Gender Differences in Endothelial Progenitor Cells and Cardiovascular Risk Profile

The Role of Female Estrogens

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Objective—Endothelial progenitor cells (EPCs) participate in vascular homeostasis and angiogenesis. The aim of the present study was to explore EPC number and function in relation to cardiovascular risk, gender, and reproductive state.

Methods and Results—As measured by flow-cytometry in 210 healthy subjects, CD34+KDR+ EPCs were higher in fertile women than in men, but were not different between postmenopausal women and age-matched men. These gender gradients mirrored differences in cardiovascular profile, carotid intima-media thickness, and brachial artery flow-mediated dilation. Moreover, EPCs and soluble c-kit ligand varied in phase with menstrual cycle in ovulatory women, suggesting cyclic bone marrow mobilization. Experimentally, hysterectomy in rats was followed by an increase in circulating EPCs. EPCs cultured from female healthy donors were more clonogenic and adherent than male EPCs. Treatment with 17β-estradiol stimulated EPC proliferation and adhesion, via estrogen receptors. Finally, we show that the proangiogenic potential of female EPCs was higher than that of male EPCs in vivo.

Conclusions—EPCs are mobilized cyclically in fertile women, likely to provide a pool of cells for endometrial homeostasis. The resulting higher EPC levels in women than in men reflect the cardiovascular profile and could represent one mechanism of protection in the fertile female population. (Arterioscler Thromb Vasc Biol 2008;28:997-1004)

Key Words: gender ■ endothelium ■ cardiovascular risk ■ stem cells ■ cord blood

Endothelial progenitor cells (EPCs) are bone marrow–derived cells actively involved in cardiovascular homeostasis.1 In basal conditions, peripheral blood EPCs provide a circulating pool of cells that repair the ongoing endothelial damage. In the setting of ischemia, EPCs are mobilized from bone marrow to peripheral blood, home to the ischemic sites and stimulate compensatory angiogenesis.2 Reduced levels of circulating EPCs have been demonstrated in the presence of classical risk factors for cardiovascular disease;3 furthermore, EPC depletion has been shown to predict the development of adverse events.4,5 Therefore, EPCs are an integrated component of the cardiovascular system and are considered a novel biomarker of cardiovascular health.6

Women in the reproductive age are exposed to a lower cardiovascular risk than age-matched men.7 This is generally attributed to the differences in sex hormones and, specifically, to the protective cardiovascular properties of female estrogens.8,9 Besides the effects on plasma lipids and on the vessel wall, other mechanisms may link estradiol to a favorable cardiovascular profile. Preliminary experience has proposed that the hormonal status may modulate EPCs,10,11 but definite data and mechanistic insights in humans are still lacking. Aims of this study were to assess the relationships between EPCs, gender, reproductive state, and cardiovascular risk. Therefore, we explored: (1) gender gradients in EPC number and function in relation to surrogate indexes of cardiovascular risk; (2) EPC variations during menstrual cycle, and (3) effects of 17β-estradiol on EPC function using in vitro and in vivo models.

Materials and Methods

For complete methods, please see the supplemental materials, available online at http://atvb.ahajournals.org.

Study Population

The study was approved by the local ethic committee, and informed consent was obtained from all subjects. In a population of 210

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healthy subjects (104 males and 106 females) we determined cardiovascular parameters, sex hormones, high-sensitive C reactive protein (hsCRP), carotid intima-media thickness (c-IMT), and flow mediated dilation (FMD) of the brachial artery. We enrolled also 5 ovulatory and 5 anovulatory young women and determined their levels of circulating EPCs, plasma soluble c-kit ligand (skitL), and sex hormones during follicular, ovulatory, and luteal phases. Additionally, we collected blood samples from 12 male and 9 female prepuberal children, from 10 male and 10 female subjects aged ≥65, and from umbilical cord of 21 male and 21 female newborns.

Quantification of Circulating Progenitor Cells

Human progenitor cells were analyzed for the expression of surface antigens with direct flow cytometry as previously described using fluorescein isothiocyanate (FITC)-conjugated anti-CD34, PE-conjugated anti-KDR, and activated protein C (APC)-conjugated anti-CD133 mAbs (supplemental Figure 1). Human EPCs were defined as CD34+KDR+ cells, according to recent population-based studies. Rat progenitor cells were quantified using PE-conjugated anti-mouse sc-a-1, FITC-conjugated anti-c-kit, and FITC-conjugated anti-rat CD31 mAbs.

Cell Culture, Characterization, and Functional Assays

EPC isolation and culture were performed as previously described. To confirm their endothelial phenotype, cells were studied for the uptake of acLDL, binding of Ulex-lectin, and expression of CD34, CD31, KDR, vWF, CXCR4, and CD18. Expression of estrogen receptor and EnOSs were also assessed. Cell colonies were counted as an indicator of EPC proliferation. In separated experiments, growth medium was supplemented with 1-10-100 nmol/mL 17β-estradiol (E2). Cells cultured under 10 nmol/L E2 were cotreated with a nonselective ER inhibitor (ICI 182.780) or a ERα-selective inhibitor (MPP). The adhesive capacity of EPCs was evaluated on a monolayer of human umbilical vein endothelial cells (HUVECs), as previously described. To explore EPC function in vivo, we used a rat model of ischemia-reperfusion (IR) injury. Sections of IR muscles injected with EPCs were analyzed to quantify capillary density, the total number of EPCs and of apoptotic EPCs, as well as the number of EPCs integrated into the host microvasculature.

Table. Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Fertile Women (n=64)</th>
<th>Young Men (n=59)</th>
<th>Postmenopausal Women (n=42)</th>
<th>Age-Matched Men (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>41.2±0.7</td>
<td>44.3±0.9*</td>
<td>57.2±1.0†</td>
<td>55.7±0.9†</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>81.0±1.3</td>
<td>96.2±1.4*</td>
<td>85.8±2.1</td>
<td>96.3±1.3*</td>
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<tr>
<td>Body mass index, kg/m²</td>
<td>22.4±0.4</td>
<td>26.9±0.5*</td>
<td>24.4±0.6†</td>
<td>26.8±0.4*</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>118.1±1.5</td>
<td>129.7±1.6*</td>
<td>124.8±2.7†</td>
<td>128.2±1.6</td>
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<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>80.8±1.1</td>
<td>88.2±1.2*</td>
<td>80.4±1.4</td>
<td>86.9±1.3</td>
</tr>
<tr>
<td>LDL-C, mg/dl</td>
<td>108.5±4.0</td>
<td>139.6±3.6*</td>
<td>140.5±4.9†</td>
<td>136.1±3.9</td>
</tr>
<tr>
<td>HDL-C, mg/dl</td>
<td>60.4±1.6</td>
<td>46.2±1.3*</td>
<td>61.0±2.8</td>
<td>49.7±1.8</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>71.0±3.8</td>
<td>109.8±7.0*</td>
<td>95.2±7.0</td>
<td>111.2±8.6</td>
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<tr>
<td>Plasma glucose, mg/dl</td>
<td>84.3±1.4</td>
<td>93.3±1.7*</td>
<td>90.8±1.4†</td>
<td>97.4±1.8</td>
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<td>Smoking habit, %</td>
<td>21.8</td>
<td>11.8</td>
<td>19.0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*Statistically significant women vs age-matched men; †statistically significant vs the younger category of the same gender.

Results

EPC Levels Are Related to Cardiovascular Risk and Reproductive State

The study sample was representative of a healthy middle-aged general population. After recruitment, women were retrospectively divided into fertile (n=64) and postmeno-

EPC Gender Gradient Fluctuates in Lifetime

We determined EPC levels in cord blood of newborns, and in peripheral blood of prepuberal children and elderly individuals to establish whether these cells vary with age. We found that female newborns had far more CD34+KDR+ EPCs than males. Interestingly, this gender difference was inverted in prepuberal children, in favor of males. In the elderly, males and females were matched for age and risk factors, and there

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was no gender difference in EPC levels (Figure 1k). Across all ages, no significant gender gradients were found in the level of circulating CD133$^+$H110H110$^+$KDR$^+$ and CD34$^+$CD133$^+$KDR$^+$ cells (not shown).

EPC Are Mobilized During the Hormone/Menstrual Cycle

We hypothesized that increased EPC levels in the female fertile population were related to the menstrual/hormonal cycle. For this purpose, we prospectively enrolled 5 ovulatory and 5 anovulatory young women to determine their levels of circulating EPCs and plasma skitL. Variations of E2 and progesterone (P) were consistent with the presence/absence of ovulation in the 2 groups. In cycling women, a 2-fold increase in CD34$^+$KDR$^+$ EPCs in the ovulatory phase was paralleled by a significant increase in skitL. There was no significant variation of EPCs and skitL in anovulatory women across phases. There were no significant differences in the absolute levels of EPCs during follicular phase between ovulatory and anovulatory women (Figure 2).

Circulating EPCs Increase After Hysterectomy in Rats

To evaluate the impact of endometrial regeneration on EPC levels, we measured sca-1$^+$c-kit$^+$ and sca-1$^+$CD31$^+$ cells before and after hysterectomy in rats. Hysterectomy was performed with care to preserve anatomically and functionally the ovaries, and to favor maintenance of the ovarian
hormonal cycle. Two weeks later, sca-1/c-kit and sca-1/CD31 cells were increased significantly, suggesting that removal of the target organ with preservation of the hormonal cycle prevented EPCs to home at sites of endometrial regeneration/remodeling, thus increasing their levels in the circulation (supplemental Figure II).

Genuine Phenotype of Isolated Human EPCs

Cell Characterization
Human EPC isolation was performed according to a validated protocol. During growth in endothelial medium, a subset of PBMCs form colonies of endothelial cells which, after 2 weeks, display function and phenotype of endothelial cells: all survived cells bind lectin, take up LDL and are brightly positive for the surface expression of CD34, vWf, CD31, and KDR. Isolated EPCs were also positive for CXCR4 (SDF-1 receptor) and CD18 (ICAM receptor), which have been previously shown to be relevant to EPC function. Finally, cultured EPCs expressed eNOS (Figure 3a through 3d).

EPCs Express Estrogen Receptors
Immunofluorescence and Western blot analyses showed that EPCs express estrogen receptor α (Figure 3 days). Real-time polymerase chain reaction (PCR) revealed that the mRNAs of both α and β isoforms were present in cultured EPCs. The relative expression of both ER isoforms normalized to the housekeeping gene 18S was more abundant in male than in female EPCs cultured from peripheral blood and cord blood. Although expression of ER α and β isoforms did not differ in female EPCs, male EPCs form peripheral blood expressed much more ERα than ERβ, whereas ERβ was more abundant in male EPCs from cord blood (supplemental Figure III).

Gender Differences in EPC Function and Effects of E2

EPC Colony Formation
In basal conditions, the number of endothelial colonies 15 days after plating was significantly higher in samples derived from women than from men. Culture supplementation with E2 progressively increased colony yield from male samples, an effect that was prevented by ER inhibitors. E2 had little effect on female samples, but ER inhibition substantially decreased female EPC colonies from baseline in the setting of both E2-stimulated and unstimulated conditions (Figure 4a).

Adhesive Properties of EPCs
In basal conditions, female EPCs showed increased adherence to a mature endothelial layer with respect to male EPCs. After exposure to E2, adherence of male EPCs progressively increased, matching adhesion of female EPCs at 1 to 10 nmol/L E2. Adhesive properties of female EPCs increased significantly versus baseline when exposed to 100 nmol/L E2. Nonselective (ICI) and α-selective (MPP) ER inhibitors prevented the effects of E2 on EPC adhesion (Figure 4b).

In Vivo Vasculogenic Capacity of EPCs
To provide further evidence for gender differences in EPC function, we explored the vasculogenic capacity of male and female EPCs in a rat model of ischemia/reperfusion injury (Figure 4c and 4d). Two weeks after implantation into ischemic rat muscles, a similar number of male and female EPCs were found in the cryosections, and injected cells were not apoptotic (supplemental Figure IV), suggesting no difference in cell survival. However, the number of chimeric vessels (containing human CMTMR-labeled EPCs lining the vessel lumen), as well as the total capillary density, were higher with transplanted female than male EPCs (Figure 4 days).

Discussion
The main findings in this study are the following: (1) gender differences in EPC number and function correlate with surrogate indexes of cardiovascular risk; (2) EPC gender gradient is present at birth and fluctuates during lifetime; (3) worsening of the cardiovascular risk profile after menopause is associated with EPC decline; and (4) EPCs are regulated by E2 in vitro and in vivo.

The low cardiovascular risk in fertile women is attributable to the antiatherosclerotic effects of estrogens, which improve endothelial function.7,8 Fertile women included in our study had a healthier risk profile than men and postmenopausal women. Parallely, FMD and c-IMT indicated a better vascular homeostasis in females than in males, and fertile women had higher levels of circulating CD34 KDR EPCs than men. This difference was likely related to gender per se rather than to the effects of concomitant risk factors, as shown by the
independent association between gender and EPCs in the multivariate analysis. The impact of risk factors was stronger in women, and the EPC gender gradient was tapered in the presence of at least 2 cardiovascular risk factors. After menopause, there were no gender differences in the risk profile, FMD, c-IMT, and CD34\(^+\) KDR\(^+\) EPCs. This is in compliance with a recent small study showing lower CD34\(^+\) KDR\(^+\) EPCs in postmenopausal versus premenopausal women.\(^{10}\) Given that EPCs are actively involved in endothelial healing and reflect the global cardiovascular health,\(^{6}\) the gender-related difference in EPCs represents a plausible explanation for the difference in endothelial function and c-IMT. After menopause, EPC reduction, attributable to aging, to the change in the reproductive state, and to the worsened risk profile, may cause endothelial dysfunction and predispose to atherosclerosis. Our data contradict a recent small study showing that, in comparison with men, postmenopausal women had higher EPCs,\(^{11}\) which were however isolated with an outdated method (CFU-ECs) which is now known to select a subpopulation of monocytes/macrophages, instead of endothelial cells.\(^{16}\) It is worth remarking that a clear gender difference in EPC levels has not been reported previously because very few studies have been conducted with healthy subjects, so that any gender difference was masked by the underlying cardiovascular risk and advanced age.

Studying ovulatory women, we found that EPCs and soluble c-kit ligand vary in phase with menstrual/hormonal cycle. As bone marrow membrane–bound c-kit ligand is cleaved by SDF-1–stimulated MMP9 activity during progenitor cells mobilization,\(^{17}\) we suggest that EPCs are mobilized to the peripheral circulation of fertile women on a monthly basis. These results confirm and integrate a recent study by Masuda et al, showing cyclic E2-regulated bioactivity of EPCs.\(^{18}\) In this view, the higher steady-state levels of circulating EPCs in fertile women than in men may reflect their cyclic mobilization, possibly related to the vascular regeneration and remodeling taking place in the endometrium. Endometrial homing is likely to be directed by the local production of growth factors and chemokines (such as VEGF and SDF-1) that favors recovery after menstrual discharge, and governs vascular proliferation and remodeling. As an indirect demonstration that EPCs are mobilized for endometrial recruitment, we show in rats that removing the uterus while preserving the ovarian cycle increased sca-1\(^+\) CD31\(^+\) EPCs, which probably accumulated in the circulation because they could not find their target organ. Consistently with this scenario, after human HLA- or sex-mismatched bone marrow transplantation, marrow cells contributed for up to 50% of stromal cells and to 14% of endometrial endothelial cells.\(^{19,20}\)
Figure 4. Comparison between male (M) and female (F) EPCs in terms of colony formation (a) and adhesion to a HUVEC monolayer (b) in basal conditions and after exposure to E2, with and without nonselective (ICI) and α-selective (MPP) ER inhibitors. *P<0.05 in comparison with basal conditions; †P<0.05 comparing male with female EPCs. c, Representative sections of postischemic rat muscles injected with human CMTMR (red)-labeled EPCs. Capillaries stained in green with CD31. Dual positivity indicates that EPCs were physically integrated into the vessel wall (yellow signal, arrows and boxes). In the background, phase contrast photographs. d, Postischemic capillary muscle density, total CMTMR-labeled cells, and percentage of chimeric vessels (CMTMR/CD31) after transplantation of female or male EPCs. *P<0.05 comparing female with male.
whereas the contribution of bone marrow–derived EPCs to cyclic endometrial vascular turnover has been recently demonstrated in mice.18

Uncertainties still exist on the exact antigenic definition of EPCs.21 For ex vivo human studies, we defined EPCs as CD34+KDR+ cells because this phenotype was previously shown to correlate with cardiovascular risk and subclinical atherosclerosis better than other antigenic combinations,12,22 and proved as an independent predictor of future cardiovascular events.5,6 Additionally, CD34+KDR+ cells are believed to represent adult hemangioblasts and behave like EPCs in vivo.23,24 Consistently, CD34+/KDR+ were selectively modulated, whereas other putative EPC phenotypes, such as CD133+KDR+ and CD34+/CD133+KDR+ cells, showed no gender gradients throughout ages.

We isolated EPCs from healthy young male and female donors to analyze gender differences in EPC function. There is no consensus on the protocols to isolate EPCs, but our culture method underwent an extensive validation, by showing that cultured cells display antigenic and functional characteristics of endothelial cells, including eNOS expression (which is considered a stringent criterion),25 thus corresponding to “true late EPCs,” rather than to the so-called “monocytic EPCs.”26 Moreover, being CD34+KDR+CD31+CD18+CXCR4+CD18+ and acLDL lectin+ cells isolated in vitro represent a subpopulation of the CD34+KDR+ population addressed in the ex vivo study. We found that female blood cells gave rise to a higher number of endothelial colonies than male cells, suggesting a gender difference in EPC generation. Moreover, a gender difference was clearly seen in the capacity of EPCs to adhere to a mature endothelium, a fundamental property required to reconstitute in vivo the dysfunctional intimal layer. Finally, using a rat model of ischemia/reperfusion injury, we show that female EPCs, when transplanted into male rat ischemic muscles, stimulated compensatory angiogenesis more efficiently than male EPCs, and were more frequently integrated into the host microvasculature. We were able to detect only a small proportion of chimeric vessels bearing human EPCs, which could not explain the strong increase in capillary density after ischemia. The intramuscular route of administration is believed to cause early apoptosis of injected cells, and may explain in part this paradox. Nonetheless, our results agree with previous observations that the proangiogenic effect of EPCs occurs in a paracrine fashion and not only through the direct integration into new vessels.27

The quantitative and functional differences in EPCs between young men and women, together with the observation that EPCs are mobilized during the hormonal cycle, strongly indicated that EPCs are influenced by female sex hormones. In fact, available experimental studies support a link between estrogens and EPCs. In ovariectomized animals, estrogen deficiency decreased circulating EPCs, whereas estrogen replacement increased EPC levels and stimulated EPC-mediated reendothelization after carotid injury.28,29 Moreover, E2 enhanced functional and anatomic recovery after experimental myocardial infarction through recruitment of EPCs.30 We confirm that E2 has a potent ability to stimulate human EPCs. Available data indicate that these effects are mediated through inhibition of apoptosis, stimulation of telomerase, and bone marrow mobilization.28,31,32 Herein, we report that KDR expression on CD34+ cells was higher in fertile women than in men, although it was similar in postmenopausal women and in age-matched men. This may indicate that E2 promotes differentiation of circulating CD34+ progenitor cells into CD34+/KDR+ EPCs in vivo, but we failed to confirm this in vitro, as E2 did not modify the expression of endothelial markers during EPC culture (supplemental Figure V).

We demonstrate that EPCs express both ER isoforms and that E2 dose-dependently enhances EPC generation and adhesion in vitro. Interestingly, male EPCs were more responsive to the mitogenic effects of E2, whereas female EPCs seemed to be maximally stimulated in basal conditions. To explore possible reasons for this different responsiveness to E2, we quantified gene expression of ER isoforms: male EPCs expressed larger amounts of ERs than female EPCs, with a prevalence of the vasculoprotective α isoform, which may mediate the potent functional stimulation induced by E2, perhaps through upregulation of VEGF.30 Nonselective and α-selective ER inhibition prevented the ability of E2 to promote generation and adhesion of EPCs, confirming that these effects are mediated by classical ER pathways. Interestingly, ER inhibition abolished the higher generation of female EPCs in both basal conditions and E2-stimulation, suggesting a sort of “constitutive” activation of ERs in female cells, as pharmacological inhibition downregulates both ligand-dependent and -independent ER activity.33

As sex hormone concentrations vary not only during the fertile cycle in women, but also with age, we investigated EPC gender differences throughout human ages. We report a marked difference in EPC levels in the cord blood of newborns in favor of females. The predominance of ERβ over ERα in male cord blood EPCs may be responsible for the lower levels of circulating EPCs: in fact, the 2 receptor isoforms act as antagonists and their relative expression drives downstream events that determine the net effect of E2.33 This may represent a sort of prenatal imprinting of the endogenous vascular regenerative potential. In prepuberal children, EPC gender difference was inverted, in favor of males, suggesting that female EPCs are more sensitive to the fall in sex hormone concentrations during the prepuberal phase. Finally, there was no EPC gender gradient in the elderly. As already known from other studies,34 we confirm that age is one major determinant of EPC level in the multivariate analysis, but EPC level tended to be relatively stable over time in males, whereas it widely fluctuated in females. This trend, apparently inconsistent with the observation that E2 influenced male EPCs more than female EPCs in vitro, could be attributed to a differential expression of ER isoforms, and to the different effects of continuous versus cyclic hormonal stimulation in men versus women.

In conclusion, we provide a series of data indicating that EPCs are regulated by sex hormones in humans. Cyclic EPC mobilization may be related to endometrial regeneration; the resulting gender gradients in EPC number and function reflect the cardiovascular protection of the fertile female population. After menopause, on cessation of both ovarian
and endometrial function, female EPCs decrease to the levels of coeval males, thus hampering vascular homeostasis and increasing cardiovascular risk.

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None.

References
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MATERIALS AND METHODS

**Study population.** As part of a population-based study, 210 healthy subjects (104 males and 106 females) were recruited from a local community of office employees on the basis of an agreement with the University of Padova. The study was approved by the local the local Institutions and informed consent was obtained from all subjects. Exclusion criteria were the following: known cardiovascular disease, pulmonary disease, acute illness of infection, recent (within 6 months) surgery, immunological disease or immunosuppression, pregnancy, lactation. Fertile women were studied in their self-reported follicular phase. Clinical data were recorded to determine cardiovascular risk factors: hypertension (defined as a systolic blood pressure higher that 140 mmHg or a diastolic blood pressure higher that 90 mmHg), obesity (defined as a body mass index higher than 30 kg/m² or a waist circumference higher that 102 for men and 88 cm for women), diabetes (defined using both fasting and two-hour post-challenge plasma glucose concentrations), smoking habit (of one or more cigarettes per day), family history (defined as early cardiovascular disease in one or more first-degree relatives) and hyperlipidemia (defined as a LDL-cholesterol concentration higher than 160 mg/dl or a triglyceride concentration higher that 200 mg/dl). None of the subjects was taking medications at the time of the study. In all statistical analysis except for that reported in figure
parameters that define cardiovascular risk factors were kept as continuous in order to avoid biases deriving from dichotomization. Menopause was defined as absence of menses for at least six months, without other apparent causes of amenorrhea. As a biomarker of subclinical inflammation, C-reactive protein was measured using the high-sensitive technique (hs-CRP). 17β-estradiol, progesterone and free testosterone were measured radioimmunologically using commercially available kits (Diagnostic System Laboratories, Webster, Texas, US) according to manufacturer’s instructions.

In addition to this population-based study, we prospectively enrolled from the local community 5 ovulatory and 5 anovulatory (because of low-dose oral hormonal contraception) women aged 24-31 years and determined their levels of circulating EPCs, plasma soluble c-kit ligand (skitL, also known as stem cell factor, SCF) by ELISA assay (R&D Systems) and sex hormones during follicular, ovulatory and luteal phases.

Finally, after obtaining consent from parents, we collected blood samples from male (n=12) and female (n=9) prepuberal children (median age 9 years, range 2-14), who underwent a primary screening program for celiac disease, and resulted normal. They were healthy as reported by their primary care physicians. Children with any medical problem or developmental/growth alteration were excluded.

We also recruited a sample (10 men and 10 women) of subjects aged ≥65 years from patients referred to the outpatient clinic for cardiometabolic evaluation, provided they were completely free from cardiovascular disease. The characteristics of this sample are resumed in supplemental Table II.

**Collection of cord blood.** After obtaining informed consent from the mother, blood samples were drawn from the vein of term male (n=21) and female (n=21) infant’s umbilical cord, during delivery with cesarean section, according to the local practice. Pregnancies with
relevant problems such as infections, premature birth, pre-eclampsia, gestational diabetes, fetal malformations, and altered cardiotocography were excluded. Gestational age (mean±SD) was 38.2±1.2 weeks. All newborns had normal 1-5 minutes Apgar scores and there was no evidence of in-hospital disease.

**Measurement of carotid intima-media thickness (c-IMT).** c-IMT was measured by quantitative high-resolution B-mode ultrasound of the far wall of the right and left common carotid arteries. The measurements were carried out as previously described according to a validated procedure,¹ using a HDI-5000 SONO CT ultrasound machine (Philips Medical System/ATL S.p.a.) equipped with a Phased array 4-7 MHz transducer. The flow divider between the internal and external carotid arteries was identified and the common carotid arteries were explored starting 1 cm below the flow divider. Measurements were done by tracing the leading edge of the lumen-intima and the media-adventitia interfaces. Maximum right and left IMT were averaged to obtain the c-IMT measure. No subject had carotid plaque, defined as c-IMT >1.5 mm.

**Measurement of flow-mediated dilation (FMD) of the brachial artery.** Endothelial function was evaluated by measurements of FMD of the brachial artery in response to hyperemia according to previously described and validated methodology.² The right brachial artery was imaged above the antecubital fossa using B-mode ultrasound, and flow was measured using pulsed-wave Doppler. Simultaneous electrocardiographic recordings were obtained to trigger ultrasound vascular images to avoid artefacts due to heart beats. Baseline measurement was followed by 5-min occlusion of arterial flow, achieved by inflation of a pneumatic cuff distally to the probe to suprasystolic pressure (250 mm Hg). The brachial artery was visualized continuously following deflation of the pneumatic cuff for 3 min of
hyperemia. Brachial artery diameters were measured at baseline (by averaging 5 random
diameters taken within 2 cm of artery length) and every 10 seconds during hyperemia to
identify the maximal vasodilation. FMD was calculated as the percent ratio of maximal to
baseline diameters. The entire procedure was digitally stored and measurements were
performed by a single trained operator.

Quantification of circulating progenitor cells. Progenitor cells were analyzed for the
expression of surface antigens with direct two- or three- color flow cytometry (FACS Calibur,
Becton Dickinson) as previously reported. Briefly, before staining with specific monoclonal antibodies, cells were treated with fetal calf serum for 10 min, and the samples washed with buffer containing phosphate-buffered saline and 0.5 percent bovine albumin. Then, 150 µl of peripheral blood were stained with 10 µl of FITC-conjugated anti-human CD34 mAb (Becton Dickinson), 10 µl of PE-conjugated anti-human KDR mAb (R&D Systems) and 10 µl of APC-conjugated anti-CD133 mAb (Miltenyi Biotech). Control isotype IgG1 and IgG2a Abs were obtained from BD. The frequency of peripheral blood cells positive for the above reagents was determined by a 2D side scatter-fluorescence dot plot analysis, after appropriate gating, stained with the different reagents. We gated CD34+ or CD133+ peripheral blood cells in the mononuclear cell fraction and then examined the resulting population for the dual expression of KDR. Triple positive cells were identified by the dual expression of KDR and CD34 or CD133 in the CD133 or CD34 gates, respectively (supplemental Figure I). For FACS analysis, 5x10^5 cells were acquired and scored using a FACS Calibur analyzer (BD). Data were processed using the Macintosh CELLQuest software program (BD). The instrument set-up was optimized daily by analyzing the expression of peripheral blood lymphocytes labeled with anti-CD4 FITC/CD8 PE/CD3 PECy5/CD45 APC four color combination. The same trained operator, who was blind to the clinical status of the patients,
performed all the tests throughout the study. Human EPCs were defined as CD34⁺KDR⁺ cells, according to recent population-based studies.⁴,⁵

**Cell culture.** EPC isolation and culture were performed as previously described. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient (Sigma-Aldrich, St. Louis, MO) from buffy coats of healthy blood donors aged <40 years. Cells were plated on six-well fibronectin-coated dishes (BD) at a density of 6x10⁶ cells per well. Cells were grown in supplemented endothelial cell growth medium (Clonetics, Baltimore, MD) for 15 days. Culture medium was changed first on day 4 and then every two days until phenotypical characterization was performed. Starting from day 1, growth medium was supplemented with 1-10-100 nM 17β-estradiol. With this culture system, attaching cells rapidly assume an endothelial-like shape and, starting from day 3 to 6 of culture, cells proliferate in clusters/colonies made up of a central core of rounded cells surrounded by radiating spindle-shaped cells, which are considered endothelial colonies. Colonies were quantified on day 15 after plating in ten randomly selected microscopic fields. It is currently agreed that cultures prolonged for two weeks allow positive selection of true late endothelial progenitor cells, that should represent the only cell type remaining two weeks after plating. At the end of the culture protocol, isolated cells tend to reach confluence, and form a cobblestone monolayer (supplemental Figure VI).

**Characterization of cultured EPCs.** At day 15 of culture, cells were characterized to confirm their endothelial phenotype. After washing with PBS, cells were incubated at 37°C with 1 μg/ml DiI-acetylated low density lipoproteins (DiI-AcLDL, Molecular Probes, Eugene, OR) for 1 hour, followed by dark incubation with 15 mg/ml FITC-conjugated Ulex lectin (Sigma-Aldrich) for 2 hours. Nuclei were stained in blue with Hoechst 33258 (Sigma-
Aldrich). EPCs were defined as cells double positive for AcLDL and lectin. For further characterization, we stained putative EPCs with other endothelial markers. Cells were fixed and incubated with anti-von Willebrand Factor (vWF) (Dako Cytomation, Glostrup, Denmark), anti-KDR (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-CD31 (Chemicon International) antibodies and secondary Cy2 anti-rabbit (Chemicon International, Temecula, CA) and Cy2 anti-mouse (Chemicon International) antibodies. Positive cells were then visualized under a fluorescent microscope. To have further methodological confirmation of endothelial phenotype, cultured EPCs were detached using EDTA and analyzed by flow cytometry for the expression of KDR (R&D Systems), CD34, CD31, CXCR4 and CD18 (BD).

To monitor for endothelial differentiation during the culture period, cell treated with 0-1-10-100 nM E2 were examined at day 0 (freshly isolated PBMCs before plating) at day 4, 11, and 15 after plating. At each time point cells were gently detached with EDTA and examined for the surface expression of CD34, KDR, and CD31, as above described.

**Expression of Estrogen Receptor.** EPCs were studied for the expression of estrogen receptor (ER) using three different techniques. Immunofluorescence (IF) was performed with cells stained with a mouse anti-human ERα monoclonal antibody (AbCam ab2746, Cambridge, UK), which does not react with the β isoform according to an extensive literature provided by the supplier, and a secondary PE-conjugated anti-mouse antibody and counterstained with FITC-conjugated lectin as above described. Nuclei were stained in blue with Hoechst 33358 (Sigma-Aldrich). IF showed both cytoplasmic and nuclear staining. Western blot (WB) was performed using the same anti-ERα antibody and EPC protein extracts. Finally, to study the relative expression of the α and β isoforms, we performed real-time polymerase chain reaction (PCR): RNA was extracted using a commercially available kit (RNABle, Eurobio, France) with 1 ml of product per 5x10^6 cells. Reverse transcription of
RNA was performed with Gene Amp RNA PCR Kit, essentially as described by the manufacturer (Gene Amp RNA PCR Kit, Perkin Elmer, USA). RNA (1 µg) was reverse transcribed using random hexamer primers and MuLV reverse transcriptase in a Perkin Elmer 2400 thermalcycler (15 min at 42°C, 5 min at 99°C and 5 min at 5°C). Amplification was performed on the Taqman 7300 (Applied Biosystems, Foster City, CA) using TaqMan® Universal PCR Master Mix, gene-specific TaqMan probes and primers and a standard 2-steps thermal cycler protocol (95°C for 15 sec, 60°C for 1 min, repeated 40 times). Specific primers were from Applied Biosystems (Hs00174860_m1 for ERα, Hs00230957_m1 for ERβ, Hs99999901_s1 for 18S). Relative quantification of gene expression was calculated by the comparative C_T method and normalized to the eukaryote housekeeping gene 18S.

**eNOS protein expression.** eNOS protein expression was performed using western blot analysis. Total protein extracts were obtained by cell lysis with a ice-cold buffer (Tris HCl 20 mM, NaCl 150 mM, EDTA 5.0 mM, Niaproof 1.5%, Na_3PO_4 1.0 mM, SDS 0.1%, PMSF 0.5mM) added with proteases inhibitors. Protein concentration was determined using the BCA Assay (Pierce, Rockford, IL); proteins were then separated by SDS-PAGE, transferred onto nitrocellulose membranes (Hybond ECL, Amersham, Uppsala, Sweden) and blocked overnight with no-fat milk (5% in Tween-PBS). Membranes were probed with primary polyclonal antibody anti-NOS3 antibody (SantaCruz, Biotechnology, CA). After incubation with proper secondary antibodies HRP-conjugated (Amersham Biosciences, Uppsala, Sweden), immunoreactive proteins have been visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, USA).

**Adhesion assay.** We evaluated *in vitro* the functional property of EPCs to adhere to mature endothelium, as previously described. For this purpose, a monolayer of human
umbilical vein endothelial cells (HUVECs, Clonetics) was prepared 48 hours before the assay by plating $2 \times 10^5$ cells/cm$^2$ (passage 5 to 8). EPCs were labeled with DiI-LDL as above described and $1 \times 10^5$ cells were added to each well and incubated for 2 hours at 37°C. Non-attached cells were gently removed with PBS, and adherent EPCs were fixed with 4% p-formaldehyde in PBS and counted in 10 random fields.

**Estrogen receptor blocking.** To study the contribution of estrogen receptor (ER) isoforms $\alpha$ and $\beta$ in the effect of E2 on EPCs, we used a non-selective ER inhibitor (ICI 182.780) and a ER$\alpha$-selective inhibitor (1,3-Bis(4-Hydroxyphenil)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole, MPP). ICI 182.780 is a steroidal estrogen antagonist that was designed to be devoid of estrogen agonist activity. It has been demonstrated to completely inhibit the ability of the ER to activate or repress transcription in a ligand-dependent or -independent manner.$^7$ In previous studies, ICI 182.780 was shown to inhibit the favourable effects of E2 on EPC proliferation, migration and apoptosis.$^8$ MPP is obtained by conjugating the PPT molecule (an ER$\alpha$ agonist) with a basic side chain, turning into a selective ER$\alpha$ antagonist. MPP is the most selective ER$\alpha$ inhibitor known thus far.$^9,10$ These molecules are used *in vitro* on the understanding that they have shown consistent anti-estrogenic effects *in vivo*. From this point of view, the pharmacological approach may be more clinically relevant than a ER gene knockout. To perform experiments, starting from day 1 in culture, cells were treated with ICI (1 $\mu$M) or MPP (10 $\mu$M) either in the presence or in the absence of E2. Proliferation and adhesion were examined as described. E2 was used at the sex-specific minimal efficacious concentration to stimulate EPC: 10 nM for male cells, and 100 nM for female cells.
In vivo angiogenesis assay. To explore EPC function in vivo, we employed a rat model of ischemia-reperfusion (IR) injury. Briefly, male Sprague-Dawley rats (n=5/group) underwent a 2-hour hind limb IR injury as follows. Animals were anesthetized by inhaled isofluorane. Distal limb perfusion was monitored with a laser Doppler flowmeter (Periflow, Perimed Italy). A 9 mm-large digit cuff (Perimed, Italy) was placed around the thigh and connected to a standard manometer. The cuff was inflated with air until the laser Doppler recorded biological zero flow and the pressure on the manometer was above 200 mmHg. Perfusion and pressure in the cuff were monitored during the experiment to assure continuous and stable ischemia: additional cuff inflations were performed when pressure fall occasionally below 200 mmHg or perfusion tended to rise. After 2 hours, the cuff was deflated to allow reperfusion. Meanwhile, cultured EPCs were washed with PBS and stained with the orange dye CMTMR (5-(and-6)-(4-chloromethyl-benzoyl-amino)-tetramethylrhodamine) (Molecular Probes) 0.5 µg/ml. Immediately after reperfusion, the tibialis anterior (TA) muscle was exposed and $2 \times 10^6$/kg labelled EPCs were injected intramuscularly. Animals were killed two weeks after EPC implantation and the TA muscles were harvested. Five µm muscle cryosections were observed under a fluorescent microscope to visualize CMTMR-labelled EPCs. Nuclei were stained with Hoescht. Elongated cellular structures double positive for CD31 and CMTMR were considered chimeric vessels bearing human EPCs. To confirm the integration of injected cells into the host endothelium, sections were also visualized under a confocal microscope (Confocal Platform Leica TCS SP5, Leica Microsystems, Milan, Italy), as shown in figure 3c. Density of neovessels, total number of EPCs for section and number of EPCs incorporating into host vessels were determined. In a pilot preliminary study (n=3 animals), we examined hindlimb perfusion during the 2-hour ischemia and two-week reperfusion using a Laser Doppler Imager.
(PeriScan, Perimed, Italy). This allowed us to confirm that reperfusion is complete and that no tissue ischemia persists at the end of the protocol (supplemental Figure VII).

**In situ detection of apoptotic cells.** To establish whether EPCs underwent apoptosis after intramuscular injection, we used a fluorescein-based in situ detection kit (ApopTag Plus, Chemicon International) according to the manufacturer’s instructions. Briefly, tissue cryosections were fixed with 1% p-formaldehyde in PBS pH 7.4, then washed twice with PBS and post-fixed with ethanol:acetic acid 2:1. After application of the equilibration buffer (15 µl/cm²), sections were incubated with TdT enzyme and reaction buffer for 1 hour at room temperature and, after washing, with the anti-digoxigenin conjugate (13 µl/cm²). Nuclei were counterstained with Hoechst, and apoptotic cells were visualized under a fluorescent microscope using standard fluorescein excitation and emission filters.

**Induction of hysterectomy and EPC count in female rats.** Female Sprague-Dawley rats, weighting 200 to 225 g, were obtained from Charles River (Calco, Italy). Rats (n=3) were housed with food and water available ad libitum in an environmentally controlled animal facility with a 12-hour light/dark cycle. All procedures conformed to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23, revised 1996). For hysterectomy, rats were anesthetized intraperitoneally with a mixture of ketamine hydrochloride (40 mg/kg) and xylazine (20 mg/kg). After a midline incision to expose the abdominal and pelvic cavities, the uterine vessels were ligated and care was taken to preserve collateral supplies. Each uterine horn was excised at the junction with the fallopian tube. The abdomens were then sutured and the animals returned to their cages. Postoperative recovery was uneventful. Rats were then killed and ovaries harvested to verify maintenance of ovulation. Control coeval female rats (n=3) underwent sham operation,
consisting of laparotomy, exposition of abdominal viscera and suture. Blood samples were
drawn at baseline and 14 days after surgery (hysterectomy or sham operation).

Circulating rat EPC were quantified with two color flow cytometry. Briefly, 150 µl of
peripheral blood were stained with 10 µl of PE-conjugated anti-mouse sca-1 mAb (Cederlane,
Hornby, ON, Canada), 10 µl of FITC-conjugated anti-mouse c-kit mAb (BD Biosciences) and
10 µl of FITC-conjugated anti-rat CD31 mAb (BD Biosciences). The frequency of peripheral
blood cells positive for the above reagents was determined by a 2D side scatter-fluorescence
dot plot analysis, after appropriate gating, stained with the different reagents. We first gated
sca-1⁺ peripheral blood cells in the mononuclear cell fraction and then examined the resulting
population for the dual expression of c-kit or CD31. Total sca-1⁺, c-kit⁺ and sca-1⁺c-kit⁺, sca-
1⁺CD31⁺ populations were determined. Rat EPCs were defined as Sca-1⁺CD31⁺ cells.

**Statistical analyses.** Data are expressed as mean±SEM. Results from flow cytometry
are reported as cells/10⁶ cytometric events. Comparison between two groups was performed
using two-tail Student’s t test and the χ²-test was used for dichotomous variables. Bonferroni
correction was applied to account for multiple testing. To identify variables independently
correlated with EPC count, a multiple stepwise regression analysis was performed, including
the 210 subjects of the population-based study, without segregation for menopausal status.
Interaction between age and gender was assessed using a univariate Generalized Linear
Model (GLM). Parameters that define cardiovascular risk factors were always kept as
continuous variables, except for the determination of the number of risk factors used for the
analysis reported in panel 1D. Statistical significance was accepted at p<0.05.
REFERENCES


**Table I.** Multiple stepwise linear regression analysis to identify variables independently correlated with CD34\(^+\)KDR\(^+\) cell count (dependent variable). Parameters that concur to determine cardiovascular risk factors were kept as continuous explanatory variables whenever possible in order to avoid biases of dichotomization. The GLM showed significant interaction between age and gender.

<table>
<thead>
<tr>
<th>Variables in the equation</th>
<th>Variables not in the equation</th>
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</thead>
<tbody>
<tr>
<td><strong>β</strong></td>
<td><strong>p</strong></td>
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<tr>
<td>Gender (male)</td>
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<tr>
<td>Age</td>
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<td>Systolic Blood Pressure</td>
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<tr>
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<tr>
<td>Smoking habit</td>
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<tr>
<td>Waist circumference</td>
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<tr>
<td>Body Mass Index</td>
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<tr>
<td>Plasma glucose</td>
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Table II. Characteristics of the sample of elderly subjects. No significant difference was found between men and women. The p-values were calculated using Student’s t test of chi square, where appropriate.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Women (n=10)</th>
<th>Men (n=10)</th>
<th>p</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>73.0±5.1</td>
<td>75.5±1.9</td>
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<tr>
<td>Waist circumference (cm)</td>
<td>88.9±12.3</td>
<td>94.1±3.4</td>
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<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>24.7±3.9</td>
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<td>Systolic blood pressure (mmHg)</td>
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<td>126.1±4.8</td>
<td>0.54</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>87.5±4.4</td>
<td>85.0±3.8</td>
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<tr>
<td>LDL-C (mg/dl)</td>
<td>127.7±11.8</td>
<td>121.0±10.6</td>
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<tr>
<td>HDL-C (mg/dl)</td>
<td>45.7±4.6</td>
<td>45.9±4.9</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>154.4±20.8</td>
<td>165.3±22.7</td>
<td>0.73</td>
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<tr>
<td>Plasma glucose (mg/dl)</td>
<td>95.2±4.4</td>
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<td>0.53</td>
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<td>Family history (%)</td>
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<td>Smoking habit (%)</td>
<td>0.0</td>
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FIGURE LEGENDS

**Figure I.** The gating strategy used to enumerate human progenitor cells by flow cytometry. After morphological gate on mononuclear cells, CD34 or CD133 cells were identified and then assayed for the expression of KDR. Triple positive CD34⁺CD133⁺KDR⁺ cells were identified at the intersection of the CD34⁺KDR⁺ and CD133⁺KDR⁺ phenotypes.

**Figure II.** A) The gating strategy used to enumerate total sca-1⁺, total c-kit⁺ and sca-1⁺c-kit⁺, sca-1⁺CD31⁺ cells by flow cytometry in peripheral blood of female rats before and after isolated hysterectomy. B) sca-1⁺, c-kit⁺, sca-1⁺c-kit⁺ and sca-1⁺CD31⁺ cells significantly increased after hysterectomy. No variation was seen in sham-operated rats (not shown) *p<0.05 (paired Student’s t test).

**Figure III.** Expression of estrogen receptor (ER) α and β in male (M) and female (F) EPCs cultured from peripheral blood of young adults or cord blood of term newborns. EPCs were from 3 independent donors in each group. cDNA dilution for ER amplification was 1:5, that for the housekeeping gene 18S was 1:100. Assays performed at least in duplicate.

* *p<0.05 comparing male with female cells.

**Figure IV.** *In situ* fluorescein-based detection of apoptotic cells. Tissue sections were analysed for cell apoptosis using the ApopTag kit. Apoptotic cells were detected by their green fluorescent. Human EPCs were red labelled with CMTMR and injected intramuscularly immediately after ischemia/reperfusion injury to study their *in vivo* vasculogenic potential. Nuclei were counterstained in blue with Hoechst, on the background of phase-contrast images. Very rarely, injected EPCs showed signs of apoptosis and no differences were found in the
apoptotic rate between male and female cells. a) Section of tibialis anterior muscle injected with EPCs, two weeks after ischemia-reperfusion injury: the arrow indicates a representative example of an apoptotic EPCs identified for the green/red double signal. b) Section of tibialis anterior, two days after ischemic injury: some apoptotic muscle fibers and interstitial cells are present (arrows, green cells). c) Negative control: section of non-ischemic tibialis anterior. d) Positive control: arrows indicate apoptotic cells (green) within a section of testis tubuli, as provided by the manufacturer of the kit.

**Figure V.** Expression of EPC markers during cell culture was not significantly modulated by supplementation with 1-10-100 nM E2. No significant differences were detected between male and female cells (not shown).

**Figure VI.** A pilot study (n=3 animals) to monitor perfusion during the 2h/14d ischemia/reperfusion (I/R) injury in rats. During the 2h-ischemia, perfusion fell significantly from baseline. After reperfusion, blood flow returned to normal values within 7 days, and remained normal for the following 7 days of monitoring. Mean±SD.

**Figure VI.** A) Visual comparison between putative EPCs cultured using either the EndoCult™ Liquid Medium Kit (according to the instructions provided by the manufacturer, Stem Cells inc.) or the custom culture protocol described in the current study and used previously by our group (male donor). The left panels show that CFU-EC obtained with EndoCult™ rapidly dissolve into a steady slowly-proliferating, non-confluent cell population. Right panels show how during the two-week culture protocol implemented in our study, adherent cells form colonies that progressively dissolve into a confluent monolayer of endothelial-shaped cells. B) Flow cytometry assessment of cells cultured according to the two
methods. Cell obtained with EndoCult™ have a lower side and forward scatter, are negative for CD34 and weakly positive for CD31 and KDR. Cells isolated according to the procedure described in this study include a well-defined population of larger cells, that are brightly positive for CD34, CD31 and KDR.
FIGURE I
FIGURE II

A

BASAL

HYSTERECTOMY

B

Sca1+

cKit+

Sca1+CD31+

Sca1

c-Kit

Sca1

c-Kit

Cell count
FIGURE III

Relative expression vs 18S

Peripheral blood EPCs  Cord blood EPCs

- ERalpha
- ERbeta

* Statistically significant difference
FIGURE IV

a

b

c
d
FIGURE V

CD31

KDR

CD34

Days after plating
FIGURE VI

A

<table>
<thead>
<tr>
<th>Day 5</th>
<th>EndoCult™</th>
<th>EPCs</th>
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B

SSC vs FSC

CD34

CD31

KDR
FIGURE VII

Baseline Ischemia         Acute reperfusion 14 days

Baseline  Ischemia  Acute reperfusion  14 days