,23
MLP
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MPP
42,8
C
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E2
E3
E4
D
MLP number
CTL
E1
E2
E3
E4
E
MPP number
CTL
E1
E2
E3
E4
F
CTL
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late dead
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Alive
99,1
early Dead
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27,2
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early apoptotic
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10µM E2
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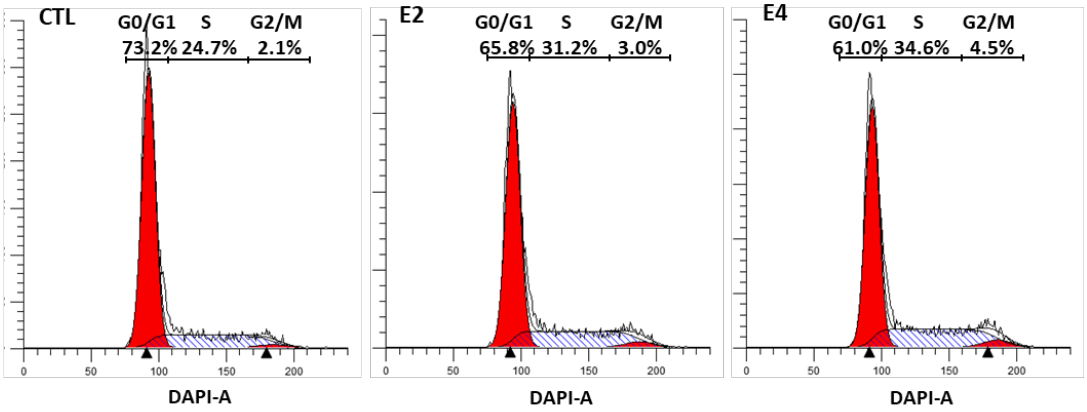
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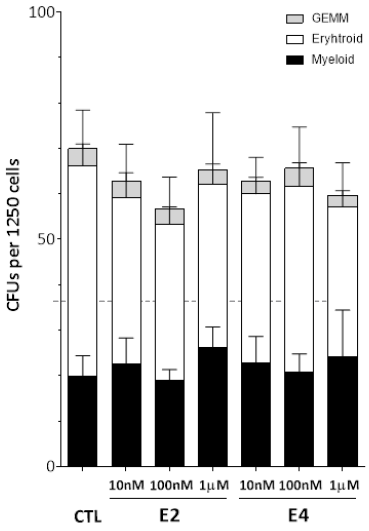
E2
G0/G1 S G2/M
65.8% 31.2% 3.0%
E4
G0/G1 S G2/M
61.0% 34.6% 4.5%
0 50 100 150 200
DAPI-A

I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Myeloid
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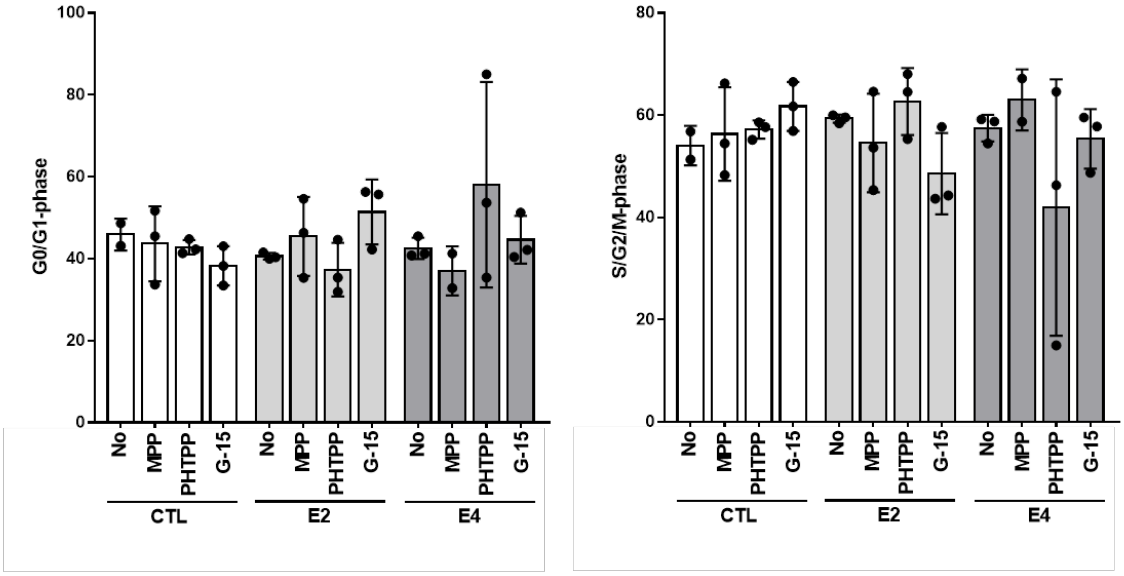
50
0
10nM 100nM 1µM 10nM 100nM 1µM
CTL
E2
E4

J


G0/G1-phase
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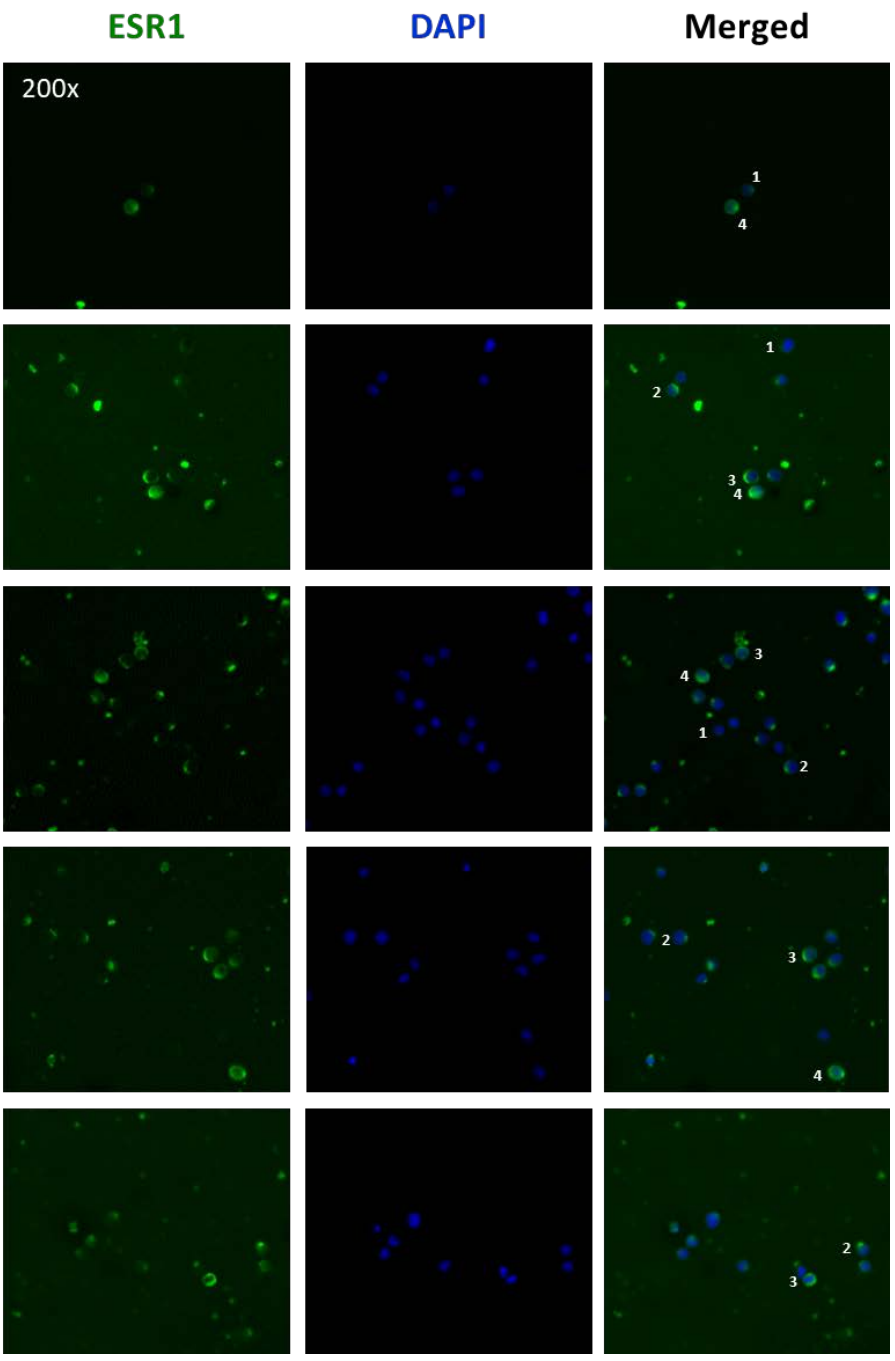
80
60
40
20
0
No MPP PHTPP G-15 No MPP PHTPP G-15 No MPP PHTPP G-15
CTL
E2
E4
S/G2/M-phase
80
60
40
20
0
No MPP PHTPP G-15 No MPP PHTPP G-15 No MPP PHTPP G-15
CTL
E2
E4

K



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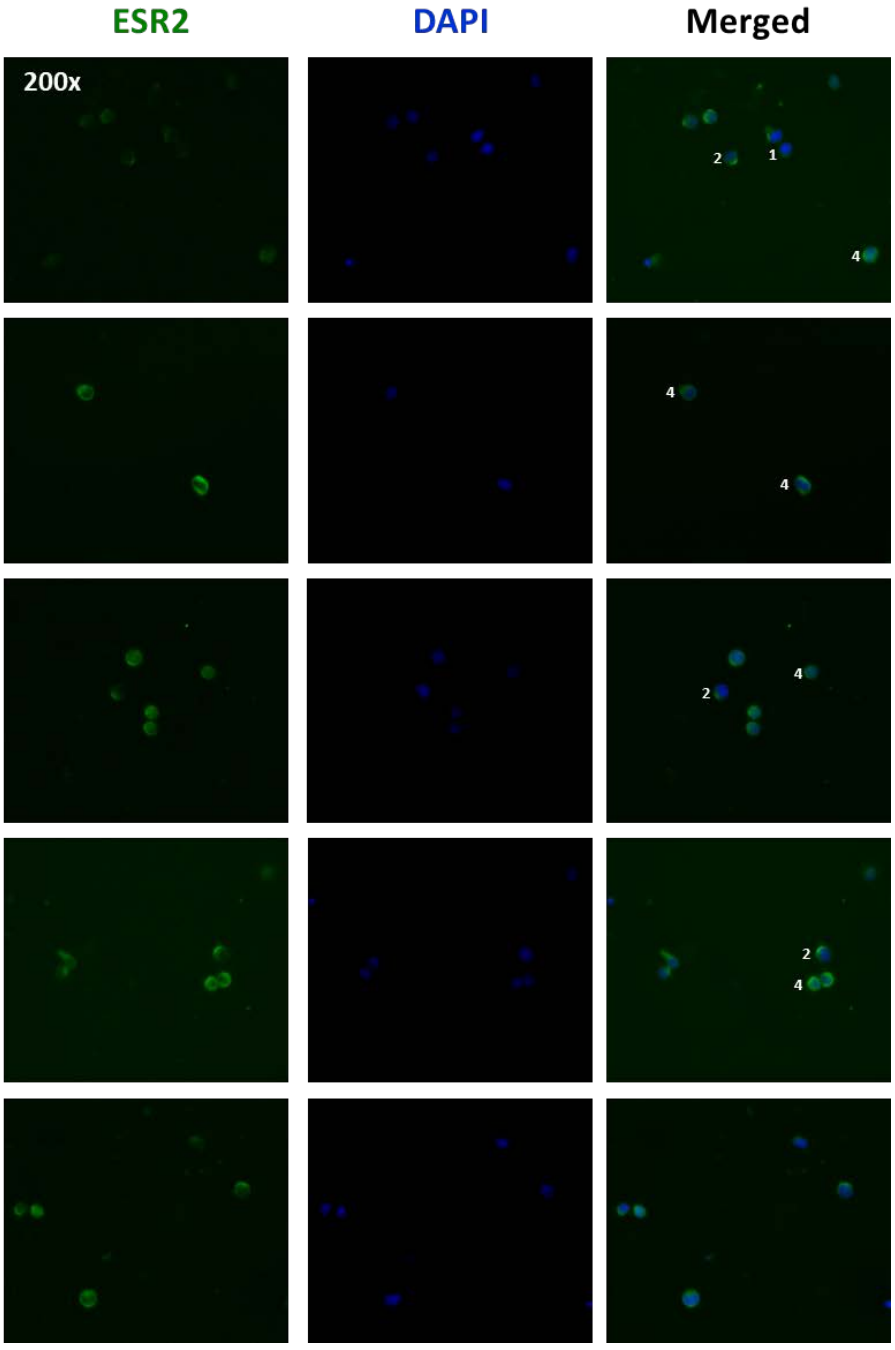
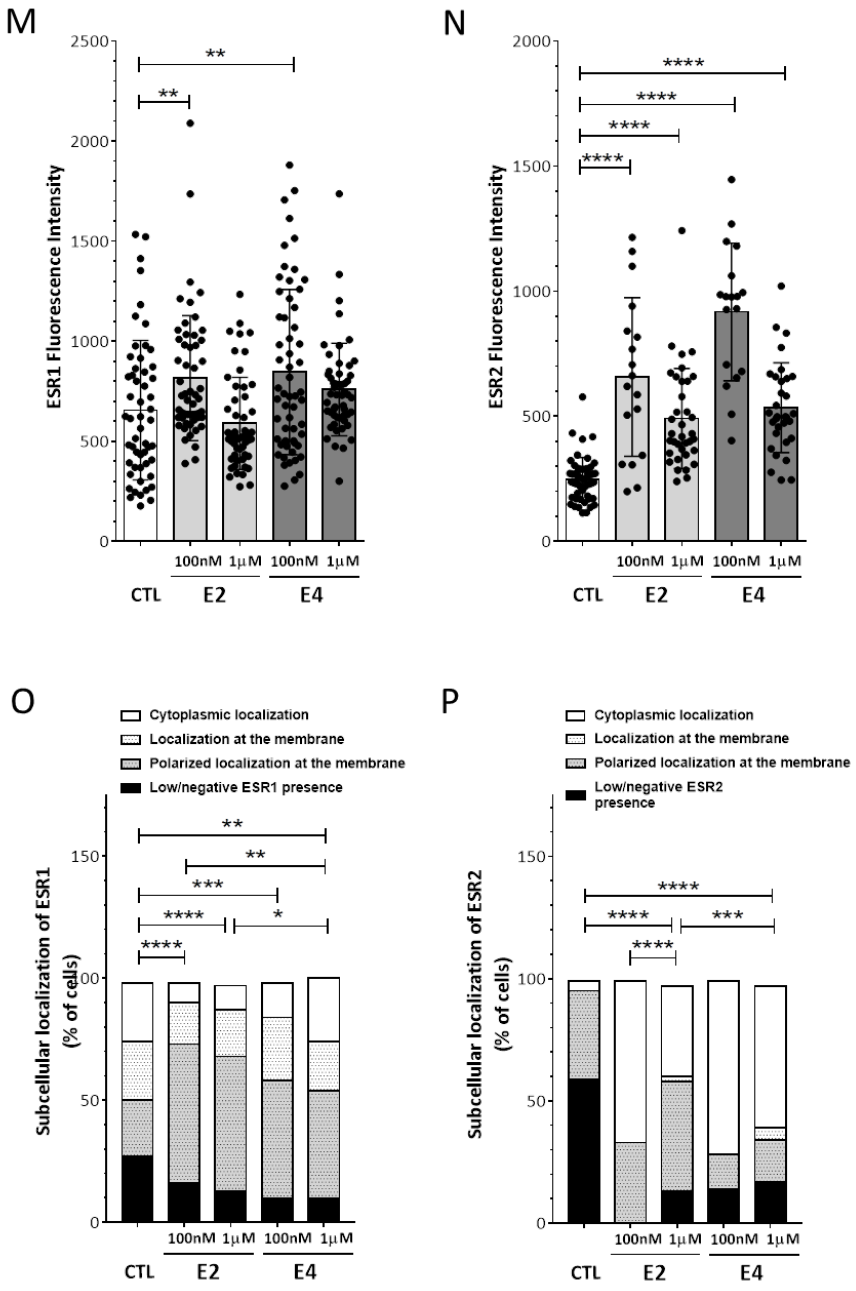


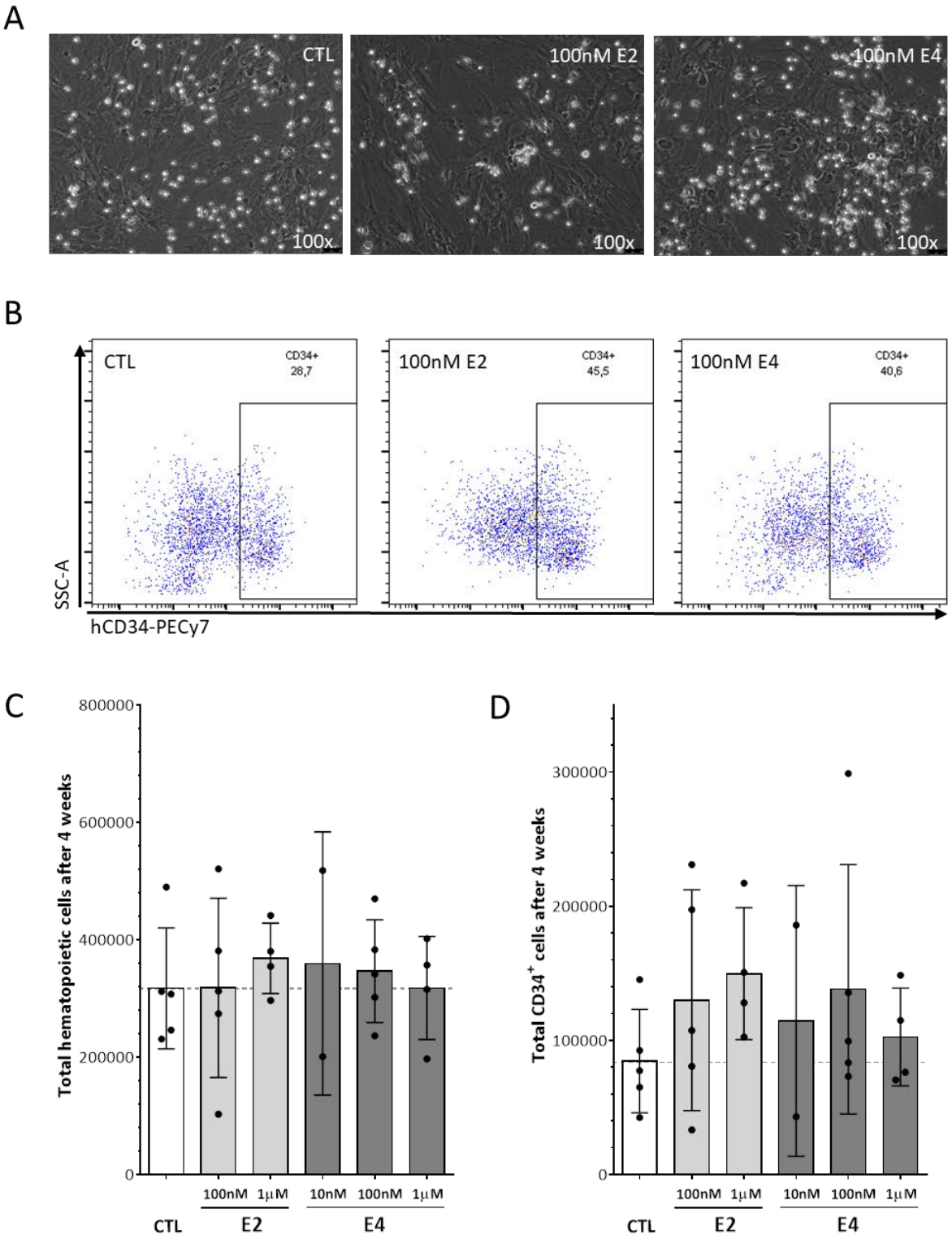


Supplemental Figure 3.

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,

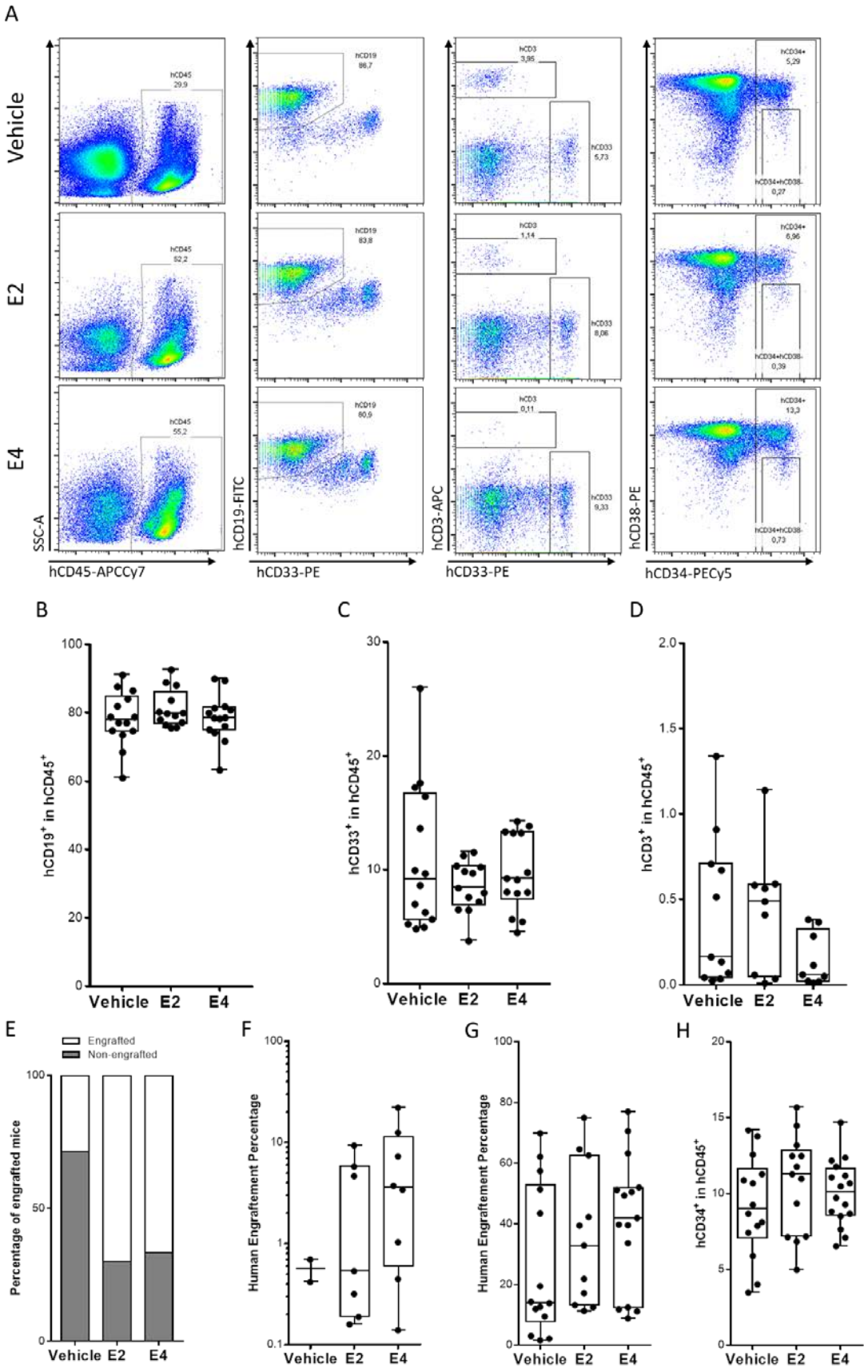
1μM, 10μM, 100μM and 500μM) of the natural estrogens (E1, E2, E3 and E4) were used. (D) Number of the MLPs within of estrogen-treated HSPCs after 4 days in culture. Different concentrations (10nM, 100nM, 1μM, 10μM, 100μM and 500μM) of the natural estrogens (E1, E2, E3 and E4) were used. (E) Number of 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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Vehicle
E2
E4
SSC-A
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hCD19-FITC
hCD33-PE
hCD3-APC
hCD33-PE
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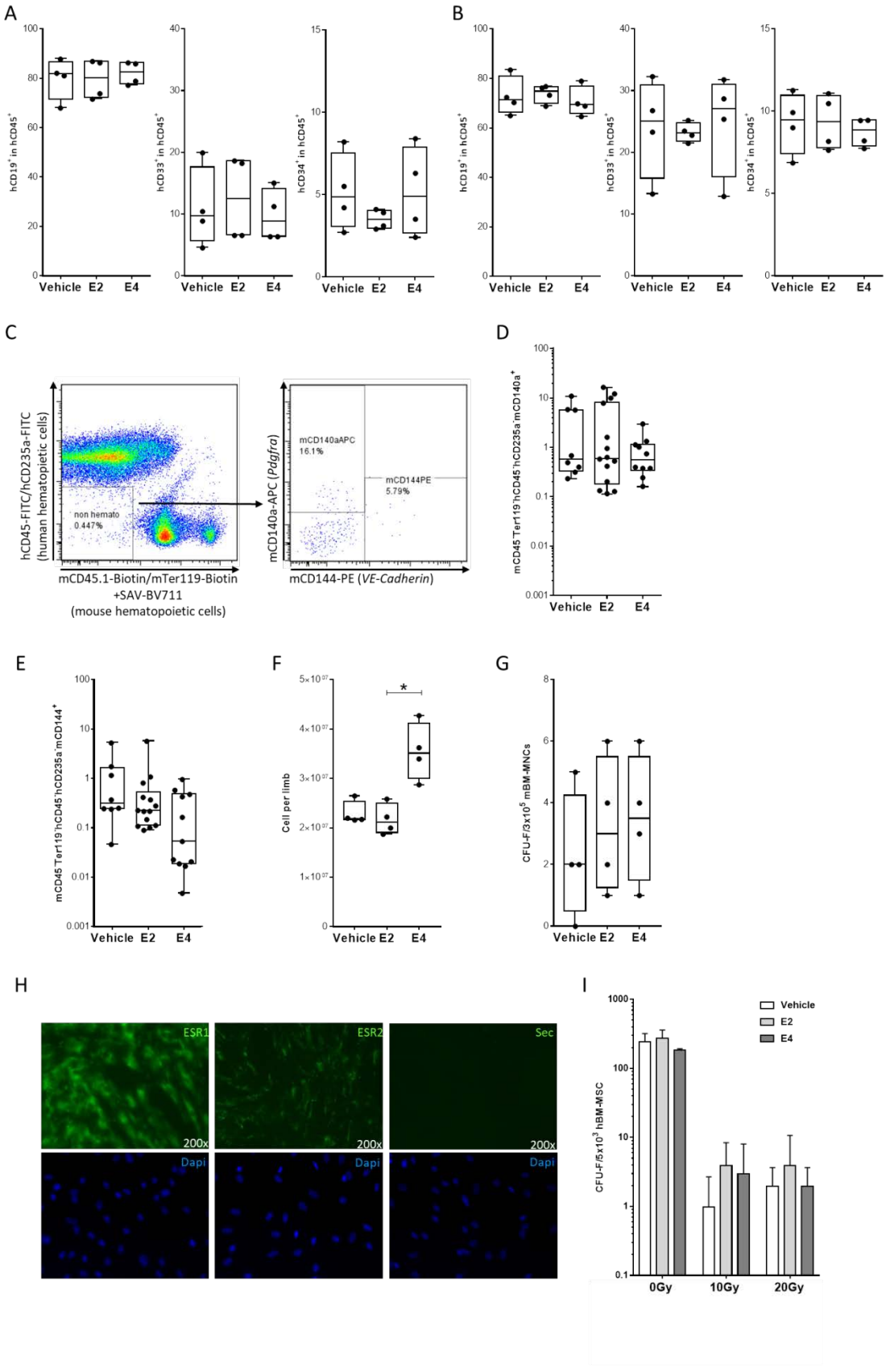
hCD34-PECy5
B
hCD19+ in hCD45+
C
hCD33+ in hCD45+
D
hCD3+ in hCD45+
Vehicle E2 E4
E
Engrafted
Non-engrafted
Percentage of engrafted mice
F
Human Engraftement Percentage
G
Human Engraftement Percentage
H
hCD34+ in hCD45+

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.


A
B
C
D
E
F
G
H
I
Vehicle
E2
E4
hCD19+ in hCD45+
hCD33+ in hCD45+
hCD34+ in hCD45+
hCD45-FITC/hCD235a-FITC
(human hematopoietic cells)
non hemato
0.447%
mCD45.1-Biotin/mTer119-Biotin
+SAV-BV711
(mouse hematopoietic cells)
mCD140a-APC (Pdgfra)
mCD140aAPC
16.1%
mCD144PE
5.79%
mCD144-PE (VE-Cadherin)
mCD45-Ter119-hCD45-hCD235a-mCD140a+
mCD45-Ter119-hCD45-hCD235a-mCD144+
Cell per limb
CFU-F/3x10^5 mBM-MNCs
ESR1
ESR2
Sec
200x
Dapi
CFU-F/5x10^3 hBM-MSC
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.