Estradiol Stimulates Capillary Formation by Human Endothelial Progenitor Cells: Role of ER-α/β, Heme Oxygenase-1 and Tyrosine Kinase

Supplementary Materials and Methods

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Running Title: Estradiol promotes EPC angiogenesis via ER-α

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Isolation and Culture of Endothelial Progenitor Cells (EPCs): EPCs were isolated and cultured as previously described[1]. Sample collection for the study was approved by the institutional ethics commission at University Hospital Zurich and written consent was obtained prior to blood collection. Briefly, peripheral blood (10ml) was collected in heparinized tubes from women undergoing in vitro fertilization treatment. White blood cells were isolated by gradient centrifugation using Biocoll (1.077 g/ml; Biochrom AG, Germany) and washed twice with buffer (2 mM EDTA in phosphate-buffered saline). CD34 positive cells were selectively separated using magnetic beads coated with CD34 antibodies. The purity of the isolated CD34 positive cells was confirmed by staining with antibodies to AC133, a marker for stem cell glycoprotein that is selectively expressed in CD34 positive progenitor cells. CD34 positive cells were resuspended in phenol red-free endothelial basal medium (Lonza Group Ltd, Switzerland) supplemented with EGM-2 SingleQuot Kit (Lonza Group Ltd, Switzerland) and 20% fetal calf serum, plated at a density of $2.5x10^6$ cells/well in 24-well fibronectin-coated plates (BD Biosciences, USA) and cultured under standard tissue culture conditions. Following 4-6 weeks in culture, EPCs were plated in 75 cm^2 flasks, and upon confluency cells were characterized for specific endothelial cell markers (von Willebrand factor, PECAM-1 and MCAM-1) using endothelial cell characterization kit (Chemicon–Millipore, Billerica, US) and re-plated at split ratios of 1:4 **(for antibody details see Table-1 below).**

Capillary Formation Studies: To assess EPC-induced microvessel formation, EPCs in 3rd passage were serum starved overnight in DMEM-F12, phenol red-free medium (Gibco, Invitrogen Corporation, USA) supplemented with 0.4% steroid-free serum. Subsequently, 200 µl aliquots containing 50,000 cells and the various experimental agents were layered on 8-well chamber slides coated with matrigel (BD Biosciences, USA) and incubated under standard tissue culture condition.[2,3] After 4 hours, microvessel formation was analyzed using an Olympus inverted microscope (4x magnification) and photomicrographs. The capillary length was randomly measured at 10 separate locations and the average compared to the untreated control. DMSO at a final concentration of 0.1% was used as a vehicle-treated control.

Lumen and Sprout Formation: To investigate whether estradiol induces lumen and sprout formation in EPC derived endothelial cells we utilized collagen gel and Cytodex3 beads in fibrin gels, respectively and as previously described[4,5,6]. Briefly, for lumen formation studies, EPC-EC were seeded into collagen gels (3.75mg/ml) in EGM2 medium and incubated for 24-hrs as follows: (a) control; (b) VEGF (40ng/ml); (c) estradiol (E2,10nmol/L); or (d), positive control (phorbol myristate actetate [PMA;50ng/ml] + VEGF). Cultures were subsequently fixed in 2%paraformaldehyde, stained with toluidine blue, photographed and the number of lumens counted microscopically (original magnification 100x). For sprout formation assays, EPC-EC-coated Cytodex3 beads in fibrin gels were cultured in EGM2 medium as follows: (a) without VEGF; (b) with VEGF (15ng/ml); (c) with estradiol (E2,10nmol/L); or (d) with VEGF+E2. After 7 days the gels were photographed and the number of sprouts counted under a microscope (original magnification 100x).

Protein Expression Studies: Western blotting was employed to assess the role of various proteins in mediating the angiogenic effects of estradiol on EPCs. Briefly, EPCs in 3rd to 5th passages were grown to sub-confluence in 35mm^2 culture dishes and were serum starved for 12

hours. Subsequently, EPCs were treated with different agents for either 15 minutes for HO-1 expression studies, 10 minutes for tyrosine kinase receptor (TRK) activity and phosphorylated vascular endothelial growth factor recetor-2 (p-VEGFR-2), 30 minutes for phosphorylted Akt (Akt-P) and phosphorylated ERK (ERK-P), or 8 and 24 hours for VEGFR-2 . Cells were washed with ice cold buffer and lysed in 50 μl of lysis buffer (Cell signalling Technology Inc., USA). Protein was assayed using the BCA protein assay kit (Pierce, USA), and the expressions of ERs, HO-1, Akt-P, ERK-P and VEGFR-2 were analyzed by Western blotting. TRK activity and phosphorylated-VEGFR-2 in cell lysates were measured using Antibody Beacon tyrosine kinase assay kit (Molecular Probes, Eugene, OR) and DuoSet sandwich ELISA kit (Abingdon, OX14 3NB, UK), respectively. VEGFR-2 expression on EPCs was also analyzed by flow cytometery and Western blotting. VEGF levels in supernatants of EPCs treated with estradiol for 8 hours were assayed using ELISA kit (Invitrogen Corp., Camarillo, CA, USA).

Statistics: Data were analyzed by analysis of variance, and statistical significance ($p<0.05$) calculated using Fisher's LSD test.

References:

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Table S1: List of antibodies used for EPC and endothelial cell characterization.

Antibody	Company	Cat #	Concentration	Dilution used
Anti-HO-1	Abcam plc, Cambridge, UK	Cat # ab13248	1 mg/ml	4 μg/ml
IRDye 680 conjugated Goat Anti-Mouse IgG	Li-Cor Biosciences, Lincoin, US	Cat # 926-32220	1 mg/ml	1:10000
Anti-β-actin	Sigma-Aldrich Chemie GmbH, Buchs, CH	Cat # A-5441		1:5000
Anti-ERα	Alexis Biochemicals	Cat #210-201-C050	1 μg/μl	1:1000
Anti-ERβ	Alexis Biochemicals	Cat #210-180-C050	1 μg/μl	1:1000
IRDye 680 conjugated Goat Anti-Rabbit IgG	Li-Cor Biosciences, Lincoin, US	Cat # 926-32221	1 mg/ml	1:10000
Anti-AC133-APC	Miltenyi Biotec, Bergisch Gladbach, D	Cat # 130-090-826	100 μg/ml	1:10
Anti-CD34-FITC	Miltenyi Biotec, Bergisch Gladbach, D	Cat # 130-081-001		1:10
Endothelial cell characterization kit	Chemicon (Millipore), Billerica, US	Cat # SCR023	CD146: 1 mg/ml VWF: 1 mg/ml CD31: 0.1 mg/ml	CD146 1:500 vWF 1:5000 CD31 1:50
Anti-Mouse IgG – FITC	Sigma-Aldrich Chemie GmbH, Buchs, CH	Cat # F-2012		1:64
Anti-Rabbit IgG – FITC	Sigma-Aldrich Chemie GmbH, Buchs, CH	Cat # F-0382		1:80
Anti-Phospho-Akt (Thr 308)	Cell Signaling Technology Inc.,Beverly, US	Cat # 9275		1:1000
Anti-Akt	Cell Signaling Technology Inc., Beverly, US	Cat # 9272		1:1000
Anti-phospho-MAP Kinase 1/2 (Erk1/2)	Upstate Biotechnology, Lake Placid, US	Cat # 07-467	0.28 mg/ml	1:1000
Anti-MAP Kinase/Erk1/2-CT	Upstate Biotechnology, Lake Placid,US	Cat # 06-182	0.55 mg/ml	1:1000
Anti VEGFR-2	Calbiochem, Merck KgaA, Darmstadt, D	Cat # 676486	1 mg/ml	1:1000

Figure S1

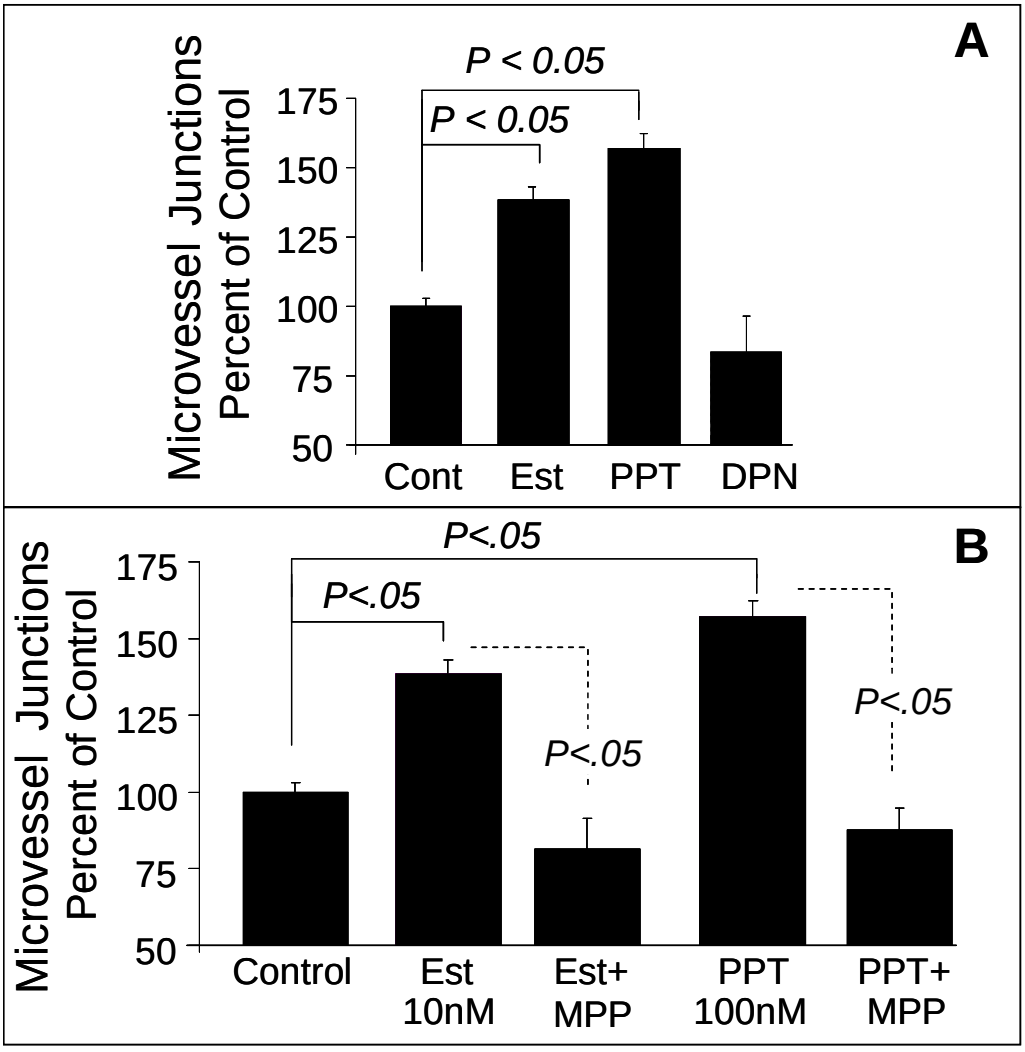

Figure S1: (A) Bar graph showing the effects of estradiol (Est; 10nmol/L), PPT (ERα agonist; 100nmol/L) and DPN (100nmol/L) on capillary formation by EPCs. Serum starved EPCs were plated at a density of 50'000 cells/200µl / well on matrigel coated 8-well multichamber slides. After 4-hrs the capillary formation was assessed by randomly counting the number of sprouting junctions at 4 different locations under a microscope and 4x magnification. (B) Bar graph showing the effects of estradiol (10nmol/L) and PPT (100nmol/L) on EPC-induced capillary formation in the presence and absence of ERα antagonist MPP (1µmol/L). Values represent means±SEM.

Figure S2

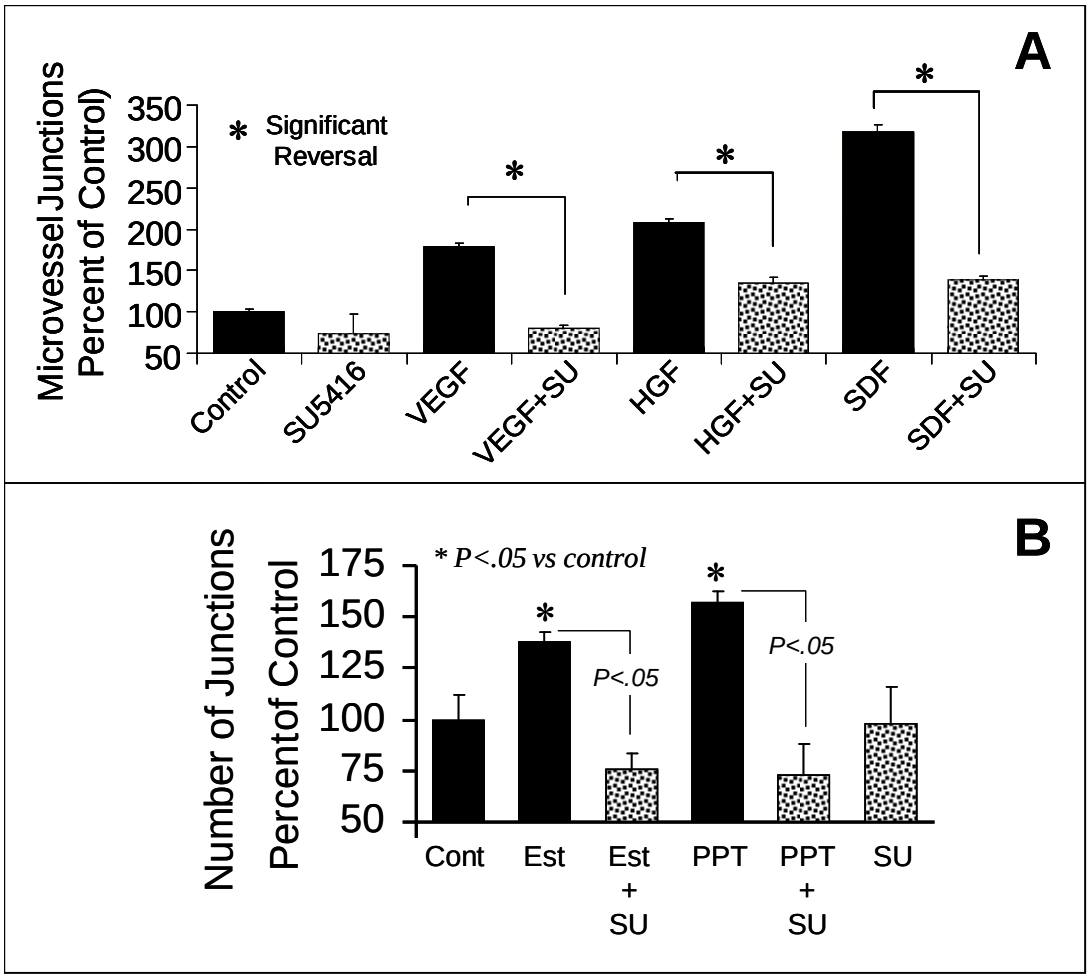

Figure S2: (A) Bar graph showing the effects of tyrosine kinase stimulators vascular endothelial growth factor (VEGF**;**100ng/mL), hepatocyte growth factor (HGF, 100ng/mL) and stromal cell-derived growth factor (SDF-1 100ng/mL) on capillary junction formation by EPCs in the presence and absence of tyrosine kinase inhibitor SU5416 (SU; 5µmol/L). Serum starved EPCs were plated at a density of 50'000 cells/200µl / well on matrigel coated 8-well multichamber slides. After 4 hrs the capillary junction formation was assessed by randomly counting the number of sprouting junctions at 4 different locations under a microscope and 4x magnification. (B) Bar graph showing the modulatory effects of tyrosine kinase inhibitor SU5416 (SU; 5µmol/L) on estradiol (10nmol/L) and PPT (100nmol/L) induced capillary junction formation in EPCs. Values represent means±SEM. * $p<0.05$ versus vehicle treated controls.

Figure S3

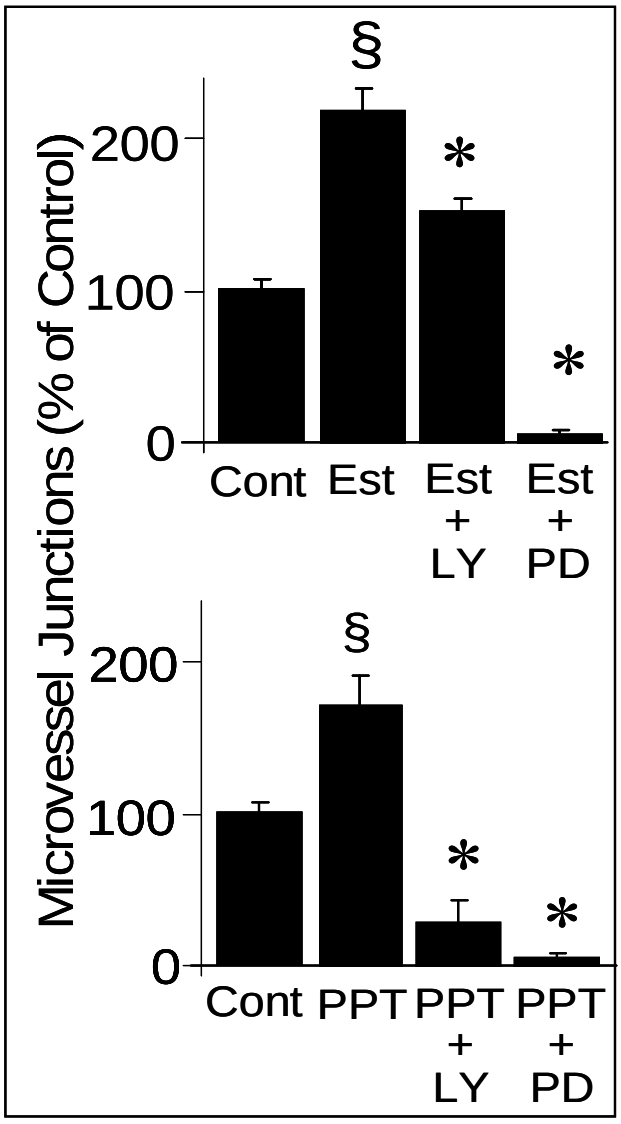

Figure S3: Bar graph showing the inhibitory effects LY294002 (Akt inhibitor; LY; 10μmol/L) and PD98059 (ERK1/2-P inhibitor; PD; 10μmol/L) on estradiol (Est; 10nmol/L) and PPT (100nmol/L) induced capillary junction formation by EPCs. EPCs were pretreated for 15-minutes with either LY or PD and subsequently Est orPPT added for another 4-hrs and capillary formation analyzed microscopically at 4x mag. * $p<0.05$ vs Estradiol or PPT alone.

Figure S4

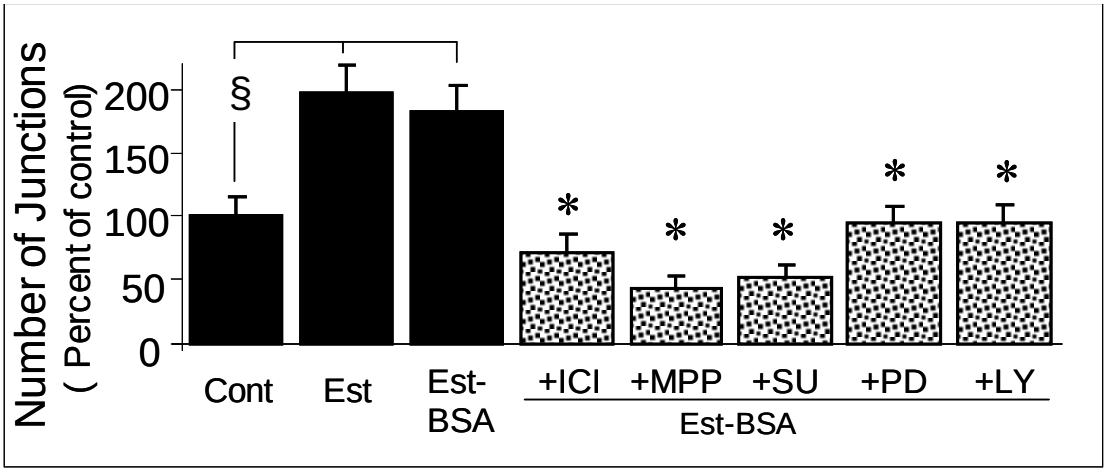

Figure S4: Bar graph showing the modulatory effects of tyrosine kinase inhibitor SU5416 (SU; 5μmol/L), LY294002 (Akt pathway inhibitor; LY, 10μmol/L) and PD98059 (ERK1/2 pathway inhibitor; PD, 10μmol/L) and MPP (ERα antagonist, 1μmol/L) on BSA-estradiol (Est-BSA, 10nmol/L) induced capillary formation in EPCs. Serum starved EPCs were plated at a density of 50,000 cells/200μl/well on matrigel coated 8-well multichamber slides. Cells were pretreated for 15-minutes with MPP, SU, LY, or PD and subsequently BSA-estradiol was added for an additional 4-hours and capillary junction formation assessed by randomly counting the number of sprouting junctions at 4 different locations under a microscope at 4x magnification. Values represent means±SEM. $^{\S}p<0.05$ versus vehicle treated controls; *$p<0.05$ versus Est-BSA.