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## Effects of Androgens on Endothelial Progenitor Cells in Vitro and in Vivo

**Short title:** "Androgens and endothelial progenitors"

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**ABSTRACT**

Beneficial or detrimental effects of androgens on the cardiovascular system are debated. Endothelial progenitor cells are bone marrow-derived cells involved in endothelial healing and angiogenesis, which promote cardiovascular health. Estrogens are potent stimulators of endothelial progenitor cells, and previous data indicated that androgens may improve biology of these cells, as well. Herein, we show that testosterone and its active metabolite dihydrotestosterone exert no effects on expansion and function of late endothelial progenitors isolated from peripheral blood of healthy human adult males, while they positively modulate early "monocytic" endothelial progenitors. In parallel, we show that castration in rats is followed by a decrease in circulating endothelial progenitor cells, but that testosterone and dihydrotestosterone replacement fails to restore endothelial progenitor cells toward the normal levels. This is associated with persistently low estrogen levels after androgen replacement in castrated rats. In a sample of 62 healthy middle-aged men, we show that circulating endothelial progenitor cell levels are more directly associated to estradiol rather than to testosterone concentrations. Our data collectively demonstrate that androgens exert no direct effects on endothelial progenitor cell biology in vitro and in vivo.

## INTRODUCTION

In the last decade, our knowledge on vascular homeostasis has significantly changed thanks to the discovery of endothelial progenitor cells (EPCs) in the adult human blood [1]. EPCs originally reside in the bone marrow and other putative niches [2], they can be mobilized in the peripheral circulation in response to many stimuli and, once in the bloodstream, actively take part in endothelial repair and formation of new blood vessels [3]. Many experimental studies have substantiated this model, suggesting that vascular homeostasis depends not only on resident cells, but also on the relevant contribution of circulating EPCs [4]. Reconstitution and maintenance of an intact endothelial layer is a crucial homeostatic function of the cardiovascular system because endothelial damage is the earliest step in the atherogenetic process [5]. Angiogenesis is another fundamental event in health and disease, as it is involved in physiologic conditions, such as body growth and menstrual cycle, and in the setting of pathological conditions, such as ischemic syndromes and wound healing. Being closely linked to endothelial repair and angiogenesis, EPCs represent an integrated component of the cardiovascular system [6]. Consistently with this view, it has been shown that subjects with risk factors for or established CVD have a depletion of circulating EPCs [7]. Much interestingly, patients with lower levels of EPCs in the bloodstream have a higher risk of cardiovascular events [8-10]. These data have suggested that depletion of EPCs is a pathogenic event that, through the inability to maintain an intact endothelium and to promote angiogenesis, translate risk factors into the development and progression of CVD [5]. Moreover, the comprehensive role of EPCs in CVD suggested that EPC level may be used as a surrogate marker of vascular damage and overall cardiovascular risk [11].

Cardiovascular risk gradients are primarily driven by gender: women in the reproductive age have a much lower incidence of cardiovascular events than age-matched

men [12]. This is generally attributed to the vasculoprotective actions of female estrogens, which are mediated by favourable effects on lipid profile, inflammatory and haemostatic parameters [13]. Interestingly, experimental studies show that  $17\beta$ -estradiol (E2) mobilizes EPCs from the bone marrow and enhance EPC function [14, 15]. We have definitely demonstrated that women have higher levels and better functions of EPCs than age-matched men, thanks to the cyclic EPC stimulation for endometrial regeneration [16]. Therefore, sex hormone-driven gender-specific modulation of EPCs may be one mechanism that accounts for the gender gradient in cardiovascular risk. There are some data suggesting that EPCs may be also regulated by androgens. In one study, hypogonadal men showed depletion of *ex vivo*-determined circulating EPCs, which were restored by pharmacological treatment with testosterone [17]. In another study, potential favourable effects of androgens on EPCs were shown *in vitro* [18]. However, these findings were likely influenced by the methods used to enumerate and isolate EPCs [19, 20], which have profound effects on study results [20, 21].

Herein, we explored the role of androgens in EPC biology by showing: i) the relationships among EPC levels, testosterone and E2 concentrations in healthy men; ii) the effects of testosterone and its active metabolite dihydrotestosterone on early EPCs and late EPCs isolated *in vitro* using a standardized and extensively characterized protocol; iii) the effects of testosterone deficiency on circulating EPCs in a rat model of primary hypogonadism *in vivo*.

## MATERIALS AND METHODS

**Patients.** Healthy men were recruited from a local community of middle-aged individuals who responded to an advertising for health screening. Exclusion criteria were any recent (within 3 months) acute or chronic disease or infection, known cardiovascular disease (ruled out by minimal criteria, including history and physical examination), immunological disease. On the basis of these criteria, 62 healthy men were included. We recorded age, smoking habit (of one or more cigarettes), body mass index (calculated from height and weight), waist circumference, systolic and diastolic blood pressure. Blood samples were drawn for the determination of plasma fasting glucose, lipid profile (total cholesterol, HDL cholesterol, triglycerides concentration), sex hormones (17 $\beta$ -estradiol and total testosterone), and progenitor cells. Informed consent was obtained from all subjects. The study protocol was in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and has been approved by the local Ethics Committee.

**Cell culture.** For isolation of late EPCs, buffy coats of young ( $\leq 40$  year old) male healthy blood donors were used, and culture was performed as previously described [11, 16]. Briefly, peripheral blood mononuclear cells (PBMCs) were separated using Ficoll density gradient (Sigma-Aldrich, St. Louis, MO). Cells were plated on 6-well fibronectin-coated plates (BD) at a density of  $6 \times 10^6$  cells per well, and 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. Phenotypical characterization was performed at day 15. To study the effects of androgens on culture EPCs, starting from day 1, growth medium was supplemented with 1-10-100 nM T or DHT. As a positive control, E2 at 1-10-100 nM was used. 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 or small colonies made up of a central core of rounded cells surrounded by radiating spindle-shaped cells. Starting from day 10-12 cells also organize in large colonies of cobblestone confluent cells, which are considered endothelial colonies. Clusters were quantified every three days starting from day 6 to day 15 in ten randomly selected microscopic fields. It is currently agreed that cultures prolonged for two weeks allow positive selection of true EPCs, that should represent the only surviving cell type [19, 20]. The AUC of the number of EPC clusters over days 6-15 is taken to represent the clonogenic expansion capacity.

Early EPCs were cultured from PBMCs of young male healthy blood donors, as previously described [22]. Briefly, cells were plated on 6-well fibronectin-coated plates at a density of  $6 \times 10^6$  cells per well, and grown in supplemented endothelial cell growth medium (Clonetics, Baltimore, MD) without medium change. After 4 days, cells were washed and stained with DiI-acetylated low density lipoproteins and Ulex lectin (see bottom). Cells double positive for DiI-AcLDL and lectin were considered early EPCs and counted in ten randomly selected high power fields.

**Characterization of cultured EPCs.** After 15 days, late EPCs were characterized to confirm the 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). Dual positivity for these markers has been previously suggested as a necessary but not sufficient criterion to identify EPCs. 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 CD34 (a stem cell marker), KDR (R&D Systems) and CD31 (BD). As an *in vivo* functional assay, we injected isolated EPCs into male Sprague-Dawley rat (Charles River, Wilmington, MA) muscles subjected to 2 hour ischemia/reperfusion injury, as previously described [16, 23]. Meanwhile, cultured EPCs were detached with EDTA, 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. Muscle capillaries were stained using an anti-rat CD31 (Chemicon International) antibody on 5µm muscle cryosections, and observed under a fluorescent microscope. Nuclei were counter-stained in blue with Hoescht. Elongated cellular structures containing both rat CD31- and CMTMR- positive cells were considered chimeric vessels bearing human EPCs.

**Adhesion assay.** We evaluated *in vitro* the functional property of late EPCs to adhere to mature endothelium, as previously described [11, 16]. 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<sup>2</sup> at early passages. EPCs were labelled with DiI-LDL as described above 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.

**Expression of androgen receptor.** Late EPCs were assayed for the expression of androgen receptor (AR) using immunofluorescence (IF) and Western blot (WB). IF was performed with cells stained with the mouse anti-human AR monoclonal antibody F39.4.1, directed against the N-terminal part of the AR protein, amino acids 301–320 (Santa Cruz Biotechnology), and a secondary FITC-conjugated anti-rabbit antibody. Nuclei were stained in blue with Hoechst 33358 (Sigma-Aldrich). For WB, EPCs were detached with EDTA, and 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<sub>3</sub>PO<sub>4</sub> 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 antibody anti-AR (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). A positive control was provided by a prostate protein extract. We also extracted proteins for WB from HUVECs (Clonetics, USA) 4<sup>th</sup>-5<sup>th</sup> passage, and PBMCs immediately after isolation.

**Aromatase activity.** As an indirect assay of aromatase activity, we incubated confluent late EPCs with 0-1-10-100 nM testosterone for 24 hours and then measured E2 concentrations (RIA estradiol assay, Immunotech SAS, Marseille, France) in the surnatant

culture media. As a negative control, a cell-free system was prepared, consisting of plates filled with culture media without cells, incubated for 24 hours with identical testosterone concentrations.

**Rat castration.** To study *in vivo* the effects of androgen deprivation and restoration, we used a rat model of castration followed by androgen replacement. The protocol was approved by the local competent Institutions. Briefly, adult male Sprague-Dawley rats underwent bilateral castration under ketamine/xylazine anaesthesia (50 mg/kg and 20 mg/kg *i.p.*, respectively). A sham operation consisted of opening the scrotum, manipulating gonads and suturing the overlying skin. After a 4-week period of recovery, blood samples were drawn for determination of sex hormones (RIA estradiol and total testosterone assays, Immunotech SAS, Marseille, France) and rat progenitor cell count. Then castrated rats were implanted subcutaneously with a silicone tube (inside diameter, 1.7 mm; outside diameter, 3.4 mm; length, 10 mm; Dow-Corning, Midland, MI, USA) containing crystals of either T (n=3) or DHT (n=3). Two weeks after beginning of androgen treatment, blood was taken by heart puncture under deep urethane anaesthesia.

**Progenitor cell counts.** Circulating progenitor cells were analyzed by flow cytometry on peripheral blood samples as previously described in detail [7, 20]. Briefly, after red cell lysis, 10  $\mu$ l of whole blood was incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 and PE-conjugated anti-KDR. We first gated CD34<sup>+</sup> cells and then assessed this population for KDR expression in the mononuclear cell fraction (Figure 1).  $1 \times 10^5$  cells were always acquired and human EPCs were defined as CD34<sup>+</sup>KDR<sup>+</sup>. Rat progenitor cells were defined as Sca-1<sup>+</sup>c-kit<sup>+</sup> and Sca-1<sup>+</sup>CD31<sup>+</sup> cells using PE-conjugated anti-mouse sca-1, FITC-conjugated anti c-kit or FITC-conjugated anti-rat CD31 mAbs, and the same gating

strategy used for human cells (Figure 5). We have previously shown that the rat Sca-1+/c-kit+ cell population is enriched with putative rat EPCs [23], and the Sca-1+CD31+ phenotype can be taken to represent the rat homolog of the human CD34+KDR+.

**Statistical analysis.** Data are expressed as mean  $\pm$  standard error (SEM) unless where specified. Progenitor cell count is expressed as cell number /  $10^6$  cytometric events. Comparison between two or more groups was assessed with Student's t test and ANOVA, respectively. The Least Significance Difference (LSD) test was used for multiple testing. The Chi square was used for frequencies and categorical variables. A multiple regression analysis was built with EPC level as depending variables and sex hormones concentrations as explanatory variables to look for independent associations. SPSS ver 13.0 was used and statistical significance was set at  $p=0.05$ .

## RESULTS

**EPCs and sex hormones in humans.** The 62 men under study were representative of a middle-aged (mean±SD 46.8±7.4 years) population with low prevalence of cardiovascular risk factors. In the whole group, there was a non-significant correlation trend between CD34+KDR+ EPC levels and E2 ( $r=0.20$ ;  $p=0.10$ ) or T ( $r= -0.19$ ;  $p=0.12$ ) concentrations. A significant correlation was found between EPC levels and the T/E2 ratio ( $r=-0.33$ ;  $p=0.006$ ). Then, men were divided into four groups according to median levels of E2 and T concentrations: group 1) high T with high E2 ( $n=20$ ); group 2) high T with low E2 ( $n=14$ ); group 3) low T with high E2 ( $n=14$ ); group 4) low T with low E2 ( $n=14$ ). Clinical characteristics of these four groups are resumed in Table 1. Among groups, there were minimal differences in BMI and lipid profile, while the other cardiovascular risk parameters were comparable. In terms of EPC levels, the only significant difference was between group 2 and 3 ( $48.5\pm 13.2$  versus  $82.0\pm 29.7$ ;  $p=0.035$ ; Figure 1). A multiple regression analysis including all variables listed in table 1 as determinant of EPC levels showed that E2 was positively associated with EPC, independently of T (standardized  $\beta=0.28$ ;  $p=0.05$ ). Taken together, these data suggest that E2 is a stronger determinant of EPCs than T.

**The phenotype of late EPCs.** To isolate late EPCs, we employed a culture method without pre-plating. This assay leads to purification of a population of blood-derived cells with a true endothelial phenotype and proliferative potential. During the first days of culture, the cell population is heterogeneous. Subsequently, medium change and removal of non-adherent cells allows a progressive selection of cells with an endothelial-like morphology, organizing in clusters and colonies (Figure 2A-B). After 15 days of culture, virtually all cells in the plate display an endothelial behaviour, as demonstrated by the uptake of acetylated

LDL, binding of Ulex lectin and expression of typical endothelial markers, such as KDR (VEGFR-2), CD31, and vWf (Figure 2C-J). We confirmed the expression of CD31 and KDR using both immunofluorescence and flow cytometry (Figure 2N-R). These cells *in vitro* were also positive for CD34, revealing as very similar to the EPC phenotype determined *ex-vivo* with flow cytometry (CD34+KDR+). To have a final functional demonstration that isolated cells behave like EPCs *in vivo*, we injected them into rat muscles subjected to ischemia/reperfusion injury. Two weeks after the ischemic damage, injected cells were clearly incorporated into the endothelial layer of the host vasculature, suggesting that they take part in angiogenetic processes (Figure 2M).

**Androgens have no effects on late EPC expansion in vitro.** In a separate set of experiments (n=3 each), the medium used to culture late EPCs was supplemented with 1-10-100 nM T, DHT, or E2. Cell clusters were counted every three days after plating, as a measure of cell proliferation. While the area under curve (AUC) of number of clusters over time reflects the proliferative kinetics, cell clusters at day 15 is taken to represent the EPC yield and the end of the culture protocol. Both these parameters were not significantly influenced by T and DHT supplementation at any concentration. Conversely, 10 nM E2 were sufficient to increase the AUC of EPC expansion and cluster numbers at day 15 (Figure 3A-B), upper and middle panels).

**Androgens have no effects on late EPC adhesion in vitro.** As a functional assay, we studied the ability of EPCs to adhere to a mature endothelial layer. This is a critical property for EPCs to exert their vasculoprotective action by means of both reendothelialization and neoangiogenesis. Culture supplementation with T or DHT had no effect on EPC adhesion, at

any concentration. Conversely, 10 nM E2 was sufficient to increase the adhesive property of EPCs to a mature layer of HUVECs (Figure 3C).

**Androgens increase early EPCs.** As a control experiment, we isolated early EPCs, which were defined as DiI-AcLDL and lectin double positive cells 4 days after plating. We show that supplementation with 1 nM T, 1-10 nM DHT and 10-100 nM E2 during the 4-day culture protocol significantly increased the generation of early EPCs from PBMCs, as compared to the unsupplemented experiments (Figure 3D).

**Androgen receptor and aromatase activity in late EPCs.** By using IF we show that cultured EPCs express the androgen receptor (Figure 2K), but WB shows a low expression as compared to prostate extract (Figure 4A). Aromatase activity was very low as well: incubation of EPCs with 10 nM testosterone for 24 hours allowed achievement of about 0.03 nM E2 concentration in the culture medium (Figure 4B). However, it should be noted that, in the absence of significant effect of androgens on cultured EPCs, the issue of aromatase activity is of limited value.

**Effect of castration on rat circulating EPCs in vivo.** It has been suggested that androgen deprivation in hypogonadal men is associated with EPC depletion. We meant to verify this observation using an animal model of primary hypogonadism to simulate androgen deprivation. Four weeks after castration, circulating levels of Sca-1+c-kit<sup>+</sup> cells and Sca-1+CD31<sup>+</sup> cells dropped markedly versus baseline. As expected, there was also a decline in T and E2 concentrations. A 2-week T supplementation was able to restore T concentrations towards physiologic levels, while it had modest and non-significant effects on E2 levels. Supplementation with either T or DHT was unable to restore progenitor cell levels toward the

baseline values (Figure 5). No differences were seen in EPC levels after sham-operation versus baseline (not shown).

## DISCUSSION

The roles of androgens in the cardiovascular system are not clearly defined. In clinical studies, both androgen excess and deficiency have been reported to exert negative effects on cardiovascular parameters and endothelial function [24, 25]. However, these studies may be confounded by the association among androgen deficiency, estrogen deficiency and classic cardiovascular risk factors. EPCs represent an integrated part of the cardiovascular system, as they are involved in endothelial homeostasis and angiogenesis [5]. Alterations in EPC biology are recognized as modifiable pathogenic biomarkers of cardiovascular risk and, at the same time, therapeutic targets of drugs with vascular effects. Ways to increase EPC levels are actively pursued through different strategies ranging from available medications to the frontiers of cell therapy. EPC assays have been used to test the “endotheliotropic” activity of many drugs including statins, ACE inhibitors, glitazones, and estrogens [16, 26-28].

In this study, we show that androgens (T and its active metabolite DHT) exert no direct effect on late EPCs *in vitro* and *in vivo*, as evidenced by standardized assays, including colony formation, adhesion, and response to androgen replacement *in vivo*. These findings apparently contradict a previous report showing modest but significant increases in EPC proliferation, migration and colony formation after treatment with a synthetic androgenic agonist [18]. There may be several reasons for this discrepancy, including the use of a different androgen receptor agonist, but the most important issue seem to be the cell culture

method. Foresta et al. isolated putative EPCs according to the Colony Forming Unit assay first described by Hill (CFU-Hill) [29], which correspond to the so-called early “monocytic” EPCs. Early EPCs are believed to reflect a population of immune cells mimicking endothelial cells [19, 30, 31]. These endothelial-like cells express CD45 and CD14 and have a limited proliferation capacity in culture, thus representing blood monocytes or macrophages. In the present study, we isolated late EPCs according to a highly standardized culture protocol, and extensively characterized these cells *in vitro* and *in vivo*. These late EPCs are CD34+KDR+CD31+AcLDL+Lectin+CD45-CD14- and therefore phenotypically different from monocytic cells. They also behave like EPCs *in vivo*, being able to incorporate in the host microvasculature after transplantation into ischemic limbs. While late EPCs isolated in the present study fulfil most criteria required to define true EPCs, including *in vivo* activity, the most recent methodological reviews suggest early EPCs have little in common with endothelial cells and should not be considered true EPCs [19, 20, 32]. Indeed, we confirm that androgens stimulates early EPCs, while we show that androgens have no effects on late EPCs. Interestingly, as noted previously [21], different methods to study EPCs can yield different or even opposite results. On the contrary, E2 seems to stimulate function of both early and late EPCs *in vitro* from male and female donors [14, 15, 33], and its effects on EPCs has been substantiated also *in vivo* [16, 34]. Moreover, E2 regulates EPC cycle for endometrial regeneration, providing fertile women with a larger EPC pool for cardiovascular homeostasis [16]. These data collectively suggest that estrogens are more important than androgens in the regulation of endogenous EPCs in humans.

One point in favour of the effect of androgens on human EPCs *in vivo* is the observation that hypogonadal men display low levels of circulating EPCs [17]. However, those hypogonadal men had low plasma concentrations of both E2 and T. Therefore, EPC reduction could not be definitely attributed to T deficiency. The observation that T



replacement in hypogonadal men was followed by restoration of a normal EPC pool is insufficient to support a direct effect of T on EPCs, because T treatment also increased E2 concentrations, expectedly through aromatase activity.

In the present study, we developed an experimental model of primary hypogonadism by performing castration in adult male rats. As seen in humans, rat hypogonadism was associated with low T and E2 levels and a profound reduction of circulating Sca-1+c-kit<sup>+</sup> and Sca-1+CD31<sup>+</sup> progenitor cells, which represent rat EPCs. However, replacement therapy with infusion of T or DHT was unable to restore EPC levels. The most reasonable explanation of this negative result is that T replacement was able to partially restore T but not E2 concentrations, while DHT replacement could not generate E2, as DHT is not substrate of aromatase. The incomplete restoration of T concentrations by exogenous T would be unlikely to explain the sustained profoundly reduced EPC levels, even if T had a direct effect on EPCs, because a partial effect on EPCs would be expected. The different gonadotropin level in the experimental model as compared with the human condition is not likely to influence the effects of androgen deficiency on EPCs, as long as a direct effect of gonadotropins on EPCs is not assumed.

Taken together, these experimental findings suggest that androgens have no direct effects on human true EPCs, and that E2 is likely to be the hormonal drive of EPC regulation in men as well as in women. To have a further support of this notion in humans, we selected a sample of men from the general population. While we found no significant correlation between EPCs and either T or E2, there was a significant negative correlation between EPCs and the T/E2 ratio, revealing a possible complex interplay between estrogens and androgens in males, possibly influenced by aromatase activity. This negative correlation might actually reveal an even detrimental effect of T on circulating EPCs. Moreover, when subjects were divided into four groups according to high or low levels of E2 and T, it appeared that high E2

with low T was associated with higher EPC levels than high T with low E2. Finally, a multivariable analyses identified E2 as a determinant of EPC levels independently of T. These data in healthy men support our experimental results indicating that androgens do not stimulate EPCs. If androgens eventually exert negative effects on EPCs remains to be determined. This is clearly not an argument against the desirable effects of androgen replacement in hypogonadal men, but does not support the use of androgens to stimulate EPCs.

In conclusion, we found no evidence in support of a direct effect of androgens on EPC biology. As noted in other settings, the methods used to study EPCs are critically important and may largely affect study results [20, 21]. Importantly, as already demonstrated in women, our results indicate that E2 rather than T is likely to be the major hormonal drive of EPC kinetics in men.

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**Table 1.** mean  $\pm$  standard deviation. Comparisons were performed with post-ANOVA LSD. \* significantly different versus group 4; \*\* significantly different versus group 1. \*\*\* significantly different versus group 2.

Characteristic	High E2 / High T	Low E2 / High T	High E2 / Low T	Low E2 / Low T
Number	20	14	14	14
Age (years)	47.7 $\pm$ 6.8	45.9 $\pm$ 7.1	45.4 $\pm$ 7.0	48.0 $\pm$ 9.1
Smoking habit (%)	15	21	0	7
Family history (%)	55	43	36	57
Body mass index (kg/m <sup>2</sup> )	26.4 $\pm$ 4.0	26.4 $\pm$ 3.6	28.8 $\pm$ 3.8*	25.9 $\pm$ 4.0
Waist circumference (cm)	97.0 $\pm$ 8.0	96.8 $\pm$ 10.4	100.6 $\pm$ 11.1	94.5 $\pm$ 11.0
Systolic blood pressure (mm Hg)	131.0 $\pm$ 10.3	129.3 $\pm$ 10.1	130.9 $\pm$ 16.6	127.1 $\pm$ 7.3
Diastolic blood pressure (mm Hg)	90.4 $\pm$ 9.3	86.9 $\pm$ 7.4	88.9 $\pm$ 10.4	85.7 $\pm$ 7.6
Plasma glucose (mg/dL)	94.3 $\pm$ 10.4	93.9 $\pm$ 10.4	98.2 $\pm$ 17.9	91.0 $\pm$ 15.0
Total cholesterol (mg/dL)	197.7 $\pm$ 28.4	201.3 $\pm$ 30.4	210.8 $\pm$ 23.7	214.5 $\pm$ 18.4
HDL-cholesterol (mg/dL)	49.4 $\pm$ 11.7	42.3 $\pm$ 6.8**	44.0 $\pm$ 6.5	43.9 $\pm$ 11.1
LDL-cholesterol (mg/dL)	128.1 $\pm$ 22.4	138.5 $\pm$ 28.5	143.7 $\pm$ 25.9	147.6 $\pm$ 22.3**
Triglycerides (mg/dL)	101.0 $\pm$ 48.3	102.5 $\pm$ 41.6	115.6 $\pm$ 50.5	114.8 $\pm$ 55.3
17 $\beta$ -estradiol (pg/mL)	19.2 $\pm$ 2.4	13.9 $\pm$ 2.4	18.6 $\pm$ 2.1	13.6 $\pm$ 2.3
Total testosterone (pg/mL)	20.7 $\pm$ 4.7	16.8 $\pm$ 3.3	11.5 $\pm$ 1.5	11.0 $\pm$ 2.8
CD34 <sup>+</sup> KDR <sup>+</sup> EPCs (/10 <sup>6</sup> events)	61.6 $\pm$ 22.1	48.5 $\pm$ 13.2	82.0 $\pm$ 29.7***	61.2 $\pm$ 22.8

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## FIGURE LEGENDS

**Figure 1. Progenitor cell count in healthy men.** Left-hand panels show the gating strategy used to enumerate CD34+KDR+ cells in healthy men. First, CD34+ cells are identified in the ungated white blood cell population. Then, expression of KDR is assayed in the CD34+ cell gate in order to identify and quantify CD34+KDR+ cells. CD34+KDR+ cells were lower in men with high T and low E2 compared to subjects with low T and high E2, \* $p \leq 0.05$ ; SSC, side scatter; T, testosterone; E2, 17beta-estradiol. Where not specified, x- and y-axis represent arbitrary fluorescence units.

**Figure 2. Characterization of EPCs cultured from healthy men.** A) Clusters / small colonies of EPCs at day 12. B) Cobblestone monolayer of EPCs at day 15. C-J) EPCs isolated from healthy male blood donors with a prolonged standardized protocol uptake acetylated LDL, bind Ulex-lectin, and stain positive for CD31, KDR and vWf. K) Cultured EPCs at 15 days also stain positive for the androgen receptor. L) Representative microphotograph of red (Cmtmr)-labelled EPCs adhering on top of a HUVEC monolayer (nuclei are counterstained in blue). M) A red (Cmtmr)-labelled EPC incorporated into the host capillary network (FITC-CD31) after transplantation into an ischemic rat muscle (nuclei are counterstained in blue). N-R) The antigenic phenotype of EPCs was confirmed by flow cytometry as CD45-CD14-CD31+KDR+CD34+.

**Figure 3. Effects of androgens on EPC expansion and adhesion.** A) T and DHT supplementation have no effects on the number of EPC colonies in culture. B) A quite similar trend was evident for the AUC of EPC colonies over the 15 days of culture. C) T and DHT had no effect on adhesion of EPCs to a HUVEC monolayer. E2 served as positive control. D)

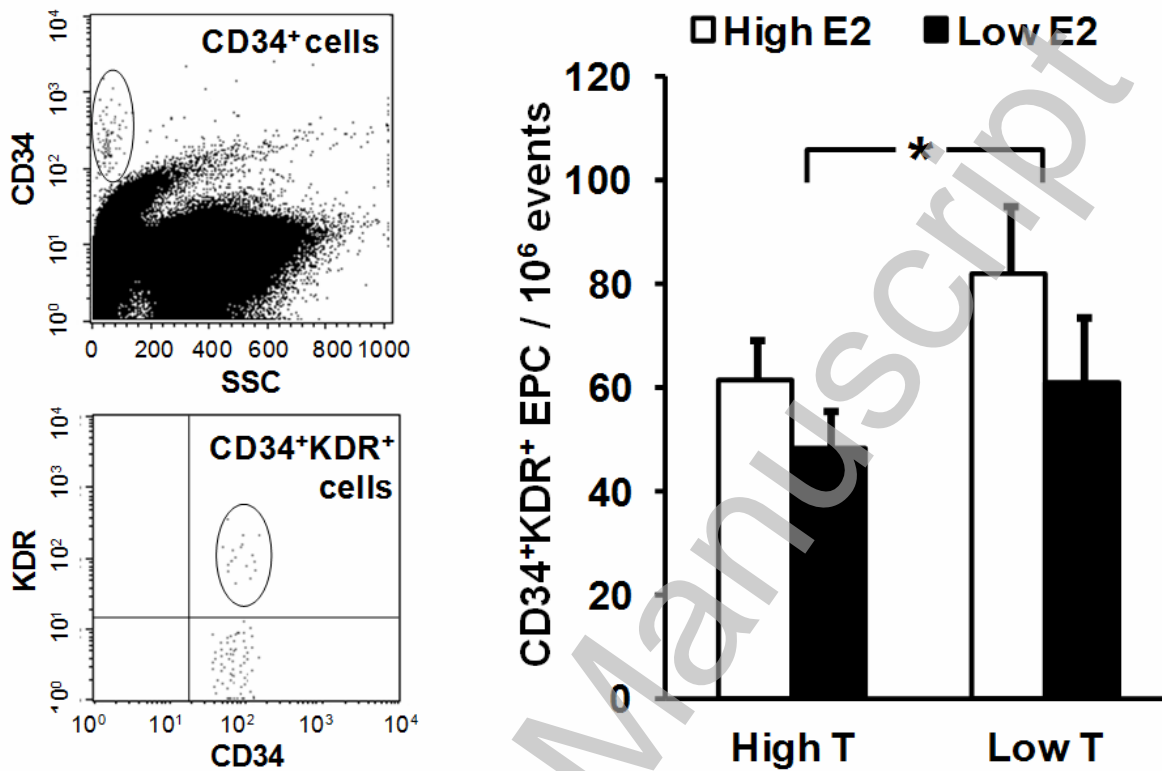


E2, T and DHT increased generation of early DiIAc-LDL+Lectin+ EPCs. \* $p \leq 0.05$  versus basal. T, testosterone; DHT, dihydrotestosterone; E2, 17 $\beta$ -estradiol.

**Figure 4. Expression of androgen receptor and aromatase activity in EPCs.** A) Western Blot analysis of androgen receptor in cultured human EPCs (prostate extract as positive control). EPCs express the androgen receptor (see figure 2K), but protein quantification via WB demonstrates very low expression as compare to positive control. B) Non-significant and non-linear conversion of testosterone (T) into 17 $\beta$ -estradiol (E2) by human EPCs in culture, suggesting low or absent aromatase activity.

**Figure 5. Effects of rat castration on sex hormones and EPCs.** Four weeks after castration, T concentrations were significantly reduced, and were partly restored by T replacement. Castration also lowered E2 levels, but T and DHT replacement were unable to restore E2 levels. Castration was followed by a profound reduction of Sca-1+/c-kit+ and Sca-1+/CD31+ rat EPC, while T and DHT supplementation were unable to restore their circulating levels. Lower panels show the gating strategy used to enumerate rat EPCs according to the fluorescence intensity of PE-Sca-1, FITC-CD31 and FITC-c-kit antibodies. \* $p \leq 0.05$  versus basal; †  $p \leq 0.05$  versus castration. T, testosterone; DHT, dihydrotestosterone. X- and y-axis of flow cytometry plots show arbitrary fluorescence units.

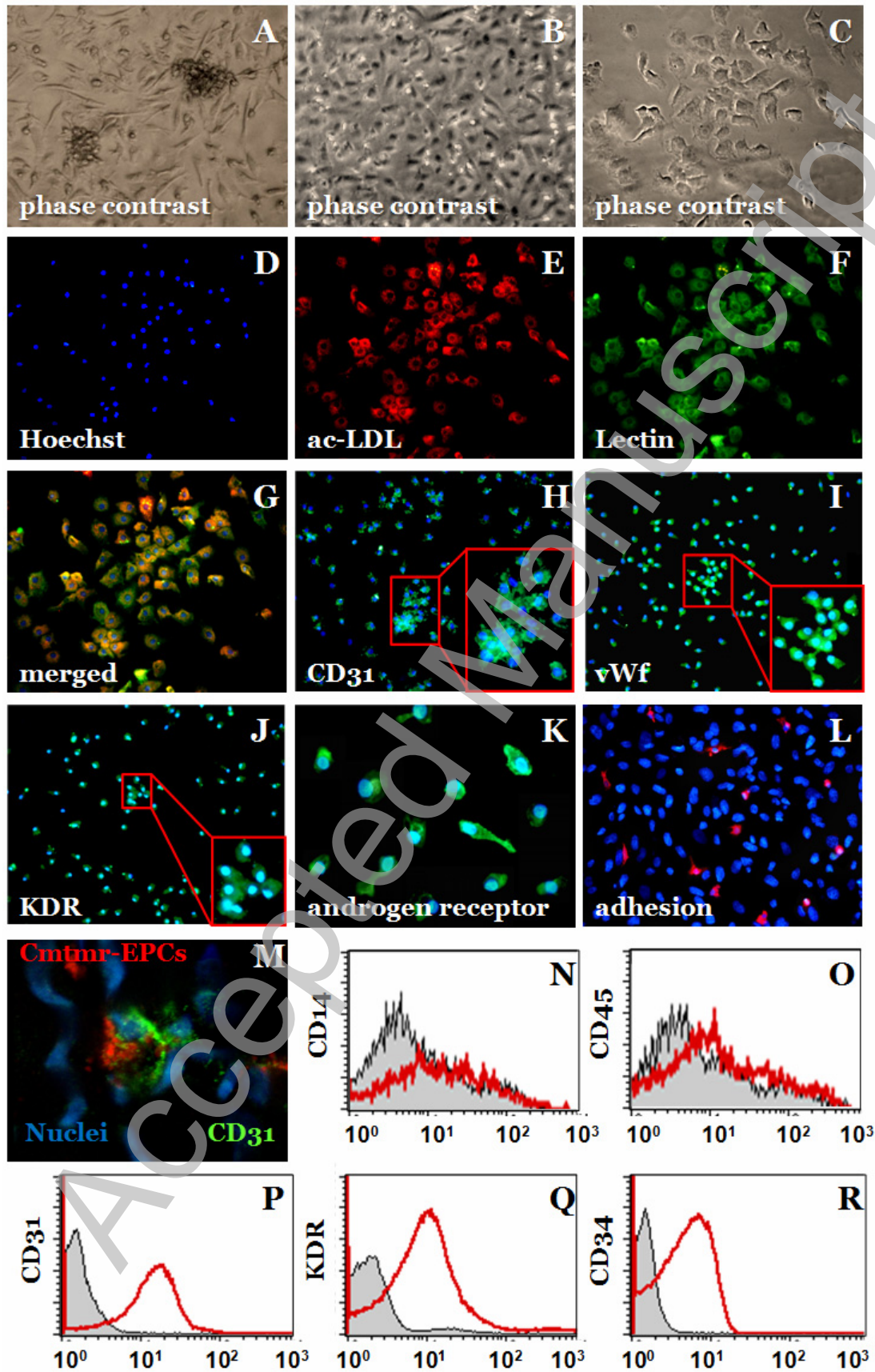
FIGURE 1



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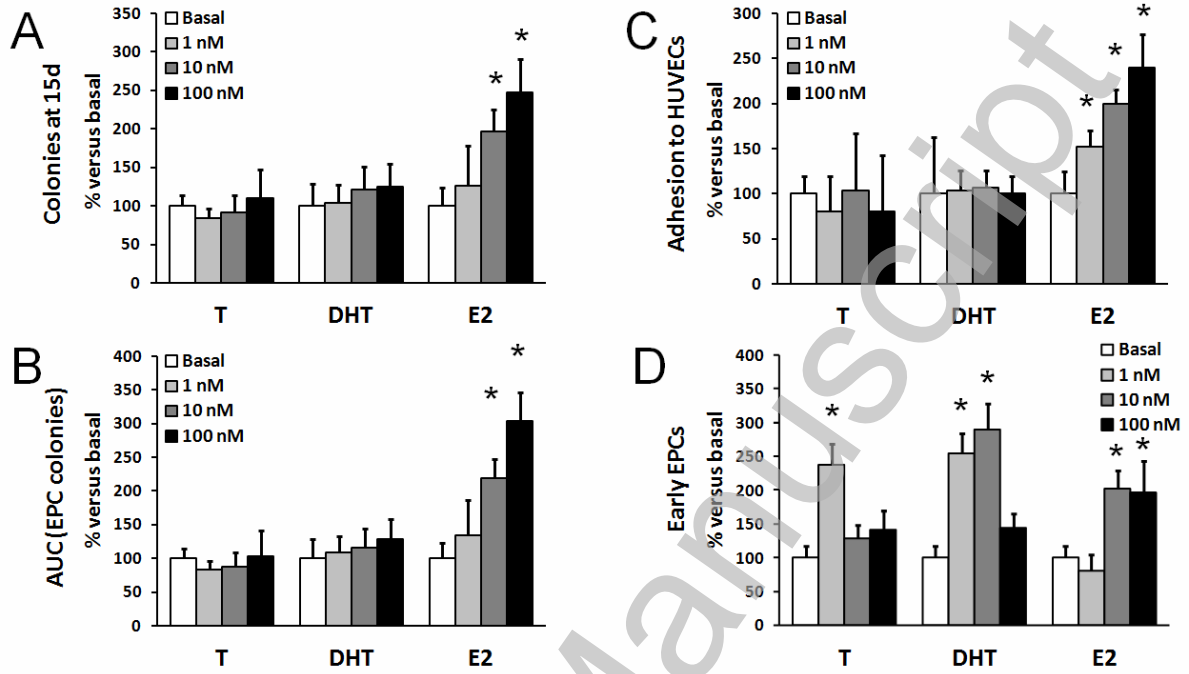
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FIGURE 2



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FIGURE 3



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FIGURE 4

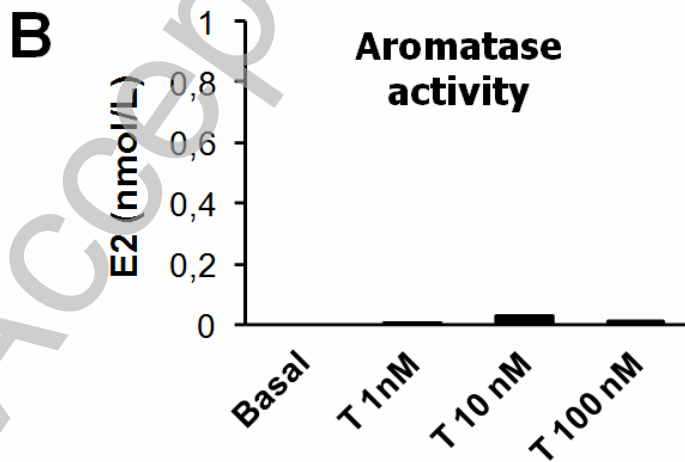
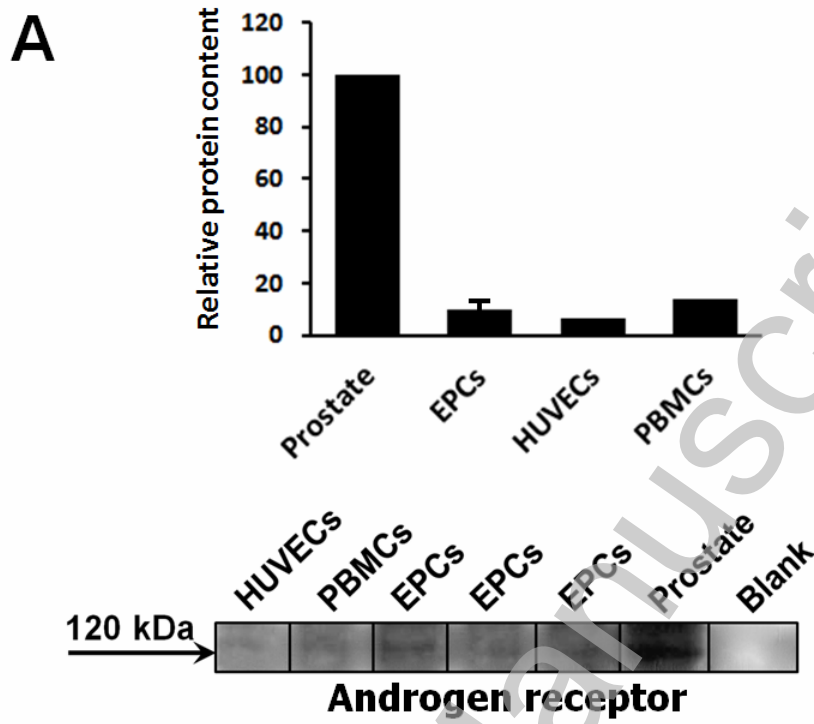
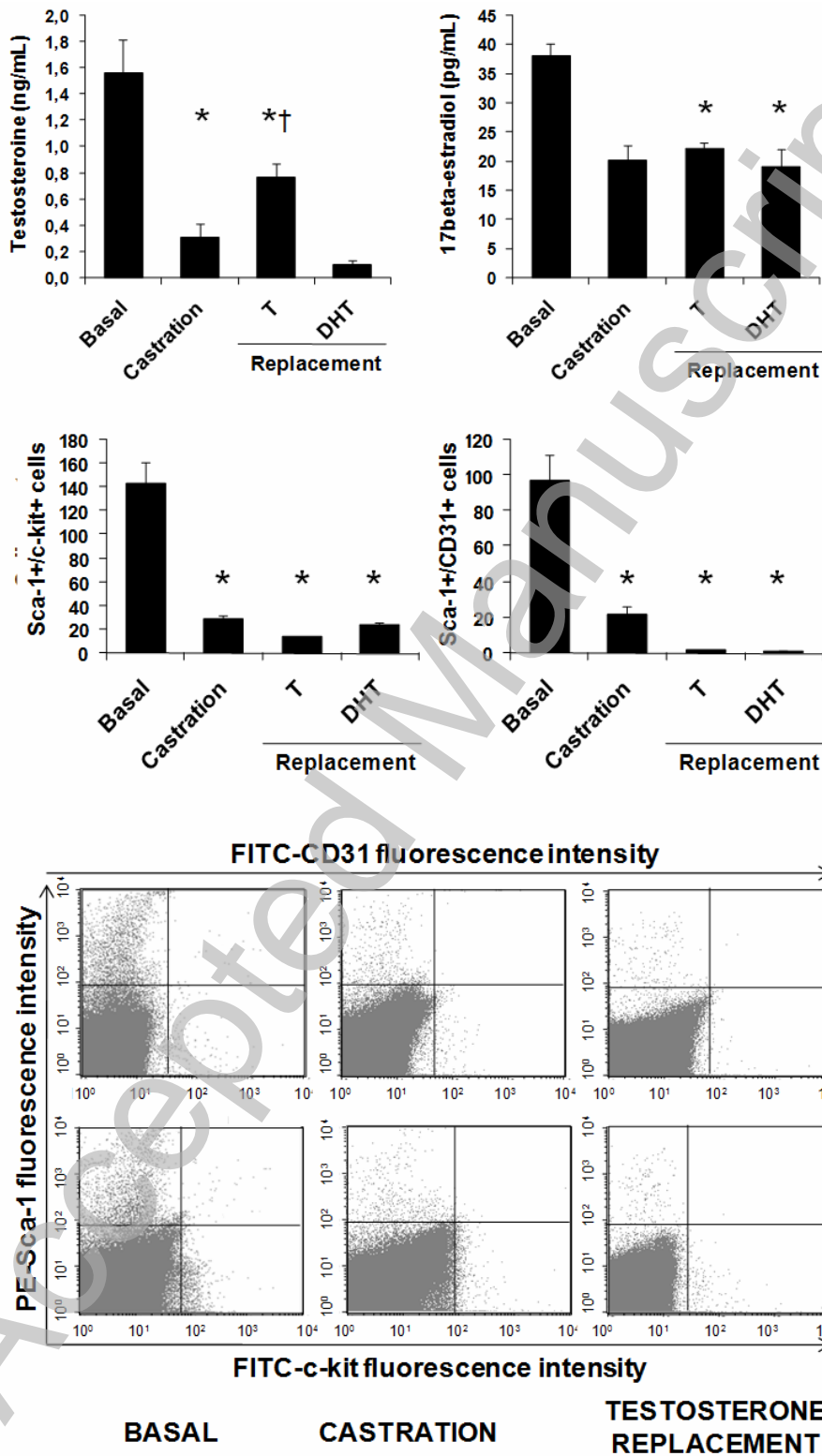


FIGURE 5



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