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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

[Link to publication in University of Groningen/UMCG research database](#)

Estradiol increases hematopoietic stem and progenitor cells independent of its actions on bone

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ABSTRACT

Hematopoietic stem and progenitor cells reside in vascular and endosteal niches in the bone marrow. Factors affecting bone remodeling were reported to influence numbers and mobilization of hematopoietic stem cells. We therefore analyzed the effects of estradiol acting anabolic on bone integrity. Here we observe that estradiol increases progenitor cell numbers in the vascular but not in the endosteal compartment independent of its estrogen receptor α-dependent anabolic bone effects. Hematopoietic progenitors capable of reconstituting lethally irradiated mice are increased by enhanced cell cycle entry, leading to a diminished long-term reconstitution potential after serial transplantation. We demonstrate that estradiol action on stromal cells potently favors hematopoietic progenitor/stem cell frequency accompanied by enhanced expression of cell adhesion molecules. Finally, estradiol treatment enhances retention of hematopoietic stem cells in the vascular niche of the bone marrow. We describe for the first time the mechanism of estrogen action on hematopoietic stem and progenitor cells.

Key words: estradiol, bone mass, endosteal and vascular niche, short-term, long-term, progenitors, CD34, adhesion molecules.

Citation: Illing A, Liu P, Ostermay S, Schilling A, de Haan G, Krust A, Amling M, Chambon P, Schinke T, and Tuckermann JP. Estradiol increases hematopoietic stem and progenitor cells independent of its actions on bone. Haematologica 2012;97(8): 1131-1135. doi:10.3324/haematol.2011.052456

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Introduction

Hematopoietic stem cells (HSCs) and their down-stream progenitors (HSPCs) are dependent on their specific micro-environment, the *niche*, to balance self-renewal and differentiation.[1] HSCs reside in the bone marrow (BM) either in the BM cavity in contact with endothelial, perivascular, sinusoidal, reticular and CAR cells (CXCL12-abundant reticular cells),[2] i.e. the vascular niche, or close to the endosteal surface in direct contact with osteoblasts (SNOs)[3,4] and osteoclasts, i.e. the endosteal niche.

Alterations in osteoblast numbers and bone mass correlate with HSC numbers.[3,4] Therefore, the number of HSCs could be centrally controlled by hormones affecting bone mass. Estrogens are well known modulators of bone mass[5] and long-term treatment leads to an increase in endosteal bone mass.[5,6] To investigate whether estradiol also influences HSCs, we analyzed estradiol treated mice. Although bone mass was strongly increased, there was no alteration in the numbers of HSPCs located in the endosteal niche. In contrast, the numbers of HSPCs in the vascular niche were significantly increased due to enhanced cycling of HSPCs and an upregulation of adhesion molecules in the stromal cell compartment, indicating that estradiol acts on HSPCs independent of its effects on bone.

Design and Methods

Animal treatments

Animal experiments were performed according to accepted standards of animal welfare and with the permission of the authorities of Thüringen. Estrogen treatment was given for four weeks by subcutaneous implantation of slow-release pellets resulting in a calculated dose of 0.24 mg/kg/d (0.36 mg; 60-day release; Innovative Research of America, Inc.) in 10-12 week old female C57BL/6 or CD45.1 (wild-type) mice and in ERα- and ERβ-knockout, ERα–loxP[PRunx2Cre] mice.[7-10] Mice received short-term treatment with estradiol 5 mg/kg (Sigma) i.p. daily.

Bone sections and von Kossa staining

Lumbar vertebral bodies (L3-L5) and one tibia of each mouse were processed and stained, and bone histomorphometry was performed, all as previously described.[9]

Isolation of hematopoietic cells of the vascular and endosteal niche

The flushed fraction of the BM from hindlimbs and humeri represents the cells from the vascular niche. Endosteal BM cells were isolated as previously described.[11] The digested fraction represents the cells of the endosteal niche.

Flow cytometry

Flow cytometry was performed as described.[12] Monoclonal antibodies from Natutec /eBioscience were:

Gr1-FITC, B220-FITC, CD3-FITC, CD11b-FITC, Ter119-FITC, Sca1-PE,CD117-APC, CD150-PE, CD150-APC, CD48-PE, CD48-APC, CD45.1-FITC, B220-PE, Gr1-PE, CD11b-APC, CD4-PE, CD8-APC.

Data were recorded with FACS-Canto II or FACS-Calibur (BD-Biosciences) and analyzed by Flow Jo 8.0 flow cytometry software (Tristar).

CAFC assay

Cobblestone area forming cell (CAFC) assay was performed as described[13] and frequency of HSC was calculated using Poisson statistics.[14]

Homing-assay

BM cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) as previously described[15,16] and injected (i.v.) into irradiated (8 Gy) estradiol treated recipients. After 15 h, BM was analyzed for CFSE-positive cells by flow cytometry.

Cell-cycle analysis

After LSK staining, BM cells were fixed, permeabilized, RNAse A (Invitrogen) treated, and PI (probidium iodide, Invitrogen) stained according to standard protocols and analyzed by flow cytometry.

Determination of competitive repopulation units in limited dilution analysis

Limited dilution analysis (LDA) was performed according to standard protocols using four dilutions at 1:3, starting with 540,000 cells.[14] For transplantation, lethally irradiated recipients were intravenously injected with BM cells from estradiol and control treated mice.

Serial transplantation

Serial transplantation was performed as described[17] using CD45.1 recipients receiving 5×10^6 BM cells from either control or estradiol treated mice. Four months posttransplantation, recipients were analyzed and 5×10^6 BM cells were transplanted into secondary or third recipients. Reconstitution was analyzed in the blood and BM for donor-derived B cells, T cells and granulocytes by flow cytometry.

Results and Discussion

Estradiol increases bone mass in wild-type mice but does not affect bone adherent hematopoietic progenitor (HSPCs) cells

To investigate the effects of a long-term treatment with estradiol in C57BL/6-mice, animals were treated with 6 µg estradiol per day for 30 days. This treatment leads to strongly increased trabecular bone mass (Figure 1A, *Online Supplementary Table S1*) which, given a similar number of osteoblasts per bone surface, results in an increase in total osteoblast numbers (*Online Supplementary Table S1*). This is also shown by an increase in the bone formation rate, demonstrating enhanced osteoblast activity (*Online Supplementary Table S1*). Since intermittent parathyroid hormone treatment indirectly influences HSPCs (LSK, lineage⁻/ Sca1⁺ / cKit⁺) by increasing trabecular bone,[3,4] we expected a profound effect of estradiol enhancing bone mass and concomitantly HSC numbers. Surprisingly, estradiol was not seen to have any influence on the numbers of LSK-cells in the endosteal stem cell niche (Figure 1B and C). This suggests that the increased bone mass did not influence endosteal HSC numbers. This was confirmed by unaltered HSC frequency in CAFC (cobblestone area forming cell) assay investigating endosteal bone marrow (BM) after estradiol treatment (*data not shown*).

Estradiol increases HSPCs with reconstitutive potential in the vascular niche

In contrast, LSK cells of the vascular niche were increased in percentages after estradiol exposure (Figure 1D and E) and also increased in absolute numbers (Figure 1F). In particular, CD34⁻/ˡᵒ LSK cells were increased in number (Figure 1G, *Online Supplementary Figure S1A*), whereas LT-HSCs (CD48⁻CD150⁺CD34⁻/ˡᵒ LSK) were not altered (Figure 1H, *Online Supplementary Figure S1B*). Accordingly, the CAFC-assay analyzed at Day 21 (Figure 1J) represents ST-HSCs and confirmed the increase in HSPCs in the vascular BM of estradiol treated mice. The CAFC assay analyzed at Day 35, representing LT-HSCs (Figure 1I), showed no significant changes from estradiol treatment. Thus, ST-HSCs rather then LT-HSCs are increased in estradiol treated mice.

Next, we tested whether estradiol increases HSPCs *in vivo* by a limiting dilution analysis (LDA) determining the frequency of competitive repopulating-units (CRUs). Mice transplanted with different dilutions of BM cells from estradiol treated animals showed better reconstitution after four months than mice receiving control treated BM (Figure 1K and L). LDA showed a strong increase in CRU (as measurement for HSCs, Figure 1L) in estradiol treated mice. Hence, estradiol elevates numbers of functional HSCs in the vascular niche.

Estradiol alters the cell cycle entry of LSK cells leading to a decrease in long-term repopulating HSCs (LT-HSCs)

LSK cells of estradiol treated mice are significantly stronger represented in S-phase compared to untreated mice (Figure 1M). Additionally, more LSK-cells are present in G2/M phase whereas there was a slight reduction in G0/G1 cells. In conclusion, estradiol causes more HSPCs to enter the S phase and, therefore, less progenitor and stem cells are quiescent in the G0/G1-phase.

We observed a significant decrease in donor-derived LSK cells in the BM of the recipient mice after the third transplantation with BM from estradiol treated mice (Figure 1N). Loss of reconstitution potential mainly affected formation of granulocytes but not the lymphoid lineage (Figure 1O). It has been hypothesized that there are heterogeneous stem cell populations consisting of myeloid biased LT-HSCs that are forming cells of the myeloid lineage, and lymphoid-biased LT-HSCs, preferentially forming cells of the lymphoid lineage.[18,19] This could indicate a selective suppressive effect of estradiol on long-term repopulation of myeloid biased LT-HSCs which is, however, not evident after measuring the numbers of CD48⁻CD150⁺CD34⁻/ˡᵒ LSK cells that remain unchanged (Figure 1H, *Online Supplementary Figure S1B*).
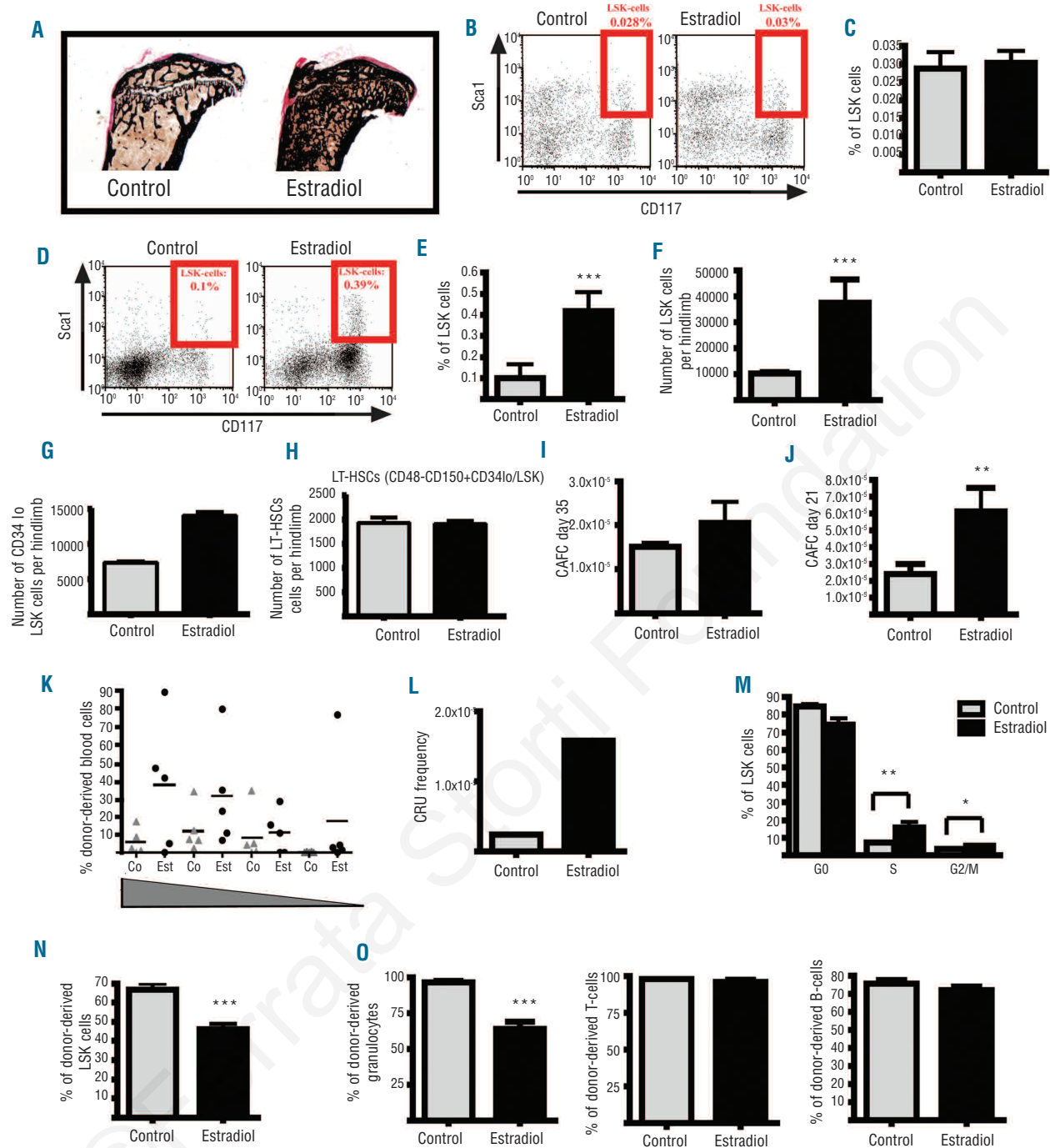
Figure 1. Estradiol increases the bone mass in wild-type mice and functional HSPCs in the vascular niche of the BM by an enhanced entry into the cell cycle resulting in early exhaustion of LT-HSCs. (A) von Kossa staining for the calcium-content of the bones. Tibias of estradiol treated mice show increased calcium-content due to increased bone mass. (B) Representative dot blots of the bone-adherent hematopoietic cells stained for lineage-negative / Sca1-positive / cKit-positive cells (LSK-cells) isolated from estradiol and control treated mice. (C) Summarized percentages of LSK-cells in the fraction of bone-adherent hematopoietic cells in control and estradiol treated mice (n=5). (D) Representative dot blots of the cells of the vascular HSC-niche stained for lineage-negative / Sca1-positive / cKit-positive cells (LSK-cells) isolated from estradiol and control treated mice. (E) Summarized percentages of LSK-cells in the vascular niche of control and estradiol treated mice (n=5). (F) Absolute numbers of LSK-cells per hindlimb in the vascular niche from control and estradiol treated mice (n=5). (G, H) Absolute numbers of CD34^{-/lo} LSK cells (G) and CD48-CD150^{+}CD34^{-/lo} LSK cells (H) per hindlimb in the vascular niche from control and estradiol treated mice (n=5). (I) Frequency of cobblestone area forming (CA-forming) colonies in the vascular niche of control and estradiol treated mice after 35 days of coculture. (J) Frequency of cobblestone area forming (CA-forming) colonies in the vascular niche of control and estradiol treated mice after 21 days of co-culture. (K) Percentages of donor-derived blood cells four months post transplantation with BM from control and estradiol treated mice in decreasing dilutions (dilutions: 540000, 180000, 60000, 20000 cells per mouse; n per dilution=5). (L) Calculated frequency of colony repopulating units (CRU), according to Poisson Statistics,[14] 4 months post transplantation with BM from control and estradiol treated mice. (M) Distribution of LSK-cells throughout G0/G1, G2/M and the S-phase of the cell cycle after treatment of mice with estradiol (n=5). (N) Percentage of donor-derived (CD45.2-positive) LSK-cells after the third transplantation with BM from control and estradiol treated mice (n=10). (O) Percentage of donor-derived (CD45.2-positive) granulocytes (Gr1/CD11b-pos. cells) and donor-derived B (CD19-positive) and T cells (CD4/CD8-positive) after the third transplantation with BM from conrol and estradiol treated mice (n=10).

Estrogen receptors ERα and ERβ are redundant for the effects of estradiol on HSPC numbers in the vascular niche

Next, we tested the involvement of estrogen receptors (ERα and ERβ)[20-22] in estradiol effects on HPSCs. Despite their well-established expression in bone, mRNA of both receptors is expressed also in HPSCs at comparable levels to that in ovaries, expressing high levels of ERα and ERβ (*Online Supplementary Figure S2A and B*). ERβ deficient mice displayed an increase in bone mass resulting in decreased cellularity, as in wild-type mice (Figure 2A), there was no alteration in endosteal HSPC numbers, and they showed increased vascular HSC numbers upon estradiol treatment (Figure 2B). In contrast, no increase in bone mass was observed in ERα-knockout mice (Figure 2C) and neither was any change seen in BM cellularity upon estradiol treatment (Figure 2D). The frequency of endosteal HSCs was also unaltered in ERα knockout mice (Figure 2E).
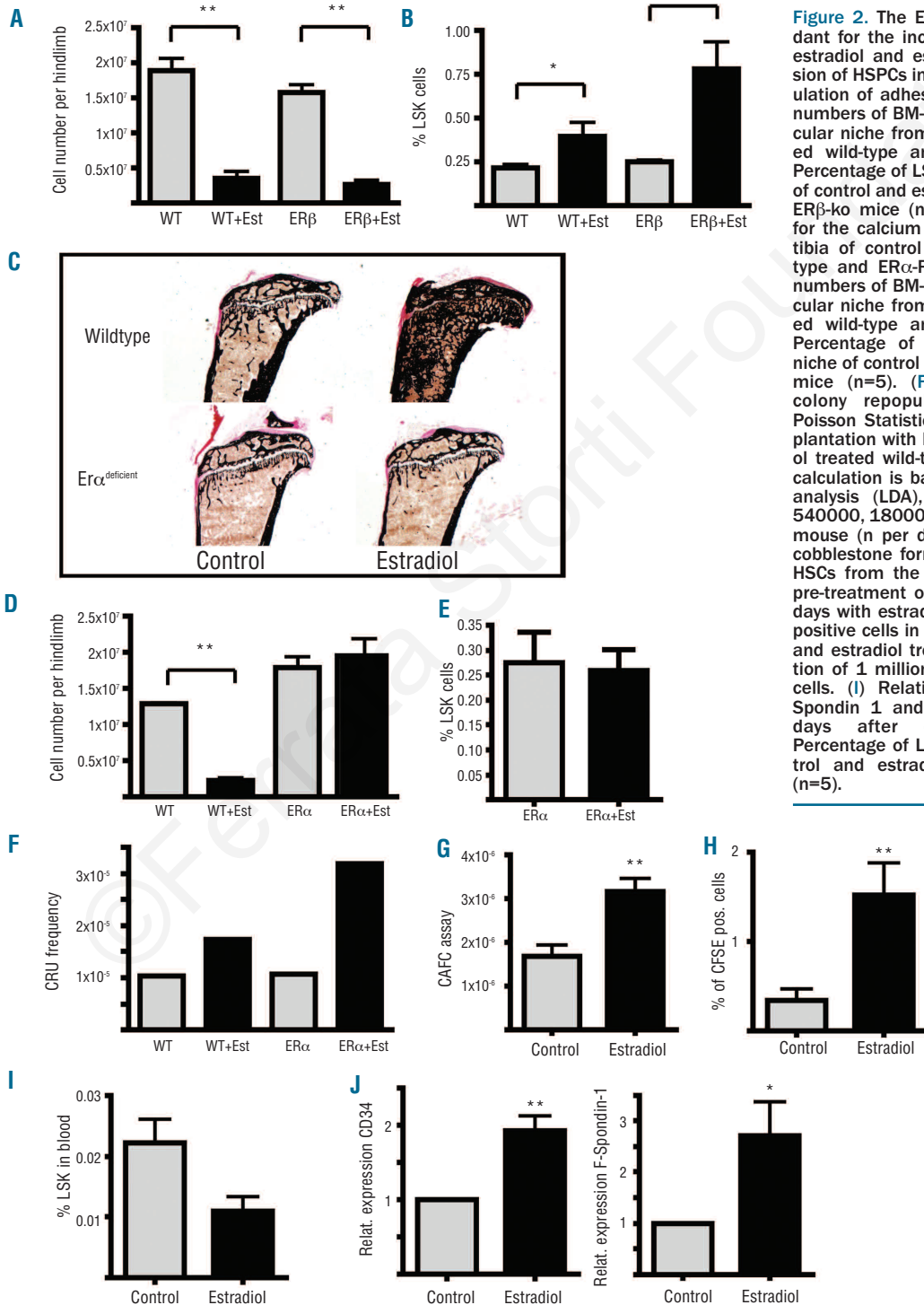

Figure 2. The ERα and the ERβ are redundant for the increase of vascular HSPCs by estradiol and estradiol increases the adhesion of HSPCs in the vascular niche by upregulation of adhesion molecules. (A) Absolute numbers of BM-cells per hindlimb in the vascular niche from control and estradiol treated wild-type and ERβ-ko mice (n=5). (B) Percentage of LSK-cells in the vascular niche of control and estradiol treated wild-type and ERβ-ko mice (n=5). (C) von Kossa staining for the calcium content of the bone on the tibia of control and estradiol treated wild-type and ERα-Runx2cre-mice. (D) Absolute numbers of BM-cells per hindlimb in the vascular niche from control and estradiol treated wild-type and ERα-ko mice (n=5). (E) Percentage of LSK-cells in the endosteal niche of control and estradiol treated ERα-ko mice (n=5). (F) Calculated frequency of colony repopulating units (CRU), using Poisson Statistics[14] four months post transplantation with BM from control and estradiol treated wild-type and ERα-ko mice. CRU-calculation is based on the limiting-dilution-analysis (LDA), recipients received either 540000, 180000, 60000 or 20000 cells per mouse (n per dilution=5). (G) Frequency of cobblestone forming areas (CAs) formed by HSCs from the BM of wild-type mice after pre-treatment of FBMD1-feeder cells for 10 days with estradiol. (H) Percentage of CFSE-positive cells in the vascular niche of control and estradiol treated mice 15 h after injection of 1 million CFSE-labeled wild-type BM cells. (I) Relative mRNA-expression of F-Spondin 1 and CD34 in FBMD1-cells, ten days after estradiol treatment. (J) Percentage of LSK-cells in the blood of control and estradiol treated wild-type mice (n=5).

Importantly, the frequency of vascular HSCs, reflected by CRUs, was also increased in ERα knockout mice (Figure 2F). These data confirm that the increase in estradiol-dependent changes in bone mass are independent of HSCs both in the endosteal and vascular compartment. Taken together, both ERs are either redundant for the phenotype resulting from estradiol treatment in the vascular HSC niche or the effects are mediated by another receptor, such as the membrane bound GPR30.[23,24]

Estradiol causes stem cell extrinsic alterations in the vascular HSC niche

To investigate the estradiol induced microenvironmental alterations we mimicked the niche by flask bone marrow Dexter-1 (FBMD1) cells, a murine preadipose stromal feeder cell line that is very efficient for maintaining HSCs *in vitro*.[13] FBMD1 cells were pre-treated for 14 days with estradiol followed by seeding of untreated wild-type BM cells in LDA. Pre-treatment of FBMD1 with estradiol leads to increased CA formation (Figure 2G) underscoring the fact that estrogen action on stromal cells can indirectly enhance HSC numbers.

Next, we performed a homing experiment with CFSE labeled untreated BM cells transplanted into estradiol treated recipients. Fifteen hours after homing, the vascular niche of estradiol treated recipients retained more CFSE positive cells than control treated recipients (Figure 2H). We conclude that estradiol does alter the microenvironmental cells resulting in increased HSC interactions as a prerequisite to an increase in HSC numbers.

Accordingly, we found a stronger retention of LSK cells in the BM upon estradiol treatment displayed by decreased LSK numbers in the peripheral blood (Figure 2I). Finally, we detected upregulation of CD34 and F-Spondin1 mRNA, both involved in cell adhesion, in estradiol treated FBMD1 cells (Figure 2J) and a tendency for enhanced expression in primary stromal cells (*Online Supplementary Figure S2C and D*). These data suggest that molecules involved in the mobilization and regulation of HSCs mediate estradiol effects in the vascular BM niche.

Conclusions

We demonstrate that estradiol leads to increased numbers of HSCs in the vascular niche but not in the endosteal niche of BM. This occurs also in the absence of increased bone mass in ERα knockout mice. Thus the increase in HSC number induced by estradiol is independent of estradiol effects on the bone. The increase in HSC numbers is due to enhanced cell cycle entry leading to HSC exhaustion and diminished long-term potential determined by serial transplantation. Here, we describe for the first time the differential effects of estrogens on HSCs in interaction with their vascular niche.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. Cell. 2004;116(5):769-78.
2. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity. 2006;25(6):977-88.
3. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425(6960): 841-6.
4. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425(6960):836-41.
5. Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocr Rev. 2010;31(3):266-300.
6. Samuels A, Perry MJ, Tobias JH. High-dose estrogen induces de novo medullary bone formation in female mice. J Bone Miner Res. 1999;14(2):178-86.
7. Antal MC, Krust A, Chambon P, Mark M. Sterility and absence of histopathological defects in nonreproductive organs of a mouse ERbeta-null mutant. Proc Natl Acad Sci USA. 2008;105(7):2433-8.
8. Dupont J, Karas M, LeRoith D. The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components. J Biol Chem. 2000;275(46):35893-901.
9. Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, et al. Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. Cell Metab. 2010; 11(6):517-31.
10. Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, et al. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. Bone. 2002;30(1):18-25.
11. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell. 2004;118(2):149-61.
12. Tuckermann JP, Kleiman A, Moriggl R, Spanbroek R, Neumann A, Illing A, et al. Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. J Clin Invest. 2007;117 (5):1381-90.
13. van Os RP, Dethmers-Ausema B, De Haan G. In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. Methods Mol Biol. 2008;430:143-57.
14. Stephen J. Szilvassy FEN, Connie J. Eaves und Cindy L. Miller. Quantitation of Murine and Human Hematopoietic Stem Cells by Limiting-Dilution Analysis in Competitively Repopulated Hosts. In: Jordan CAKaCT, ed. Hematopoietic Stem Cell Protocols. New York: Humana Press, 2002:167-87.
15. Trumpp A, Refaeli Y, Oskarsson T, Gasser S, Murphy M, Martin GR, et al. c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature. 2001;414(6865):768-73.
16. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood. 2001; 97(8):2293-9.
17. Kamminga LM, van Os R, Ausema A, Noach EJ, Weersing E, Dontje B, et al. Impaired hematopoietic stem cell functioning after serial transplantation and during normal aging. Stem Cells. 2005;23(1):82-92.
18. Muller-Sieburg C, Sieburg HB. Stem cell aging: survival of the laziest? Cell Cycle. 2008;7(24):3798-804.
19. Morita Y, Ema H, Nakauchi H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. J Exp Med. 2010;207(6):1173-82.
20. Vidal O, Lindberg M, Savendahl L, Lubahn DB, Ritzen EM, Gustafsson JA, et al. Disproportional body growth in female estrogen receptor-alpha-inactivated mice. Biochem Biophys Res Commun. 1999;265 (2):569-71.
21. Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C. Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta(-/-) mice. J Clin Invest. 1999;104(7):895-901.
22. Windahl SH, Norgard M, Kuiper GG, Gustafsson JA, Andersson G. Cellular distribution of estrogen receptor beta in neonatal rat bone. Bone. 2000;26(2):117-21.
23. Lindberg MK, Weihua Z, Andersson N, Moverare S, Gao H, Vidal O, et al. Estrogen receptor specificity for the effects of estrogen in ovariectomized mice. J Endocrinol. 2002;174(2):167-78.
24. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. Physiol Rev. 2001;81(4): 1535-65.