Doctoral Thesis

Regulation of endothelial progenitor cell growth by estradiol role of estrogen receptors and intracellular mechanisms

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Regulation of Endothelial Progenitor Cell Growth by Estradiol: Role of Estrogen Receptors and Intracellular Mechanisms

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

For the degree of
Doctor of Sciences ETH Zurich

Presented by

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2009
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Cardiovascular disease is the leading cause for mortality in both men and women in the world. Atherosclerosis is a chronic vaso-occlusive inflammatory disease which is, in part, due to accumulation of cholesterol and macrophage within the vessel wall (atherosclerotic plaque), as well as overproliferation of arterial smooth muscle cells (neointima). The pathophysiological consequences of these insults result in occlusion of the blood vessels and the eventual rupture of the plaque and infarction due to the thrombus formed. Endothelial dysfunction and damage is a major primary step in the initiation of atherosclerosis. Indeed if endothelial cells are old and injured, in presence of one or more risk factors, the damages to the endothelium cannot be repaired and this would initiate an inflammatory process leading to vaso-oclusion and vascular remodeling. Epidemiological studies provide evidence that estradiol protects against cardiovascular disease, though the exact mechanisms remain unclear. Among the beneficial effects of estradiol on the cardiovascular system, it is known to induce mitogenic effects on endothelial cells, which help maintaining the health of the endothelium. Moreover, in the last decade, a new cell type, the so called endothelial progenitor cells (EPCs), has been discovered, which is able to initiate as well as participate in processes like angiogenesis and neovasculogenesis. EPCs are bone marrow-derived cells that will finally differentiate into endothelial cells. Based on these observations, we hypothesized that, besides acting as a mitogen on endothelial cells, estradiol may induce its protective effects on the cardiovascular system by promoting growth of EPCs. In support of this hypothesis we provide evidence that estradiol activates mobilization of EPCs from the bone marrow, and stimulates their proliferation, adhesion, capillary formation as well as the nitric oxide production. The biological effects of estradiol are mainly mediated by estrogen receptors (ER) α and β. In the present study we demonstrated that the growth stimulatory effects of estradiol on EPCs are ERα mediated. We also investigated the intracellular mechanisms that may be involved in mediating the mitogenic effects of estradiol on EPCs. We demonstrated that in EPCs estradiol activates the key mitogenic signal transduction pathways MAPK (ERK 1/2) and Akt, upregulates the expression of cell cycle regulatory proteins, i.e. cyclins D1, A, E and B, promotes hyperphosphorylation of retinoblastoma protein, and downregulates the expression of p21, a cell cycle protein, which negatively regulates growth. Additionally, we also demonstrate that estradiol promotes microvessel formation via activation of Akt signal pathway and by increasing the expression of the heme oxygenase-1 protein. Moreover, since in the human body estradiol is ubiquitously metabolized to 2-hydroxy- and 2-methoxyestradiol, we also investigated the effects of this metabolism on EPC growth and functions. An alternative mechanism via which estradiol mediates its protective effects on the cardiovascular system, is by inhibiting the growth of smooth muscle cells and neointima formation, processes which contribute to vascular remodeling and vaso-oclusion. In conclusion, here we demonstrate that, in contrast to EPCs, estradiol inhibits mobilization and growth of bone marrow-derived smooth muscle progenitor cells via ERα. Moreover, it inhibits Akt pathway, downregulates cyclin expression and retinoblastoma hyperphosphorylation and upregulates the expression of p21, thus further promoting cardiovascular health.

These findings provide evidence that estradiol may induce its anti vaso-occlusive and vasoprotective actions by promoting EPC growth and inhibiting SM-like progenitor cells. Finally, the findings provide potential support for the hypothesis of progenitor cell-based therapy for cardiovascular disease treatment and prevention.
**RIASSUNTO**

Le malattie cardiovascolari sono tra le principali cause di morte di uomini e donne nel mondo. L'arteriosclerosi è una malattia infiammatoria cronica in parte dovuta ad un accumulo di colesterolo e macrofagi nelle pareti vascolari (placca aterosclerotica) e ad una proliferazione eccessiva delle cellule muscolari liscie che compongono le arterie (neointima). Questi fenomeni portano alla restrizione del lume vasale e, in caso di rottura della placca, il trombo che ne deriva provoca un infarto. Tra gli eventi che promuovono l'insorgere e lo sviluppo dell'arteriosclerosi vi sono la disfunzione e il danneggiamento dell'endotelio. Infatti, se le cellule endoteliali non riescono più a riparare i danni a causa dell'età o della presenza di uno o più fattori di rischio, inizia un processo infiammatorio. Studi epidemiologici evidenziano che l'estradiolo protegge dalle malattie cardiovascolari, sebbene i meccanismi esatti non siano stati ancora chiariti del tutto. Alcuni degli effetti benefici dell'estradiolo sul sistema cardiovascolare si possono ricondurre alla promozione della proliferazione delle cellule endoteliali, le quali sono responsabili della buona condizione dell'endotelio. Inoltre, nell'ultimo decennio, è stato scoperto un nuovo modo di cellule, chiamate cellule endoteliali progenitrici (EPC), in grado di iniziare e partecipare a processi di angiogenesi e vascolarizzazione. Le EPC derivano dal midollo spinale e maturano differenziandosi in cellule endoteliali. Per questo motivo abbiamo ipotizzato che un meccanismo coinvolto nella protezione del sistema cardiovascolare da parte dell'estradiolo, oltre all'azione mito genica sulle cellule endoteliali, sia dovuto alla promozione della crescita delle EPC. A supporto di questa ipotesi abbiamo fornito le prove che l'estradiolo promuove la mobilizzazione delle EPC dal midollo osseo, la loro proliferazione, adesione, la capacità di formare capillari e di aumentare la produzione da parte delle cellule di monossido d'azoto. Gli effetti biologici dell'estradiolo sono mediati per la maggior parte dai recettori dell'estrogeno (ER) α e β. Con questo studio abbiamo dimostrato che gli effetti mitogeni dell'estradiolo nelle EPC sono mediati in particolare dell'ERα. Inoltre, abbiamo studiato i meccanismi intracellulari che potrebbero essere coinvolti nella promozione della crescita delle EPC da parte dell'estradiolo. A riguardo, abbiamo dimostrato che, nelle EPC, l'estradiolo attiva le vie di trasmissione del segnale cellulare MAPK (ERK 1/2) e Akt, aumenta l'espressione delle cicline che controllano il ciclo cellulare (ciclina D1, A, E e B), promuove l'iper-fosforilazione della proteina del retino blastoma e diminuisce l'espressione della proteina p21, inibitrice del ciclo cellulare. Sempre a livello molecolare abbiamo dimostrato che l'estradiolo promuove la formazione di micro capillari attraverso la via di trasmissione del segnale Akt aumentando l'espressione della proteina emossigenasi-1. Inoltre, poiché l'estradiolo è metabolizzato ovunque nel corpo umano in 2-idrossi e 2-metossiestradiolo, abbiamo anche indagato gli effetti di questi metaboliti sulla crescita e sulle funzioni delle EPC. Un ulteriore meccanismo attraverso il quale l'estradiolo protegge il sistema cardiovascolare è l'inibizione della crescita delle cellule muscolari lisse, e quindi di neointima, impedendo così la vaso-occlusione. Col nostro lavoro abbiamo potuto dimostrare che l'estradiolo, al contrario di quanto accade per le EPC inibisce, attraverso l'ERα, la mobilizzazione e la crescita di cellule muscolari lisse progenitrici derivate del midollo osseo. Infatti, inibisce la via di trasmissione del segnale cellulare dell'Akt, l'iper-fosforilazione della proteina del retino blastoma, diminuisce l'espressione delle cicline e aumenta l'espressione della proteina p21, promuovendo così ulteriormente la salute del sistema cardiovascolare. Questi risultati dimostrano che l'estradiolo può indurre i suoi effetti anti-occlusivi e vaso protettivi promuovendo la crescita delle EPC e inibendo le cellule progenitrici muscolari. In conclusione, questi risultati possono offrire supporto all'ipotesi di una terapia basata sulle cellule progenitrici per la cura e la prevenzione delle malattie cardiovascolari.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-Methoxyestradiol</td>
</tr>
<tr>
<td>2-OHE</td>
<td>2-Hydroxyestradiol</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B (PKB)</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-Triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bichinonic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CMFDA</td>
<td>5-Chloromethylfluorescin Diacetate</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-Methyltransferase</td>
</tr>
<tr>
<td>CV system</td>
<td>Cardiovascular system</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMEM-F12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPN</td>
<td>Diarylpropionitrile</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
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<td>E₂</td>
<td>17β-Estradiol</td>
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<td>EBM</td>
<td>Endothelial Basal Medium</td>
</tr>
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<td>ECs</td>
<td>Endothelial cells</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGE</td>
<td>Endothelial Growth Factor</td>
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<td>EGM-2</td>
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<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
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<td>eNOS</td>
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</tr>
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<td>ER</td>
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<td>ERα</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>G1</td>
<td>Gap 1</td>
</tr>
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<td>G2</td>
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<td>G-CSF</td>
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<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<td>HO-1</td>
<td>Heme Oxygenase-1</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
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<td>HSD</td>
<td>Hydroxysteroid Dehydrogenase</td>
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<td>HUVEC 血</td>
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<tr>
<td>ICAM</td>
<td>Intracellular Cell Adhesion Molecule</td>
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<td>Abbreviation</td>
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<tr>
<td>ICI</td>
<td>ICI182,780 / Fulvestrant</td>
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<td>IGF</td>
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<td>IgG</td>
<td>Immunoglobulin</td>
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<td>IVF</td>
<td><em>In Vitro</em> Fertilization</td>
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<td>Low Density Lipoprotein</td>
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<td>MACS</td>
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<td>Minimum Essential Medium</td>
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<td>Magnesium</td>
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<td>Matrix Metalloproteinase</td>
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<td>SMCs</td>
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<td>SU5416</td>
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<td>TCA</td>
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<td>TEMED</td>
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<td>TNF-α</td>
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<td>VCAM</td>
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<td>VEGF</td>
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<td>VEGF-R</td>
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<td>vWF</td>
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1.1 **ESTRADIOL**

1.1.1 DESCRIPTION AND BIOLOGICAL EFFECTS OF ESTROGENS

Estrogens are endogenous steroid hormones synthesized from cholesterol. Among the several types of estrogens that are produced within the body (estriol, estrone and estradiol), estradiol (17β-estradiol) is the most predominant and functionally relevant.

Estrogens are responsible for the development and function of the female reproductive system. They also promote the development of secondary sex characteristics in females, regulate the menstrual cycle and have a role in the fertilization process and in the maintenance of pregnancy. Estrogens play a role in regulating the biology and physiology of other tissues apart from the ones related to the reproductive system. For example, it protects against osteoporosis in the bones; in the liver it regulates the metabolism of cholesterol and lipoproteins, and in the brain it has been shown to have neuroprotective actions and protect against Alzheimer’s disease. Moreover, in the gastrointestinal tract, estrogens reduce the risk of colon cancer and, in addition, ameliorate skin aging [rev. in (Gruber, 2002)]. They also improve arterial blood flow and have beneficial effects on the cardiovascular system (see section 1.4). However, estrogens have a powerful mitogenic action on hormone sensitive tissues, and this can result in undesirable deleterious effects. In this regard, estrogens can induce cancer of the uterine lining and of the breast tissue (Yager, 2000). Finally, even though estrogens are frequently referred as to female sex hormones, they also exert an influence on the reproductive system and on the fertility of males (Gruber, 2002).

1.1.2 SYNTHESIS

17β-Estradiol (estradiol; E2) is the main estrogenic hormone and its primary sources are the theca and granulosa cells of the ovaries, the corpus luteum or the placenta. Estrogens, as well as the other steroid hormone, progestins and androgens, are synthesized from cholesterol. The steroidogenic enzymes responsible for the biosynthesis of these hormones consist of several specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs) and steroid reductases (Miller, 1988).

Synthesis of estradiol is achieved by aromatase and can occur both from testosterone and androstenedione via two mechanisms: (i) androstenedione is converted into estrone by aromatase and this is subsequently converted into estradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD); (ii) 17β-HSD converts androstenedione into testosterone and testosterone is then transformed into estradiol by aromatase (Dubey, 2001) (Fig. 1).
In reproductive-age women, the most important site of estradiol synthesis is the ovary (Bulun, 2000), but aromatase and 17β-HSD are also expressed in many other tissues, as for example the skin, the adipose tissue, the endometrium, the vaginal mucosa, the liver and the breast tissue. They are also present in the vascular cells (Murakami, 2001). Once it is synthesized, estradiol is released into the circulation, where it can be found either in its free form (about 2-3% of total amount), or largely bound to albumin or to sex hormone-binding globulin. The concentrations of circulating estradiol range from less than the 0.36 nmol/l of the follicular phase to the 2.2 nmol/l at time of ovulation. These concentrations can rise up to about 73 nmol/l during the pregnancy. Following menopause, when the ovaries stop to function, estradiol concentration can drop to 0.02 nmol/l which is a value similar to, if not lower, that in men (Ling, 2006).

1.1.3 METABOLISM

Elimination of estradiol is largely mediated via its conversion into non-estrogenic water-soluble metabolites that are excreted in urine or feces. Estradiol is also converted into oxidative metabolites and their subsequent O-methylated form.

Metabolism of estradiol takes place mainly in the liver, however, the enzymes responsible for estradiol metabolism (CYP450 and COMT) are present also in other tissues, such as the vascular tissue, the kidneys, the gastrointestinal tract, the spleen, the brain and the pancreas (Dubey, 2001; Zhu, 1998).

Oxidative metabolism of estradiol involves CYP450, which converts exogenous and endogenous estradiol to 2-, 4- and 16-hydroxyestradiol. The hydroxylated products are then rapidly methylated by the catechol-O-methyltransferase (COMT) to 2-methoxyestradiol and 4-methoxyestradiol (Fig. 2).
Although metabolites of estradiol are less active estrogens and are water soluble and excreted in the urine, some of them have significant growth regulatory effects (Dubey, 2001).

2-hydroxyestradiol (2-OHE) is rapidly converted to 2-methoxyestradiol [its plasma half-life is 96 s (Kono, 1983)]. 2-methoxyestradiol (2-ME) has been shown to inhibit neovascularization and tumor growth (Mooberry, 2003). Moreover, it inhibits neointima formation and smooth muscle cell growth (Barchiesi, 2006).

**FIGURE 2. 17β-estradiol and its conversion into the biologically active metabolites 2-hydroxyestradiol and 2-methoxyestradiol by CYP450 and COMT.**
1.2 **ESTROGEN RECEPTORS**

Steroid/thyroid hormone receptors are members of a large family of nuclear ligand-dependent transcription factors that include the estrogen receptors (ERs) (Weigel, 1996). Upon ligand binding, ER undergoes a conformational change and dimerizes. This permits the interaction of the receptor with the so-called estrogen response element (ERE) on DNA, promoting the transcription of target genes (Kumar V., 1988; Parker, 1993). ER-mediated gene expression is also regulated by the recruitment of co-activators or co-repressors together with the whole transcriptional machinery at site of gene transcription.

ERs have a modular structure with distinct regions corresponding to functional and structural domains. There are two transcription activation domains (AF-1 and AF-2) and five other regions: (i) a variable NH2-terminal region (A-B), (ii) a conserved DNA binding domain (C), (iii) a hinge region (D), (iv) a ligand binding domain region (E) and (v) a COOH-terminal region with unknown function (F) (Fig. 3) (Brosens, 2004).

![FIGURE 3. Schematic representation of the functional and structural regions of estradiol receptors.](image)

To date, there are two known ER: ERα and ERβ. ERα was the first one to be cloned in 1986 (Walter, 1985) followed by ERβ in 1996 (Kuiper, 1996). ERα and ERβ are products of different genes; they are ~95% homologue in the DNA binding domain and only ~55% in the ligand binding domain. This variation in their ligand binding domains may contribute to the difference shown in the affinity for ligand, interaction with proteins and with selective pharmacologic agonists and antagonists (Sun, 1999; Gruber, 2002); Both ERα and ERβ genes can undergo alternate splicing, the best characterized variant being ER46 and ERβcx for ERα and ERβ, respectively (Herynk, 2004). ERα and ERβ have a different tissue-specific distribution.
1.2.1 MECHANISMS OF ER ACTION

1.2.1.1 The classical pathway

ERs are located, in absence of ligand, in the cytoplasm and nucleus of the cell, and they are held in a multi-subunit complex by heat shock proteins (Hsp). Hsp act as chaperones and maintain the receptors in an active conformation ready for ligand binding. As estrogen diffuses into the cell, it binds to its receptor, ERs undergo a conformational change, dissociate from the chaperones, dimerize, diffuse to the nucleus and eventually bind to the ERE on DNA [reviewed in (Brosens, 2004)]. Nuclear ER can also regulate gene expression without binding to DNA. In this case, ER is assumed to interact with other transcriptional factors in order to affect gene expression [reviewed in (Zhang, 2006)]. Different growth factors, as IGF-1 and EGF, have been shown to promote transcription of ERs target genes, since they activate signal transduction pathways that will, in turn, activate ERs. The mechanism for this ligand independent activation seems to be phosphorylation of the receptors [reviewed in (Brosens, 2004)].

1.2.1.2 Alternative non-genomic pathways

Beside the classical genomic mechanism of ERs action, there are also non-genomic ones, which don’t require ERs to bind on DNA in order to mediate estradiol effects (Fig. 4) (Losel, 2003). These mechanisms are referred to as alternative pathways, and they can be divided into ER-dependent and ER-independent.

1.2.1.2.1 ER-dependent pathway

Estradiol ER-mediated gene transcription takes hours to days in order to occur. However, there are also biological responses that need a short time, in the range of minutes, after estradiol stimulation in order to be elicited and cannot be explained by gene transcription. These rapid non-genomic effects have been shown to be mediated by ERs located at the plasma membrane (Kelly, 2001). In endothelial cells (ECs) ERs have been shown to be compartmentalized in the so called caveolae, which are membrane invaginations, coated with multiple proteins, among the others caveolin (Kim, 2005). In endothelial cells, examples of this rapid actions of estradiol are the activation of the endothelial nitric oxide synthase (eNOS) in the caveolae, which leads to NO production, and stimulation of Akt activity, with subsequent induction of transcription factors, signaling molecules, and genes coding for structural proteins, cytokines and enzymes (Pedram, 2002). In breast cancer cells, an example is the interaction of ERs with protein members of mitogenic signal transduction pathways, as PI3K/PKB (Akt) or ERK1/2 (MAPK), which are important in survival and proliferation (Migliaccio, 1996).

1.2.1.2.2 ER-independent pathway

Studies conducted with double ERα and ERβ knockout mice, show that estradiol effects on SMC are not abrogated by the absence of the receptors. This provides evidence that, apart from the ER-dependent mechanism, an ER-independent mechanism is involved in the mediation of estradiol effect on cells (Karas, 2001). These effects are due to conversion of estradiol into 2OHE and 2ME (see section 1.1.3). Estradiol metabolites act on an ER-independent pathway and they have been shown to inhibit growth of SMCs (Barchiesi, 2006).
FIGURE 4. Schematic simplified pictures showing different pathways of estradiol action: (A) the classical pathway with cytosolic/nuclear receptors acting as nuclear transcription factor and the alternative ER-independent metabolic pathway; (B) the alternative non-genomic ER-dependent, ER-independent, ERE-independent and ligand independent pathways.

ER: estrogen receptor; ERE: Estrogen responsive element; C: Chaperone; Co: Coactivator; CYP: Cytochrome P450; COMT: catechol-O-methyltransferase; NO: nitric oxide; eNOS: Endothelial nitric oxide synthase; Gp: G protein; AP-1 Activating protein-1; P: Phosphate group.
1.3 **CELL PROLIFERATION** *(Alberts, 2002)*

Following mitogen stimulation, cells enter the cell cycle and start to proliferate (Fig. 5). Progression through the cell cycle is regulated by the cell-cycle control system, and will lead to the production of two identical daughter cells. The cell cycle can be divided into four phases: G1 (gap 1), S (synthesis), G2 (gap 2) and M (mitosis). Quiescent cells are referred as in G0 phase.

The proteins responsible for the control of the cell cycle, and the transition between the different phases, are the so called cyclin-dependent kinases (Cdks), whose activity, in turn, is controlled by regulators called cyclins. Cdks are active only when tightly bound to cyclins. During the progression through the cell cycle, the concentration of Cdks remains constant, while changes in cyclin levels result in cyclic assembly and activation of Cdk-cyclin complex. Cyclins are divided into four classes: (i) G1/S-cyclins, which commit the cell to DNA replication, as cyclin E, (ii) S-cyclins which are required for DNA replication, as cyclin A, (iii) M-cyclins which promote cell division, as cyclin B and (iv) G1-cyclins which help complete the G1-phase and enter S-phase, as cyclin D. Cdk-cyclin D complex promotes the hyperphosphorylation of retinoblastoma protein (Rb). Hypophosphorylated Rb binds, and thus inactivates the transcription factor E2F. Hyperphosphorylation of Rb causes it to dissociate from E2F, which can then bind on DNA and activate transcription of genes encoding for proteins required for entry into the S-phase. In order to avoid mistakes and division unless every single step has been successfully completed, there are several so called checkpoints at which the cycle can be arrested. Progression will take place only if all steps required for a single phase to be concluded have been carefully fulfilled. Enter in the S-phase is controlled by the G1 checkpoint. Here the cell controls its size, the presence of nutrients, the integrity of DNA and whether the whole DNA replication machinery has been synthesized. If DNA damages are present, the gene regulatory protein p53 is activated. p53 promotes the transcription of genes that encode proteins that inhibit activation of G1/S-Cdk and S-Cdk complexes by binding to them. One of these proteins is p21, which helps blocking the entry into the S-phase. Entering the M-phase requires the cell to control the integrity of the chromosome segregation machinery. During M-phase, the spindle checkpoint ensures that all chromosomes are attached to the mitotic spindle before they are divided into the two daughter cells. Progression through G1 and G2 phases is regulated by the DNA damage checkpoint, which prevent the cell to continue to the next phase, if damages in DNA are detected.

Mutations or abnormal expression of the cell cycle proteins can lead to excessive cell division and cancer. On the other hand, targeting of cycle proteins for therapeutic treatment of endothelium dysfunction may be of large interest.
1.3.1 MITOGENIC SIGNAL TRANSDUCTION PATHWAYS

A mitogenic signal in order to activate the whole cell cycle machinery has to bind on receptors on cell membrane and activate a transduction pathway. These receptors are enzyme-linked receptors, and they are classified into six classes, the most numerous one being the receptor tyrosine kinases class. Receptor tyrosine kinases are activated by a variety of growth factors and hormones, as EGF, FGF, PDGF, HGF, IGF, VEGF, M-CSF and NGF. The binding of a signal molecule to the binding domain of the receptor on cell surface, activates the intracellular kinase domain. Once activated, a phosphate group from ATP is transferred on tyrosine side chains on the receptor itself and on intracellular signaling proteins, which will then bind to the phosphorylated receptor. MAPK and Akt proteins are part of downstream phosphorylation cascades activated by a mitogenic signal.

1.3.1.1 MAPK

Phosphorylation of MAPK leads to activation of gene regulatory proteins and also genes encoding cyclin D, required for cell proliferation. Once the growth factor has bound to the receptor tyrosine kinase, this becomes activated and activates in turn Ras protein. Ras, then, activates MAPK-kinase-kinase (Raf), which will phosphorylate MAPK-kinase (MEK) which, at the end, phosphorylates MAPK (ERK1/2). MAPK activation upon estradiol binding has been described in breast cancer cells (Migliaccio, 1996) and in endothelial cells (Russel, 2000; Klinge, 2005). Moreover, estrogen, which has antiproliferative effects on SMCs, has been shown to reduce MAPK activity in these cells (Dubey, 2000).
1.3.1.2 Akt

Akt is responsible for the estradiol induction of NO production in endothelial cells, and thus its activation promotes vasodilation which is beneficial for the cardiovascular system. Upon signal molecule binding, the activated receptor tyrosine kinase activates in turn PI3-kinase. This leads to the formation of phosphorylated inositol phospholipids in the cell membrane [PI(3,4,5)P3]. PI(3,4,5)P3 phosphorylates phosphatidylinositol-dependent protein kinase (PDK1), which in turn phosphorylates and activates protein kinase B (PKB), also called Akt. Activated Akt phosphorylates, among the others, BAD protein. Active BAD-protein binds and holds in an inactive state death-inhibitory proteins. Phosphorylation by Akt inactivates BAD, which dissociate from the death-inhibitory proteins making them active and thus promoting inhibition of apoptosis.
1.4 CARDIOVASCULAR DISEASES

1.4.1 ATHEROSCLEROSIS

Atherosclerotic cardiovascular disease is the leading death cause in the Western society, and is expected to be the number one death cause worldwide in 2020 (Lopez, 1997). It leads to chronic inflammation of vessels, their occlusion and organ damage (Ross, 1999), and is characterized by leukocyte infiltration into the intima layer of the vessels, smooth muscle cell proliferation, and neointima formation.

Arteries are composed of an outer medial layer of smooth muscle cells, an intermediate intima layer made of connective tissue, and an inner monolayer of endothelial cells, the endothelium. The endothelium has a crucial role in maintaining the homeostasis of the vascular system and its integrity is essential to prevent the initiation of atherosclerosis (Lusis, 2000; Ross, 1995). Endothelial cells regulate the permeability of plasma lipoproteins, adhesion of leukocytes, release of pro- and anti-thrombotic factors, growth factors and vasoactive substances (Rubanyi, 1993). Endothelium dysfunction, due to age or presence of risk factors, plays a central role in the pathogenesis of atherosclerosis. Injury of the endothelium leads to oxidative stress, which in turns leads to vascular inflammation because of excessive production of reactive oxygen species (ROS) (Morita, 2005). Following inflammation, endothelial cells start to secrete cytokines, which are chemoattractive for monocytes, and to express adhesion molecules for monocytes, thus facilitating their accumulation into the vessel wall (Ling, 2006). Monocytes differentiate into macrophages and oxidize LDL, and are converted into lipid-laden foam cells. Consequently, smooth muscle cell (SMCs) proliferation is increased, as well as matrix deposition, thus leading to atherosclerotic plaque formation (Ross, 1999; Hansson, 2005). These processes result in neointima formation and in the thickening of the vessel wall. Eventually, the plaque may break and thrombus obstruction of the vessel lead to ischemic stroke or myocardial infarction (Fig. 6) (Libby, 2002).

FIGURE 6. Picture showing the different steps involved in the onset and the progression of atherosclerosis. From Libby and Aikawa, 2002, Nat. Med.
1.4.2 HORMONE REPLACEMENT THERAPY

Among the risk factors promoting the onset of cardiovascular disease there is age, hypertension, diabetes, cholesterol, obesity, sedentary lifestyle and male gender [reviewed in (Umemura, 2008)].

Epidemiological studies have demonstrated the sex difference in the incidence of cardiovascular diseases (CVD), being the onset in women delayed of about 5 years compared to men. Moreover, premenopausal women suffer less of CVD than postmenopausal ones (World Health Organization, 1996; Ling, 2006). Furthermore, risk of CVD has been shown to be increased in premenopausal women undergoing a surgically induced menopause, or a naturally premature menopause (Senoz, 1996), whereas women taking hormone replacement therapy (HRT) have a lower incidence of CVD (Grodstein, 1997). However, use of HRT increases the risk of breast and endometrial cancer. To avoid these undesirable effects, some forms of progesterone are used along with estradiol as an alternative. Moreover, results from the Heart Oestrogen-Progestin Replacement Study (HERS) (Hulley, 1998) and the Women’s Health Initiative do not confirm the benefits of HRT in postmenopausal women (Writing Group for the Women's Health Institute Investigators, 2002). The lack of benefits may be due to timing of therapy start. Use of estrogens during menopause may be protective for the cardiovascular system, but cannot repair existing damages. Therefore, if estrogen is given after damage has occurred, not only the beneficial effects are not observed but the progression of the disease may be aggravated (Teede, 2007). The effect of HRT on total mortality is related to the age at which it is initiated (Rossouw, 2007). Studies in primates show that lesion formation was reduced by 70% if estrogen replacement begun at the time of ovariectomy, but if it was delayed 2 years the benefits were completely abolished (Kim, 2005).

1.4.3 EFFECTS OF ESTRADIOL ON THE CARDIOVASCULAR SYSTEM

Both in vivo and in vitro studies support the favorable effects of estradiol on the cardiovascular system. In this content, in animal models estradiol has been shown to prevent injury induced neointima formation and cholesterol induced atherosclerosis (Bakir, 2000). Moreover, in humans (young women without preexisting cardiovascular disease) estradiol prevents age related intimal thickening and lowers cholesterol [rev. in (Dubey, 2005)].

1.4.3.1 Endothelial cells

Estradiol has been shown to promote growth of endothelial cells and inhibit apoptosis. Moreover, it modulates the expression of adhesion molecules on ECs, preventing attachment of monocytes to the vessel wall (Ling, 2006). This promotes endothelium health, and as a consequence, health of the cardiovascular system. Estradiol has also been shown to regulate the production of nitric oxide (NO). NO is a key molecule in the maintenance of the homeostasis of the endothelium, and its protective effects are lost if the endothelium is damaged. In ECs it is produced by the endothelial nitric oxide synthase (eNOS) which is clustered in membrane invaginations called caveolae (Chambliss, 2000). Signal from ER is transmitted through a G-protein to PI3K, this, in turn, activates Akt, which stimulates eNOS activation (Haynes, 2003). NO thus produced in ECs diffuses to the neighboring smooth muscle cells (SMCs) causing their relaxation. Estradiol promotes NO synthesis in two ways. First, by activation of the ER-genomic (classic) pathway and consequent upregulation of eNOS
expression. Second, by binding at ERs at the plasma membrane and rapidly stimulating Akt activation, which, in turn, activates eNOS leading to NO production (Haynes, 2000). Moreover, experiments conducted with the use of agonists and antagonists for both ERα and ERβ, indicate that ERα is mediating estradiol eNOS activation (Chambliss, 2000). Estradiol has also been shown to reduce the adhesion of monocytes to ECs as well as the expression of inflammation acute-phase proteins, whose chronic increase contribute to development of CVD (Gao, 2006).

1.4.3.2 Smooth muscle cells

Estradiol also inhibits growth and proliferation of SMCs (Fig. 7), which are responsible for the neointima formation during atherosclerosis. Moreover, the estradiol metabolite 2-methoxyestradiol (2ME) has been shown to inhibit SMCs growth (Barchiesi, 2003).

FIGURE 7. Photomicrographs showing the protective effects of estradiol. Intimal thickening in ovariectomized rats treated with (A) vehicle is greater than in (B) estradiol-treated rats. From Bakir et al, 2000, Circulation.
1.5 **ENDOTHELIAL PROGENITOR CELLS**

1.5.1 **VASCULOGENESIS AND ANGIOGENESIS**

Living cells need blood supply in order to acquire oxygen and nutrients, essential for their survival.

Generation of blood vessels is classified into vasculogenesis and angiogenesis (Fig. 8).

Vasculogenesis, a process formerly considered to occur only during embryogenesis, is the assembly and alignment of angioblasts, which will then develop into endothelial cells, to build vessels de novo.

Angiogenesis, a process occurring both during embryogenesis and in the adult, is characterized by the sprouting of new vessels from existing ones by division of endothelial cells. (Carmeliet, 2000; Flamme, 1997; Newman, 1997; Risau, 1997; Simons, 2005; Skalak, 2005)

![FIGURE 8. Picture showing the processes of new blood vessel formation by vasculogenesis and angiogenesis. From Harvey and Rosenthal, 1999, Academic Press, San Diego](image)

1.5.2 **THE ENDOTHELIAL PROGENITOR CELLS**

Until the late 90s, it was thought that angiogenesis was the only process taking place in adults, being vasculogenesis a peculiarity of the prenatal period. In case of injury, it was thought that endothelial cells near site of damage migrated and proliferated under the influence of paracrine mediators released from injury site (Hirsch, 1983). In 1997 Asahara et al. discovered CD34+ cells in peripheral blood of
adults, which were able to participate in processes of vasculogenesis (Fig. 9). They termed these cells endothelial progenitor cells (EPCs) (Asahara, 1997).

These cells, similar to angioblasts, are bone marrow-derived, can proliferate, migrate, and participate in the development of vascular networks by differentiating into endothelial cells (ECs) (Rafii, 2000). EPCs express progenitor markers, as CD34 and AC133. Once they leave the bone marrow, they start to lose progenitor markers and express endothelial markers, for example von Willebrand Factor (vWF), CD31 (or PECAM-1), and vascular endothelial growth factor receptor 2 (VEGFR-2) (Peichev, 1999; Urbich, 2004; Yin, 1997). Circulating EPCs, defined ad CD34+/AC133+/VEGFR-2+ cells, comprise about 0.002% of total mononuclear cells (MNC).

At least two types of EPCs can be obtained by the in vitro culture of MNC: (i) early EPCs, which have a peak growth within 2-3 weeks after isolation and die after 4 weeks (Hur, 2004) and (ii) late EPCs which grow after 2-3 weeks in culture and have a cobblestone-shaped morphology (Ingram, 2004). There are also studies suggesting that EPCs are CD34- and CD14+. It is important to note, that this finding strongly depends on isolation and culture conditions (Kim, 2005; Romagnani, 2005). Since there is not a standard protocol for isolation [FACS analysis or processing of mononuclear cells (MNC)], culture and characterization of EPCs, such lack of consistency and controversy will remain.

EPCs have also been shown to be able to build colonies in vitro (Colony Forming Units or CFU). CFU are clusters of rounded cells surrounded by spindle-shaped cells emanating from the centre. CFU are used as an indicator of the number of circulating EPCs. Moreover, the number of CFU correlates with the number of cardiovascular risk factors (Hill, 2003). Furthermore, Matrigel assay (Hur, 2004) and adhesion to endothelial cells (George, 2003) are two methods to assess EPC function.

1.5.3 ENDOTHELIAL PROGENITOR CELLS IN CARDIOVASCULAR DISEASE AND ISCHEMIA

Endothelial dysfunction plays a key role in the onset of atherosclerosis, therefore replacement of damaged ECs will result in recovery of the endothelium. Since EPCs are able to proliferate and

![Figure 9](image-url)
differentiate into ECs, they are ideal candidates for vascular regeneration. Many studies conducted in animals show that EPCs, both culture expanded and freshly isolated, are able to restore the circulation in ischemic organs, positively influence the repair of ECs after injuries and the progression of atherosclerosis (George, 2005; Shintani, 2001; Asahara, 1999; Zhang, 2002). Similar, clinical trials indicate that EPCs may be therapeutically useful to improve blood flow of ischemic tissue and heart function (Tateishi-Yuyama, 2002; Assmus, 2002). Thus, any impairment of EPCs to endothelial repair and regeneration may correlate with the progression in atherosclerosis (Rauscher, 2003).

1.5.3.1 Mobilization of EPCs

Following ischemia, growth factors and cytokines are released, and bone marrow is stimulated to release EPCs, which will then home at the injury site and stimulate vessel growth and repair (Takahashi, 1999). NO plays an important role in the mobilization of progenitor cells from the bone marrow into the circulation (Aicher, 2003). Thus, mobilization of endogenous EPCs may be an alternative way to increase neovascularization in adults. VEGF (Asahara, 1999), SDF-1 (Askari, 2003), GM-CSF and G-CSF (Powell, 2005) have been shown to increase the levels of circulating EPCs. In general, stem cell mobilization is mediated by proteinases, as for example matrix metalloproteinases (MMPs) (Lapidot, 2002), which cleave the adhesive bonds on stromal cells of the bone marrow, which interact with integrins on stem cells (Assmus, 2002; Levesque, 2001), making them able to leave the bone marrow and enter the circulation.

1.5.3.2 Homing of EPCs

Following bone marrow-mobilization, EPCs have to home at site of injury. This process most likely depends on the expression of homing receptors by EPCs. Selectin family adhesion receptors are required in the initial events of vascular adhesion, and in particular L-selectin expressed on EPCs seems to play a critical role in the homing process (Biancone, 2004). Furthermore, P-selectin may mediate the interaction between EPCs and platelets. Platelets are the first cells to arrive and adhere at site of endothelial injury, and they have been shown to provide a matrix, which promotes adhesion and proliferation of EPCs (Lev, 2006). Moreover, in animals, platelets regulate chemotaxis, adhesion and differentiation of EPCs (Langer, 2006).

EPCs are thus incorporated at sites of ischemia and contribute to the formation of new vessels. However, the number of incorporated cells is quite low, therefore neovascularization may not only be due to EPC incorporation, but also to release of pro-angiogenic factors. Studies in this optic demonstrate that EPCs express and secrete a variety of growth factors in a paracrine manner, one of the most important being VEGF, besides IGF-1, HGF and SDF-1. These molecules stimulate the migration of mature endothelial cells and act as chemoattractants to promote migration of more EPCs (Urbich, 2005).

1.5.3.3 EPCs and cardiovascular risk factors

During atherosclerosis development, rapid restoration of endothelial integrity and function could prevent development and growth of neointimal lesions. Classical risk factors for atherosclerosis as age, male gender, hypertension, diabetes, high cholesterol, smoking, obesity and physical inactivity have also been shown to inversely correlate with the number of circulating EPCs, and in patients suffering of coronary artery disease (CAD) the number of circulating EPCs is lower than in healthy subjects and
their functionality is impaired (Umemura, 2007; Vasa, 2001). This decrease in circulating EPCs seems to be related to the decreased bioavailability of NO in the presence of risk factors (Umemura, 2008). Furthermore, continuous endothelial damage and dysfunction may deplete the reserve of available EPCs (Hill, 2003). Patients with a low baseline of EPCs show a higher death from cardiovascular causes compared to patients with a high EPC baseline (Werner, 2005). It has also been shown, that the number of circulating EPCs is increased in the early phase of acute myocardial infarction (Massa, 2005). EPCs are a source of cells for endothelial repair, and since circulating EPCs, defined as CD34+/AC133+/VEGFR-2+ cells, are about the 0.002% of total mononuclear cells (MNC) (Peichev, 1997), further reduction in their number and functionality, because of cardiovascular risk factors, is expected to have an impact on the health of endothelium.

### 1.5.3.3.1 Ageing

Even in the absence of any risk factor, atherosclerosis will naturally progress in any individual, being it an age-related disease. Ageing is associated, besides the lower rate of proliferation of vascular cells due to senescence, with diminished functional features of progenitor cells (Heiss, 2005). Advanced age is associated with low levels of VEGF in animals (Gennaro, 2003) and in humans (Scheubel, 2003), which is responsible for EPC mobilization.

EPCs are not stem cells, but rather lineage-committed progenitor cells, therefore, they are subject to replicative senescence. Estradiol has been shown to reduce EPC senescence by downregulating angiotensin type 1 receptor (Imanishi, 2005) and by promoting telomerase activity (Imanishi, 2005).

### 1.5.4 HEME OXYGENASE-1

Atherosclerosis is an inflammatory disease of the arteries. Inflammation is caused by high oxidative stress, which leads to excessive production of ROS, as a consequence of presence of cardiovascular risk factors. Therefore, protective responses of the vasculature to oxidative stress may be crucial to atherosclerosis prevention (Morita, 2005). Heme, associated with proteins as in haemoglobin and myoglobin, is critically required for oxygen transport in blood, but its free form can be quite toxic, especially in the presence of oxidants. Being a component of the blood vessels, the most exposed cell type in the body are ECs. Haemoglobin has been shown to act as a catalyst for the oxidation of LDL, which in turn causes atherosclerosis (Jeney, 2002). Moreover, in cultured cells, heme induces expression of adhesion molecules, involved in monocyte adhesion to vessel wall (Wagener, 1997). Heme oxygenase (HO) is a rate-limiting enzyme that participates to the degradation of heme into biliverdin, iron and carbon monoxide (Maines, 1997). All products of heme oxygenase activity have an important function in the vascular system, and are linked to the protection of ECs (Dulak, 2008). Three isoforms of HO have been identified in mammals (McCoubrey, 1994; Shibahara, 1993; McCoubrey, 1997). HO-1, the inducible form of HO, is usually present at low levels in the majority of cells, and it is transcriptionally upregulated as a sensitive anti-inflammatory protein by various types of oxidative stress (Morita, 2005). Deficiency in HO-1 led to an abundance of circulating heme and consequently to damage of vascular endothelium. Moreover, HO-1 has proangiogenic effects, which are related to induction of VEGF [reviewed in (Dulak, 2008)]. The other two isoforms of HO are HO-2, which is the constitutive isoform, and HO-3, which has a very little heme degrading activity and which functions remain unclear.

There is need to enhance the number of EPCs, to allow harvesting of adequate amounts of cells for therapeutic applications. Moreover, due to the limited ability of EPCs to expand in culture there is need for new experimental strategies to increase their number endogenously or in vitro. Strategies to
Endothelial Progenitor Cells

Introduction

improve retention and survival of the transplanted cells also need to be developed. There is also need to standardize the protocols for isolation, cultivation and therapeutic application for cell-based therapy (Dzau, 2005).
A key step in the formation of an atherosclerotic plaque is the overproliferation of SMCs and the neointima thickening. In the classical view, SMCs in the intimal layer are migrating from the arterial media (Ross, 1999). However, SMCs have been rarely reported to migrate into the subendothelial layer. More interestingly, MNCs have been shown to adhere to injured endothelial layer (Sata, 2003). It has also been shown, that neointimal formation is possible even in the absence of medial layer cells (Sata, 2000). Moreover, SMCs in the neointima express hematopoietic lineage markers, in contrast with SMCs in the arterial media (Zohlnhofer, 2001). These observations led to the hypothesis that SMCs in atherosclerotic lesions may originate from a population of circulating smooth muscle progenitor cells, instead deriving from the underlying media. Experimental work (Sata, 2000; Saiura, 2001; Sata, 2002) shows that, following allograft bone marrow or heart transplantation from mice expressing LacZ, neointimal cells in the recipient were LacZ positive. There are also works in humans supporting the existence of these putative SM-progenitor cells. MNC cultured in endothelial growth medium-2 (EMG-2) supplemented with platelet-derived growth factor BB (PDGF-BB) show the typical hill and valley morphology of SMC and they were positive for SMC markers (Simper, 2002; Zoll, 2008). This provides evidence that not all bone marrow responses to cardiovascular injury are beneficial, as occurs with EPCs. Neointima formation is a pathological event, which complicates chronic atherosclerosis. Thus, bone marrow-derived cells are implicated into regenerative and pathogenic processes in the cardiovascular system.

### 1.6.1 C-KIT CELLS

c-kit is a proto-oncogene which encodes a receptor tyrosine kinase (Chabot, 1988) that has been shown to be expressed on the surface of hematopoietic and stem cells including cardiac and endothelial cells (Beltrami, 2003). Within the bone marrow it is expressed on hemangioblasts, which are the precursors of EPCs (Kabrun, 1997). Stromal cells in the bone marrow express the ligand for c-kit, KitL [or stem cell factor (SCF)], in a cell-bound form, which maintain them in a quiescent state. (Flanagan, 1991). Under stress conditions, MMP-9 action leads to the release of soluble KitL, thus inducing proliferation of endothelial and hematopoietic stem cells and their mobilization to circulation (Heissig, 2002). In an animal model, it has been shown that about 66% of the cells participating into neointima formation after vascular injury were bone marrow-derived. Furthermore, these cells were c-kit*, providing evidence that SCF/c-kit signaling is a critical determinant of bone marrow cell contribution to neointimal formation following injury, since in SCF-mutant or c-kit-mutant mice the rescue of injured SMC was impaired (Wang, 2006; Wang, 2007).
1.6.2 MOBILIZATION OF SMOOTH MUSCLE PROGENITOR CELLS

Among the chemokines controlling cell trafficking, stromal derived factor-1α (SDF-1α) and its receptor CXCR4 are an important signaling axis for the mobilization of SM-progenitor cells. Moreover, M-CSF has been shown to mobilize bone marrow cells expressing CXCR4 to the circulation, where they are then recruited to injury site (Shiba, 2007). Additionally, hypoxia-inducible factor-1α (HIF-1α) induces the expression of SDF-1α (Ceradini, 2004), and its expression is upregulated in SMC after vascular injury. Therefore it is supposed that HIF-1α could be involved in SDF-1α SM-progenitor cell neointimal recruitment (Karshovska, 2007).

1.6.3 CHANGE OF PHENOTYPE

One experimental work in mice provided evidence that skeletal muscle-derived stem cells have a potential to differentiate \textit{ex vivo} into an endothelial lineage (Arriero, 2004). This is consistent with the observation of the multipotency of these cells (Cao, 2003) and with the growing evidence of their bone marrow origin [rev. in (Wagers, 2004)]. Moreover, blocking of VEGF-R in ECs has shown to first induce apoptosis and then the proliferation of surviving cells, followed by their differentiation into vascular SMCs. These proliferating cells were either CD34+ or c-kit+ (Sakao, 2007).

In conclusion, further research and a consistency in the use of definition for EPCs and SM-progenitor cells are still needed, as novel techniques to allow precise identification of these cells.
2. **HYPOTHESIS AND OBJECTIVES**

Estradiol is known to protect against vaso-occlusive disorders associated with cardiovascular diseases. At a cellular level, estradiol has been shown to promote endothelial repair by stimulating EC growth, and preventing neointima formation by inhibiting SMC growth. Because EPCs are functionally similar to ECs, and contribute to vascular endothelium repair, we hypothesize that estradiol may protect the vasculature/endothelium by promoting EPC growth. Additionally, since estradiol inhibits SMC growth and c-kit+ cells are SM-like cells and contribute to neointima formation, we hypothesize that estradiol may abrogate neointima formation by inhibiting c-kit+ cells growth.

1. The first aim of this study was to isolate, characterize and investigate the effects of estradiol on EPC growth and to dissect the role of the two different ERs in mediating these effects. Another aim was to elucidate the intracellular mechanisms via which estradiol mediates its mitogenic effects on EPCs.

2. Because EPCs have been shown to repair injuries in blood vessel and to participate in angiogenesis/neovasculogenesis processes, we investigated the role of estradiol in regulating the adhesion of EPCs to ECs, and in activating EPC induced microvessel formation. Moreover, we investigated the intracellular mechanisms mediating the effects of estradiol in these processes.

3. Circulating progenitor cells not only form EPCs, but can also develop into other cells. We observed that some CD34+ cells did not develop into ECs, but rather into SM-like cells. Hence, the third aim of this study was to isolate, characterize these c-kit+ SM-like progenitor cells and to investigate the growth effects of estradiol. Moreover, we investigated the role of ERs and the intracellular mechanisms via which estradiol regulates their growth. Because SM-like progenitors are similar to SMCs, we hypothesized an antimitogenic effect of estradiol.
# 3. MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 CELL CULTURE

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<td>BSA</td>
<td>A-3059</td>
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3.1.3 **CELL TREATMENT**

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<tr>
<td>HGF</td>
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<td>LY294002</td>
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<td>MPP</td>
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<td>Obiter Research LLC, Champaign, US</td>
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<td>PPT</td>
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<td>S-8442</td>
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<td>TNF-α</td>
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### Materials

**VEGF**  
cat# V-7295  
Sigma-Aldrich Chemie GmbH, Buchs, CH

#### 3.1.4 Protein isolation and detection

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<td>Bio-Rad, Hercules, US</td>
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<td>161-0771</td>
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<td>10x Tris/Glycine/SDS (running buffer)</td>
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### Materials

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#### Materials and Methods

### 3.1.5 IMMUNOFLUORESCENCE

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## 3.1.7 FACS

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<td>Anti-Mouse IgG1, γ-APC</td>
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<td>BD Biosciences, Franklin Lakes, US</td>
</tr>
<tr>
<td>Anti-Mouse IgG1, κ-PE</td>
<td>555749</td>
<td>BD Biosciences, Franklin Lakes, US</td>
</tr>
<tr>
<td>Anti-Mouse IgG2a, κ-FITC</td>
<td>555573</td>
<td>BD Biosciences, Franklin Lakes, US</td>
</tr>
</tbody>
</table>

## 3.1.8 NO ASSAY

<table>
<thead>
<tr>
<th>Material</th>
<th>Cat#</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-(1-naphthyl)ethylenediamine dihydrochloride</td>
<td>222488</td>
<td>Sigma-Aldrich Chemie GmbH, Buchs, CH</td>
</tr>
<tr>
<td>Ortho-phosphoric acid, 85%</td>
<td>79617</td>
<td>Fluka, Buchs, CH</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>S-9251</td>
<td>Sigma-Aldrich Chemie GmbH, Buchs, CH</td>
</tr>
<tr>
<td>Minimum Essential Medium-MEM (1x), Phenol red and nitrate/nitrite free</td>
<td>51200-046</td>
<td>Gibco Invitrogen Corporation, Carlsbad, US</td>
</tr>
</tbody>
</table>

## 3.1.9 MICROVESSEL FORMATION

<table>
<thead>
<tr>
<th>Material</th>
<th>Cat#</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basement Membrane Matrix, Phenol Red-free</td>
<td>356237</td>
<td>BD Biosciences, Franklin Lakes, US</td>
</tr>
</tbody>
</table>

## 3.1.10 ³H -THYMIDINE INCORPORATION AND CELL GROWTH

<table>
<thead>
<tr>
<th>Material</th>
<th>Cat#</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>³H-methyltimidine</td>
<td>NET027Z001MC</td>
<td>PerkinElmer Inc., Waltham, US</td>
</tr>
<tr>
<td>NaOH</td>
<td>S-8045</td>
<td>Sigma-Aldrich Chemie GmbH, Buchs, CH</td>
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</tbody>
</table>
### 3.1.11 Tissue Culture Ware

<table>
<thead>
<tr>
<th>Item</th>
<th>Catalog Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin coated plates, 24 wells</td>
<td>354411</td>
<td>BD Biosciences, Franklin Lakes, US</td>
</tr>
<tr>
<td>Cell culture dishes (35 and 60mm)</td>
<td></td>
<td>Falcon (BD Biosciences, Franklin Lakes, US)</td>
</tr>
<tr>
<td>Cell culture flasks, 75cm²</td>
<td></td>
<td>Falcon (BD Biosciences, Franklin Lakes, US)</td>
</tr>
<tr>
<td>Cell culture flasks, 25cm²</td>
<td></td>
<td>Samadeni AG, Ostermundigen, CH</td>
</tr>
<tr>
<td>Cryotubes</td>
<td></td>
<td>Nalge Nunc International, US</td>
</tr>
<tr>
<td>Western Blotting equipment</td>
<td></td>
<td>Bio-Rad Laboratories Inc., Hercules, US</td>
</tr>
<tr>
<td>Reaction tubes (14 and 50 ml)</td>
<td></td>
<td>Falcon (BD Biosciences, Franklin Lakes, US)</td>
</tr>
<tr>
<td>Reaction tubes (1.5 ml)</td>
<td></td>
<td>Eppendorf AG, Hamburg, D</td>
</tr>
<tr>
<td>Tips</td>
<td></td>
<td>TipOne (USA Scientific Inc., Ocala, US)</td>
</tr>
</tbody>
</table>

### 3.1.12 Equipment and Software

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometer</td>
<td>BD Biosciences, Franklin Lakes, US</td>
</tr>
<tr>
<td>Fluorescence and light microscope</td>
<td>Olympus America Inc., Center Valley, US</td>
</tr>
<tr>
<td>Odyssey Imaging System</td>
<td>LI-COR Biosciences, Bad Homburg, D</td>
</tr>
<tr>
<td>Sonoplus</td>
<td>Bandelin Electronic GmbH, Berlin, D</td>
</tr>
<tr>
<td>SpectraFluor Plus</td>
<td>Tecan Group Ltd., Männendorn, CH</td>
</tr>
</tbody>
</table>
3.2 METHODS

3.2.1 EPC ISOLATION

Buffers and solutions

<table>
<thead>
<tr>
<th>Complete culture medium, phenol red free</th>
<th>EBM, phenol red free; EGM-2 SingleQuot Kit; 1x antibiotic/antimycotic; 20% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation Buffer</td>
<td>2mM EDTA in PBS; sterile filtered</td>
</tr>
<tr>
<td>MACS Buffer</td>
<td>2mM EDTA, 0.5% BSA in PBS; sterile filtered</td>
</tr>
<tr>
<td>CD34 MicroBead Kit</td>
<td>FcR Blocking reagent, MicroBeads-conjugated CD34 antibody</td>
</tr>
</tbody>
</table>

Both umbilical cord blood and adult peripheral blood were used to isolate EPC.

Patient umbilical cord blood was collected in a blood bag shortly after delivery, whereas peripheral blood was collected in 15 ml heparin-containing tubes. In both cases, blood was put at 4°C to cool off.

Blood was transferred into 50 ml plastic tubes and mixed at a 1:1 ratio with isolation buffer. The blood and isolation buffer mixture was subsequently layered over a pre-established gradient of 15 ml Biocoll. Care was taken during the layering of blood to avoid mixing. Blood was then centrifuged at 500 g for 30 min (4°C) without brake.

After centrifugation four fractions were obtained (from bottom to top): red blood cells, Biocoll, white membrane with mononuclear cells (MNC) and serum. The serum fraction was aspirated and the MNC layer was carefully transferred to a new 50 ml tube using a 1000 μl pipette. Cells were then washed twice with 10 ml of isolation buffer (10 min, 1200 rpm at 4°C) and resuspended in phenol red free complete culture medium. The cells were counted and plated at a density of 2.5x10⁶ cells/well in a 24 wells fibronectin coated plate (precoated plates, Falcon; otherwise coated with 2 μg/cm² of fibronectin). Two days after isolation, non-adherent cells were collected and plated in new wells (Fig. 1).
MNC were isolated from blood as described above. After the last washing step, the pellet was resuspended in 300 μl of MACS buffer and 100 μl of Fc-R Blocking Reagent and CD34 Micro Beads (CD34 MicroBead kit, Miltenyi) were added. Cells were gently vortexed and incubated at 4°C for 30 min. Cells were washed once with 2 ml of MACS buffer (100 g, 5 min at 4°C) and then resuspended in 500 μl of MACS buffer.

Subsequently, a MS Column was inserted into the MACS separator and equilibrated with 500 μl of MACS buffer. The cells were applied onto the column and unlabeled cells were collected in a 15 ml tube. The column was washed with 3x500 μl of MACS buffer and then removed and placed on a new 15 ml tube. CD34+ cells were collected by rinsing the column with 1 ml of MACS buffer, then they were counted and plated at a density of 2.5x10⁶ cells/well in a 24 wells fibronectin coated plate (precoated plates, Falcon; otherwise coated with 2 μg/cm² of fibronectin). Cells were maintained in phenol red free complete culture medium.
Methods

3.2.1.2 CFU counting

One week after replating of MNC in phenol red free complete culture medium, or nine days after CD34+ cells isolation CFU were counted under an inverted microscope.

3.2.2 CELL CULTURE

Buffers and solutions

<table>
<thead>
<tr>
<th>Complete culture medium</th>
<th>EGM-2 Bullet Kit (EGM-2 and SingleQuot); 1x antibiotic/antimycotic; 20% FCS</th>
</tr>
</thead>
</table>

EPCs were grown in 75 cm² flasks at 37°C, 5% CO₂. Upon reaching confluency, as observed in an inverted microscope, the medium was aspirated and 5 ml HBSS w/o Ca²⁺/Mg²⁺ were added to wash the cells. 3 ml Trypsin-EDTA were added and after 5 min in the incubator, cells were detached by gently tapping the flasks. Again the inverted microscope was used to confirm complete detachment of the cells. 10 ml of growth medium were added and the cells resuspended. Cells were then plated at split ratios of 1:4. SM-like cells were grown as described for EPCs, using M231 medium supplemented with SMGS, 1x antibiotic/antimycotic and 20% FCS.

3.2.2.1 Cryopreservation of cells

Subconfluent cells were trypsinized as described above and centrifuged at 100 g, 5 min. The pellet was then resuspended in complete culture medium and 450 μl of cells were put in cryotubes. DMSO was added to a final concentration of 10% (50 μl) and cells were gradually frozen to -80°C using a Mr. Frosty box. For long-term storage, cells were put into liquid nitrogen.

3.2.2.2 Cell thawing

Cells in cryotubes were rapidly thaw under warm water and transferred to a 25 cm² flask. 4 ml of complete culture medium were then added into the flask. After reaching confluency, they were trypsinized and put into a 75 cm² flask.

3.2.2.3 Cell treatment

Buffers and solutions

<table>
<thead>
<tr>
<th>DMEM-F12-0.4% (starving medium)</th>
<th>DMEM-F12, phenol red free; 0.11% NaHCO₃, 1x antibiotic/antimycotic; 0.4% steroid free serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-F12-5% (treatment medium)</td>
<td>DMEM-F12, phenol red free; 0.11% NaHCO₃, 1x antibiotic/antimycotic; 5% steroid free serum</td>
</tr>
</tbody>
</table>
Cells were trypsinized as described in cell culture, and $60 \times 10^5$ cells were plated on 60 mm culture dishes or $20 \times 10^5$ were plated on 35 mm culture dishes. When cells reached about 70-80% confluency, they were serum-starved overnight using DMEM-F12-0.4%. The next day, starving medium was removed and cells treatment was performed in DMEM-F12-5%.

### 3.2.3 Western Blot

#### 3.2.3.1 Cell treatment

**Buffers and solutions**

| DMEM-F12-0.4% (starving medium) | DMEM-F12, phenol red free; 0.11% NaHCO₃; 1x antibiotic/antimycotic; 0.4% steroid free serum |
| DMEM-F12-5% (treatment medium) | DMEM-F12, phenol red free; 0.11% NaHCO₃; 1x antibiotic/antimycotic; 5% steroid free serum |

To evaluate the effects of estradiol on EPC growth, cells were cultured and serum-starved as described above. 1 nM, 10 nM, 100 nM or 1 μM E2 was added to the cells; DMSO was used as control (final concentration of 0.1%).

To look at the role of estrogen receptors on EPC growth, cells were cultured and serum-starved as described above. Cells were pretreated 15 min with 1 μM ICI182,780 (ERα and ERβ antagonist) or 1 μM MPP (ERα antagonist) and treated with 10 nM E2, 100 nM PPT (ERα agonist) or 100 nM DPN (ERβ agonist); DMSO was used as control (final concentration of 0.1%).

To look at the role of VEGF-R on protein expression, cells were cultured and serum-starved as described above. Cells were pretreated 30 min with 5 μM of VEGFR-2 inhibitor SU5416 prior to treatment with 10 nM of E2; DMSO was used as control (final concentration of 0.1%).

Treatment was performed for 7 min to look at phosphorylation of Akt, MAPK and eNOS; it was performed for 48 hours for all others proteins.

SM-like cells were treated as described above, but they were not serum-starved before treatment.

#### 3.2.3.2 Cell lysis

**Buffers and solutions**

| Lysis buffer | 200 μl 10x lysis buffer; 40 μl 10% SDS; 5.6 μl PMSF 100 mM; 1.75 ml dH₂O |

After treatment, medium was aspirated and cells washed twice with HBSS (with Ca²⁺ and Mg²⁺), lysis buffer was added to the dish (respectively 50 μl and 70 μl for a 35 mm or a 60 mm dish). The dish was put on ice for 5 min. Cells were then scraped and the lysate was put in a 1.5 ml Eppendorf on ice. Cells were homogenized 2x 3 seconds by sonication (Sonoplus).
3.2.3.3 BCA protein concentration assay

Buffers and solutions

<table>
<thead>
<tr>
<th>BCA assay kit</th>
</tr>
</thead>
</table>

Samples were prepared as follow:

- Dilutions:
  - 1:2 20 μl cell lysate 20 μl dH2O
  - 1:5 8 μl cell lysate 32 μl dH2O
  - 1:10 4 μl cell lysate 36 μl dH2O

Standards were prepared as follow:

- S1: albumin standard ⇒ 2000 μg/ml
- S2: 80 μl of albumin standard + 80 μl dH2O ⇒ 1000 μg/ml
- S3: 80 μl of S2 + 40 μl dH2O ⇒ 666.67 μg/ml
- S4: 80 μl of S3 + 40 μl dH2O ⇒ 444.44 μg/ml
- S5: 80 μl of S4 + 40 μl dH2O ⇒ 296.29 μg/ml
- S6: 80 μl of S5 + 40 μl dH2O ⇒ 197.53 μg/ml
- S7: 80 μl of S6 + 40 μl dH2O ⇒ 131.68 μg/ml

Triplicates of standard or sample were prepared putting 10 μl per well of a 96 well plate. Distilled water was used as blank. The substrate was prepared by mixing one part of BCA Reagent A with 1/50 part of BCA Reagent B. 200 μl of it were added to each well. The plate was shook for 30 seconds in order to mix the substrate with the samples and then it was incubated for 30 min at 37°C. After incubation the plate was cooled for 2 min at room temperature and absorbance was measured at 540 nm with SpectraFluor Plus (Tecan) and data were analyzed with Magellan6 software.

3.2.3.4 Gel electrophoresis

Buffers and solutions

<table>
<thead>
<tr>
<th>10% resolving gel</th>
<th>2 gels: 4 ml Rotiphorese Gel30; 3.75 ml 1.5 M Tris-HCl, pH 8.8; 150 μl SDS 10%; 6.2 ml dH2O; 63.8 μl APS 20%; 7.5 μl TEMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% resolving gel</td>
<td>2 gels: 6.25 ml Rotiphorese Gel30; 3.1 ml 1.5 M Tris-HCl, pH 8.8; 125 μl SDS 10%; 2.9 ml dH2O; 62.5 μl APS 20%; 5 μl TEMED</td>
</tr>
<tr>
<td>5.7% stacking gel</td>
<td>2 gels: 1 ml Rotiphorese Gel30; 1.5 ml 0.5 M Tris-HCl, pH 6.8; 60 μl SDS 10%; 3.3 ml dH2O; 30 μl APS 20%; 6 μl TEMED</td>
</tr>
<tr>
<td>6% resolving gel</td>
<td>2 gels: 3 ml Rotiphorese Gel30; 3.75 ml 1.5 M Tris-HCl, pH 8.8; 150 μl SDS 10%; 8 ml dH2O; 75 μl APS 20%; 1 μl TEMED</td>
</tr>
<tr>
<td>Running buffer</td>
<td>1% SDS, 250 mM Tris, 1.92 M Glycin (100 ml, 10x conc, Bio-</td>
</tr>
<tr>
<td>Method/Buffer Description</td>
<td>Composition</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Stripping buffer 1</td>
<td>0.1 M Glycine in PBS, pH 2-3</td>
</tr>
<tr>
<td>Stripping buffer 2</td>
<td>1 M NaCl in PBS</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>25 mM Tris, 192 mM Glycine, (100 ml, 10x con, Bio-Rad); 20% (200 ml) Methanol; dH₂O ad 1 litre</td>
</tr>
</tbody>
</table>

**HYPERFILM ECL**

At least 10 µg of protein were diluted in 5x loading buffer and 0.1 M DTT and denatured 5 min at 95°C. Samples were loaded on a 6, 10 or 15% SDS gel (depending on protein of interest) and electrophoresis was performed with the Bio-Rad gel system according to the manufacturer’s instruction. Nitrocellulose membrane and gel were briefly soaked in transfer buffer. DNA was transferred to the nitrocellulose membrane 1 hour using Wathman filter paper (3 mm, premoistened with transfer buffer). Then, membrane was blocked for 1 hour in PBS, 0.2% Tween-20, 5% milk and incubated overnight with the primary antibody in PBS, 0.2% Tween-20, 1% milk. The next day, membrane was washed 3 x 10 min at room temperature in PBS, 0.2% Tween-20, 1% milk and subsequently incubated for 1 hour with the secondary peroxidase-labeled antibody in PBS, 0.1% Tween-20, 1% milk. Then, the membrane was washed once 10 min in PBS, 0.2% Tween-20, 1% milk and twice in PBS, 0.2% Tween-20. SuperSignal West Dura or Pico were mixed 1:1 and directly added on the membrane and after 5 min incubation membrane was briefly placed on Linsoft paper tissue and wrapped into Saran transparent films. Luminescence detection was performed by exposing the membrane to a Hyperfilm ECL. Blocking, incubation and washing steps were performed under gentle agitation.

**LI-COR**

Gel electrophoresis and protein transfer were performed as described above. Then, membrane was blocked for 1 hour in PBS, 5% milk and incubated overnight with the primary antibody in PBS, 0.1% Tween-20, 1% milk. The next day, membrane was washed 3 x 5 min at room temperature in PBS, 0.1% Tween-20 and subsequently incubated for 1 hour with the secondary labeled antibody in PBS, 0.1% Tween-20, 1% milk. Then, the membrane was washed twice 5 min in PBS, 0.1% Tween-20 and once in PBS. IR detection was then performed using Li-cor. Blocking, incubation and washing steps were performed under gentle agitation.

For further detection on the same membrane of other proteins, the membrane was stripped for 20 min in stripping buffer 1, shortly washed in stripping buffer 2 and then washed 3 x 5 min with PBS, Tween-20 (0.2 or 0.1% depending on the method). After 1 hour blocking in PBS, 5% milk (and 0.2% Tween-20 for the hyper-film method), the membrane was incubated again overnight with the primary antibody.

**3.2.3.5 ERα and ERβ characterization**

In order to characterize ERα and ERβ into EPCs, 0.5 µg/ml of anti-ERα or anti-ERβ antibody were incubated overnight at 4°C with 15 µg/ml of their respective immunizing peptide in PBS, 0.1 mg/ml BSA. Proteins were detected by western blotting as described above.
3.2.4 CHARACTERIZATION – IMMUNOFLUORESCENCE

Buffers and solutions

<table>
<thead>
<tr>
<th>Complete culture medium</th>
<th>EGM-2 Bullet Kit (EGM-2 and SingleQuot); 1x antibiotic/antimycotic; 20% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>PFA 4%</td>
<td>4% paraformaldehyde in PBS, sterile filtered</td>
</tr>
<tr>
<td>Triton X-100 0.1%</td>
<td>0.1% Triton X-100 in PBS, sterile filtered</td>
</tr>
</tbody>
</table>

EPCs were grown until confluence in 8 well chamber slides (BD Biosciences) using complete culture medium. They were washed once with PBS, fixed for 10 min at 4°C with a 4% paraformaldehyde solution and then permeabilized for 10 min at room temperature with Triton X-100.

Primary antibodies were diluted in PBS as follow:

- Mouse anti-Endothelial Cell (CD146): 1/500 dilution based on 1 mg/ml, final 2 ng/ml
- Rabbit anti-VWF: 1/5000 dilution based on 1 mg/ml, final 0.2 ng/ml
- Mouse anti-CD31: 1/50 dilution based on 0.1 mg/ml, final 2 ng/ml
- Mouse anti-ICAM-1: 1/500 dilution based on 1 mg/ml, final 2 ng/ml
- Mouse anti-VCAM-1: 1/500 dilution based on 1 mg/ml, final 2 ng/ml
- Mouse anti-IgG: 1/1000 dilution
- Rabbit anti-IgG: 1/1000 dilution

100 μl of diluted primary antibodies were added per well and the cells were incubated 30 min at 4°C.

Cells were washed once with PBS and then 100 μl of diluted secondary antibody were added. Secondary antibodies were diluted in PBS as follow:

- FITC-anti mouse IgG: 1/64 dilution
- FITC-anti rabbit IgG: 1/80 dilution

Cells were washed once with PBS and let dry. They were then analyzed with an Olympus fluorescence microscope, using CellM software. HASMC (cultured in M231, supplemented with SMGF) were used as a negative control.

SM-like cells were cultured, fixed and permeabilized as described above. Following antibody dilutions were used (in PBS):

- Rabbit anti-Desmin: 1/50 dilution based on 0.2 mg/ml, final 4 ng/ml
- Mouse anti-Vimentin: 1/100 dilution based on 0.1 mg/ml, final 1 ng/ml
- Mouse anti-Smooth muscle cell actin: 1/100 based on 0.1 mg/ml, final 1 ng/ml

To characterize the progenitor markers on EPCs and SM-like cells, following antibody dilution were used:

- Anti-CD34 (only for EPCs): 1/10 dilution
- Anti AC133 (both EPCs and SM-like cells): 1/10 dilution
- Anti-CD117 (only SM-like cells): 1/10 dilution
3.2.5 Adhesion

Buffers and solutions

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>8 well chamber slide</td>
<td></td>
</tr>
<tr>
<td>96 wells plate</td>
<td></td>
</tr>
<tr>
<td>Complete culture medium</td>
<td>EGM-2 Bullet Kit (EGM-2 and SingleQuot); 1x antibiotic/antimycin; 20% FCS</td>
</tr>
<tr>
<td>DMEM-F12-0.4% (starving medium)</td>
<td>DMEM-F12, phenol red free; 0.11% NaHCO₃; 1x antibiotic/antimycinic; 0.4% steroid free serum</td>
</tr>
<tr>
<td>DMEM-F12-5% (treatment medium)</td>
<td>DMEM-F12, phenol red free; 0.11% NaHCO₃; 1x antibiotic/antimycinic; 5% steroid free serum</td>
</tr>
</tbody>
</table>

3.2.5.1 ELISA

HAEC were grown in a 96 well plate (Falcon) at 37°C, 5% CO₂ in complete culture medium, until confluence. They were then serum-starved overnight using DMEM-F12-0.4%, pretreated 1 hour with 5 μM MPP, 5 μM ICI or 10 ng/ml TMF-α and treated for 24 hours with 10 nM E2, 100 nM PPT, 100 nM DPN or 10 nM / 1 μM 2-ME in DMEM-F12-5%. EPCs were trypsinized, resuspended in DMEM-F12-5%, counted and adjusted to a concentration of 5x10⁶ cells/ml. Calcein-AM or CMFDA were added to EPCs to a final concentration of 1 μM and cells were then incubated 30 min at 37°C. Cells were centrifuged at 100 g for 5 min, resuspended in PBS and left 10 min at 37°C, in order to wash excess of dye, and centrifugated again at 100 g for 5 min. Cells were then resuspended in DMEM-F12-5% steroid free serum, medium of HAEC was aspirated and 50’000 EPCs were added per well. The plate was incubated 1 hour at 37°C, medium was aspirated from the wells and non adherent cells were washed with PBS. Fluorescence was measured at 517 nm (absorption 494 nm) with SpectraFluor Plus (Tecan) and data were analyzed with Magellan6 software (Fig. 2).

3.2.5.2 Microscopy

HAEC were grown and treated in 8 well chamber slide as described above. Labeling and adhesion of EPCs were performed as for ELISA. After washing non-adherent cells, cells on slide were fixed 5 min at -20°C with ice cold ethanol. Cells were then observed with an Olympus fluorescence microscope, using CellM software.
3.2.6 NO ASSAY

Buffers and solutions

<table>
<thead>
<tr>
<th>Stock I</th>
<th>2% Sulfanilamide, 10% H3PO4 in 50 ml dH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock II</td>
<td>0.2% Naphthylethylendiamine in 30 ml dH2O</td>
</tr>
<tr>
<td>Griess Reagent</td>
<td>Stock I and Stock II mixed 1:1, shortly before use</td>
</tr>
<tr>
<td>MEM-0.4% (starving medium)</td>
<td>Minimum Essential Medium phenol red and nitrite/nitrate free; 0.11% NaHCO3; 1x antibiotic/antimycotic; 0.4% steroid free serum</td>
</tr>
<tr>
<td>MEM-5% (treatment medium)</td>
<td>Minimum Essential Medium phenol red and nitrite/nitrate free; 0.11% NaHCO3; 1x antibiotic/antimycotic; 5% steroid free serum</td>
</tr>
</tbody>
</table>

20x10^3 EPCs per well were plated on 96-well culture plate. When the cells reached about 70-80% confluence, they were serum-starved overnight with MEM-0.4%. After starvation cells were treated for 48 hours with either 10 nM E2 or 100 nM PPT, or pretreated for 15 min with 1 μM MPP prior treatment with 10 nM E2 or 100 nM PPT. After 48 hours medium was collected, put in another 96-well plate, and mixed 1:1 with Griess Reagent. The plate was incubated 20 min at RT, and absorbance was measured at 540 nm with SpectraFluor Plus (Tecan) and data were analyzed with Magellan6 software.
3.2.7 FACS

Buffers and solutions

| FACS Buffer | HBSS w/o Ca²⁺ and Mg²⁺; 0.5% BSA; 2 mM EDTA |
| FcR | Fc Receptor Blocking Reagent |
| Complete culture medium | EGM-2 Bullet Kit (EGM-2 and SingleQuot); 1x antibiotic/antimycotic; 20% FCS |

EPCs were isolated from peripheral blood as described in 3.2.1 and frozen in complete culture medium with 10% DMSO. The day of the experiment they were thaw and washed once with 2 ml of FACS buffer at 100 g for 5 min, 4°C. They were then resuspended in 0.5 ml FACS buffer and 50 μl of FcR blocking reagent were added to each tube. Cells were incubated 30 min at 4°C and then 50 μl of cell suspension were added to a FACS falcon tube. 2 μl of antibody were added to the tubes (IgG were used as negative control). Cells were incubated with antibody 30 min, 4°C in the dark and then washed twice with 1 ml FACS buffer (300 g, 5 min, 4°C) and resuspended in 0.5 ml FACS buffer. Samples were then run in BD FACS.

3.2.8 MICROVESSEL FORMATION

Buffers and solutions

| Matrigel | Basement Membrane Matrix, phenol red free 1:1 with DMEM-F12 phenol red free w/o serum |
| DMEM-F12-5% | DMEM-F12, phenol red free; 0.11% NaHCO₃; 1x antibiotic/antimycotic; 5% steroid free serum |

Basement Membrane Matrix, phenol red free (Matrigel) was thaw overnight on ice at 4°C. The next day it was mixed at a 1:1 ratio with ice-cold serum free and phenol red free medium, and 0.25 ml were put in each well of a 8 well chamber slide using cooled pipette tips. The slide was put at 37°C to allow polymerization of the gel. In the meanwhile, EPCs were trypsinized, resuspended in DMEM-F12-5%, counted, and adjusted to a final concentration of 50'000 cells/200 μl. 200 μl (i.e. 50'000 cells) were then put in 1.5 ml Eppendorf tubes. After 30 min of pretreatment with 5 μM MPP, EPCs were treated with 10 nM E2 or 100 nM PPT for another 30 min; 10 nM DPN were used to investigate the role of ERβ; DMSO was used as control (final concentration of 0.1%). Cells were put on the Matrigel and incubated for 2 hours at 37°C, 5% CO₂. Microvessels were then analyzed using an Olympus inverted microscope.

In order to study effects of tyrosine kinase inhibitor SU5416, experiments were performed as described above. Cells were pretreated 30 min with 5 μM SU5416, and then they were treated either with 100ng/ml VEGF, HGF or SDF-1α or with 10 nM E2. To study the effects of LY294002, cells were treated with 10 μM LY294002 and then treated with 10 nM E2. In both cases DMSO was used as control (final concentration of 0.1%).
To study the effects of estradiol metabolites on microvessel formation experiments were performed as described above, by treating EPCs with different concentrations of 2-ME (from 0.01 to 10 μM).

3.2.9 ³H-Thymidine Incorporation and Cell Growth

<table>
<thead>
<tr>
<th>Complete culture medium</th>
<th>EGM-2 Bullet Kit (EGM-2 and SingleQuot); 1x antibiotic/antimycotic; 20% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-F12-0.4% (starving medium)</td>
<td>DMEM-F12, phenol red free; 0.11% NaHCO₃; 1x antibiotic/antimycotic; 0.4% steroid free serum</td>
</tr>
<tr>
<td>DMEM-F12-5% (treatment medium)</td>
<td>DMEM-F12, phenol red free; 0.11% NaHCO₃; 1x antibiotic/antimycotic; 5% steroid free serum</td>
</tr>
</tbody>
</table>

Subconfluent monolayers of EPCs were grown in complete culture medium in 24 well plates until 70-80% confluence and then serum-starved overnight in starving medium. They were then treated in starving medium supplemented with the substance to test (if pretreatment was present, this was performed 30 min before treatment). After 48 hours the treatment was repeated with freshly prepared solution, and after other 44 hours fresh treatment supplemented with ³H-thymidine (1μM Ci/well) was added to the cells. The experiments were terminated by washing the cells twice with PBS and twice with ice-cold 10% TCA. The precipitates were solubilized in 500μl of 0.3N NaOH and 0.1% SDS (50°C for 2 hours). Aliquots were mixed with 10 ml scintillation fluid and counted with a liquid scintillation counter.

For cell growth experiments, 5x10⁶ EPCs/well were plated in 24 well plates and grown in complete culture medium until 70-80% confluence was reached. They were then serum-starved overnight in starving medium and then treatment was performed in starving medium supplemented with the substance to test (if pretreatment was present, this was performed 30 min before treatment addition). Cells were then trypsinized and counted using a cell counter.

Experiments with SM-like cells were performed as described above, but in this case treatment was performed in treatment medium.

3.2.10 Statistical Analysis

Data were analyzed using ANOVA and statistical significance (p<0.05) was calculated using Fisher's least significant difference test. Values are presented as mean ± SEM.
4. RESULTS

4.1 ENDOTHELIAL PROGENITOR CELL (EPCs) CHARACTERIZATION

OBJECTIVES

The primary aim of these experiments was to confirm that cells obtained from blood were truly EPCs. To demonstrate this, the cells were characterized for both their progenitor and endothelial character using specific markers and immunofluorescence.

INTRODUCTION

Cardiovascular disease is the leading death cause in the world (Lopez, 1997). Artery damage and denudation can lead to neointima and plaque formation, with consequent obstruction of the blood vessel. One of the first steps in the onset of atherosclerosis is the damage of the endothelial monolayer lining the inner of the vessel wall. Therefore, endothelial repair is crucial for cardiovascular health, and it can occur in two ways: either (i) by division of the surrounding mature endothelial cells, or (ii) by incorporation of the so called endothelial progenitor cells (EPCs). EPCs are bone marrow-derived CD34/AC133/VEGFR positive cells (Peichev, 1997). EPCs have been shown to incorporate at sites of ischemia and to be able to restore the blood flow by the formation of new vessels. Since these cells represent only 0.002% of total mononuclear cells (MNC), corresponding at <0.01x10^-3 % of total blood cells, isolation from blood and culture expansion become crucial steps for further therapeutic use of these cells. Of importance is also the correct characterization of the cells, in order to assure the utilization of the correct cell type.

METHODS

Total mononuclear cells (MNC) were isolated from blood as described in methods (3.2.1) and cultured in endothelial basal medium, to promote the differentiation into endothelial lineage.
RESULTS

4.1.1 EXPRESSION OF PROGENITOR MARKERS BY FRESHLY ISOLATED EPCs

To confirm the progenitor character of the cells, the presence of two different markers known to be progenitor cell-specific was investigated. (a) CD34 (present on hematopoietic stem and progenitor cells, but absent on differentiated cells, it functions as cell-cell adhesion factor, and also mediates the attachment of stem cells to bone marrow extracellular matrix ([Lange, 2007])); (b) AC133 (also known as CD133 or Prominin-1, its function is still unclear, it is present only on hematopoietic stem and progenitor cells [rev. in (Shmelkov, 2005)]).

As shown in Fig.1, the cells were positive for both markers, suggesting their progenitor character.

![Image](CD34/AC133.png)

**FIGURE 1.** Microscopic picture of freshly isolated EPCs expressing both CD34 (green) and AC133 (blue).

4.1.2 EXPRESSION OF ENDOTHELIAL MARKERS BY CULTURED EPCs

To confirm that, after culture, the cells developed into the right endothelial character, an endothelial cell characterization kit from Chemicon was used. The presence of three different proteins was investigated: (a) von Willebrand Factor (vWF, a glycoprotein synthesized in endothelial cells which promotes thrombus formation by mediating adhesion of platelets to one another and to the damaged vessel wall and stabilizes factor VIII in the circulation [rev. in (Pusztaszeri, 2005)]); (b) CD31 (also known as platelet cell adhesion molecule-1 (PECAM-1), it is a major component of the intercellular junctions of endothelial cells and there is evidence that it is involved into the extravasation of leukocytes during an inflammatory process [rev. in (Pusztaszeri, 2005)] and of EC during angiogenesis [ (DeLisser, 1997)]; (c) CD146 (is an adhesion molecule localizes at cell junctions [ (Malyszko, 2005)].

As shown in Fig. 2, the cells were positive for all the three markers, confirming their endothelial character.
EPC Characterization

FIGURE 2. Microscopic pictures of EPCs expressing (A) CD31, (B) vWF and (C) CD146. HASMC were used as negative control; vWF expression is shown (D). Magnification: 10x

DISCUSSION

Prior to utilizing either fresh or cultured EPCs, we characterized them for both their progenitor and endothelial character. Immunofluorescence technique was used to confirm the expression of progenitor markers AC133 and CD34, and endothelial markers vWF, CD31 and CD146. To confirm the specificity of antibody labeling, cultured EPCs were used as negative control for progenitor markers (not shown), whereas human aortic smooth muscle cells (HASMC) were used as negative control for endothelial markers (here only control for vWF is shown). Even if there is still controversy in the exact EPC characterization (Kim, 2005; Romagnani, 2005), these results confirm the expression of progenitor markers in our cells, building the basis for the next experiments.
4.2 Estradiol Increases CFU and Circulating EPCs In Vivo

OBJECTIVES

Although repair of damaged vessels protects against cardiovascular disease, the building of new vessels (vasculogenesis) is also crucial during pregnancy, because embryo survival depends on nutrient and oxygen supply from mother to child via the placenta. Indeed development of placenta is a process similar to angiogenesis. Hence, the aim of this study was to determine whether estradiol influences the number and the colony forming potential of circulating EPCs in vivo.

INTRODUCTION

EPCs have been shown to participate in neovasculogenesis and angiogenesis processes and to repair damaged vessel walls (Asahara, 1997). These are important processes for the maintenance of cardiovascular health, since endothelial dysfunction is one of the initiating steps of atherosclerosis (Lusis, 2000; Ross, 1995). Moreover, vessel formation is also crucial in pregnancy, for the correct blood afflux through the placenta. Women, who opt for in vitro fertilization (IVF) have their estrogen levels pharmacologically regulated from low (<100 pM) to high (>1000 pM) concentrations, in order to stimulate ovulation. Estrogens have been shown to increase EPC number in mice and humans (Strehlow, 2003), moreover, male gender is a factor that negatively influences EPC levels (Fadini, 2008). Thus, one can expect that a high circulating EPC number will be advantageous regarding a pregnancy, since the probability of new vessel formation will be higher. Moreover, EPCs, which are CD34/AC133 positive cells, possess the ability, typical for hematopoietic cells, to build colonies. A colony is formed by a group of cells which all derive from a single progenitor cell (the CFU). So, counting colonies is a way to count the number of circulating cells and also look at their growing ability.

METHODS

To look at the effects of estradiol on EPC number and growth in vivo, samples of blood of IVF patients were obtained and EPCs were isolated as described in methods (3.2.1). For each patient two samples were taken; one with low and one with high estradiol levels (Fig. 3).

FIGURE 3. Schematic representation of treatment schedule and sample collection from subjects undergoing hormonal stimulation for IVF. Low E2 blood sample was collected at day of stimulation (d0) and high E2 blood samples was collected after 8 days (d8). HMG: human menopausal gonadotrophins; r-FSH: recombinant follicle stimulating hormone.
RESULTS

4.2.1 COLONY FORMING UNIT (CFU) ASSAY

To analyze the CFU potential of EPCs, cells were isolated and plated on fibronectin coated 24 well plates. After 2 days non-adherent cells were plated in new wells, and colonies per million cells plated were counted after 7 days, as described in 3.2.2, (Fig. 4). Also, the number of colonies formed in high estradiol level samples was compared to colonies of low estradiol level (control) samples.

![Representative photomicrograph](image)

**FIGURE 4.** A representative photomicrograph depicting colonies formed by EPCs after nine days in culture (magnification: 4x).

As shown in Fig. 5, compared with samples with low estradiol plasma levels (<100 pM), significantly higher CFU number were observed in samples with high estradiol plasma levels (>1000 pM). The CFU markers in high and low estradiol samples were 488.1 ± 89.6% and 100 ±19% respectively (p<0.05 vs low estradiol samples).

![Graph](image)

**FIGURE 5.** CFU by EPCs. Number of colonies per million cells plated was compared for each IVF patient between high and low estradiol level samples. n=45; *p<0.05 vs low.
4.2.2 FACS Analysis of Circulating EPCs

EPCs were identified by the combination of progenitor cell-surface antigen markers CD34/AC133. The number of these cells was counted, for each patient, in low and high estradiol level samples by flow cytometric analysis, as described in 3.2.7.

Number of circulating EPCs was also compared between high and low estradiol level samples. As shown in Fig. 6, in the blood of IVF patients the number of CD34+/AC133+ cells was significantly (p<0.05) higher when they had high plasma level of estradiol (216.3 ± 61.1%) compared to the samples with low estradiol plasma level (100 ± 18%).

![Figure 6. Bar graph representing FACS analysis of amount of circulating EPCs in IVF patients’ blood. Compared to low E2 samples, the number of EPCs increases in samples with high level E2. n=45; *p<0.05 vs low.](image)

Discussion

Using variation in the concentration of estradiol in plasma of IVF patients, the purpose of these studies was to look at the effects of estradiol on the number and growth ability of circulating EPCs *in vivo*. Our results confirm findings from other studies, and indicate an increase in both circulating cell number (Strehlow, 2003) and CFU (Fadini, 2008) in response to increase in estradiol levels. Mobilization of EPCs from the bone marrow into the circulation is correlated to NO bioavailability (Aicher, 2003). Estradiol promotes growth of EPCs, moreover, in ECs, estradiol has been shown to upregulate the NO production, thus it is feasible that the increases in circulating CD34+/AC133+ EPCs we observed in IVF patients is due to an increased NO production. Since CFU assay is a way to count EPCs and look at their growth ability, it is feasible that estradiol is promoting growth of EPCs, which is also reflected by an increase in CFU. These growth promoting effects of estradiol on EPCs may be beneficial in cardiovascular disease prevention and healing, since they may influence the protection of cardiovascular system by increasing the number of circulating EPCs available for repair and neovascularization and by improving their proliferation ability. Thus, factors as estradiol, which promotes EPCs proliferation, may be of therapeutical importance and be employed in the prevention of atherosclerosis. Furthermore, this is also important in IVF patients, because a higher number and a higher proliferative capacity of circulating EPCs will, theoretically, increase the chances of a successful pregnancy. Therefore, we need to further investigate the roles of estradiol in regulating EPC growth and the mechanisms behind this.


4.3 Estradiol Promotes Proliferation of EPCs In Vitro

OBJECTIVES

We previously demonstrated that estradiol improves EPC number and growth in vivo. The aim of these experiments was to investigate whether estradiol also affects the growth of these cells after isolation and culture in vitro.

INTRODUCTION

EPCs comprise the 0.002% of the total MNCs in blood. In particular terms, this quantity is too low to employ them directly in patients. Realistically it would be more suitable to isolate them and expand them in culture in order to have an adequate number of cells for therapeutic use. To achieve this, one has to be sure that cultured cells behave the same as the freshly isolated ones, i.e. EPCs circulating within the blood. In order to conduct further experiments using cultured cells, we first investigated whether, similar to freshly isolated EPCs, estradiol acts as a mitogen in EPCs which have been isolated and cultured.

METHODS

Cells were isolated from umbilical cord blood and grown as described in methods (3.2.1 and 3.2.2). Thymidine incorporation was performed as described under 3.2.9.

RESULTS

4.3.1 Cell culture

To qualitatively assess the effect of estradiol on growth of cells in culture, after isolation cells were divided into two fractions and either treated with 10 nM estradiol (a physiological concentration) or used as control (0.1% of DMSO). After one week cells were observed under the microscope.

![Microscopic pictures of cultured EPCs](image)

**FIGURE 7. Microscopic pictures of cultured EPCs. Compared to the control (A), estradiol induces growth of cultured EPCs (B). Magnification: 4x**
At the time of inspection, untreated cells started to adhere to the culture dish and divide, whereas estradiol treated cells (10 nM) already built a complete monolayer (Fig. 7).

4.3.2 Thymidine Incorporation; Effects of Estradiol and Its Metabolites on EPC Proliferation

Cell proliferation was also quantitatively measured by detecting the amount of incorporation of ^3H-thymidine into the DNA of dividing cells following four days of estradiol treatment, after serum starvation (0.4% FCS). Estradiol treatment concentration-dependently induced EPC proliferation (cell number) and DNA synthesis (^3H-thymidine incorporation), both in the physiological (1 and 10 nM) and in the pharmacological (100 nM and 1 μM) range (Fig. 8). The induction of cell growth reached a maximum at 10 nM for both proliferation and DNA synthesis (156.2 ± 2.3% and 139.5 ± 3.9% of untreated cells, respectively, p<0.05) and then was less pronounced at higher concentrations (128.8 ± 1.7% and 119.7 ± 2.2% of control at 1 μM, respectively, p<0.05). Accordingly to these results, we decided to use 10 nM estradiol in further experiments.

This biphasic effect of estradiol on EPC growth may be due to its conversion to 2-OHE and 2-ME metabolites, which have been shown to inhibit growth of different cell types, for example HASMCs (Barchiesi, 2006). To investigate this possibility, we performed thymidine incorporation experiments in EPCs treated with 2OHE and 2ME. As shown in Fig. 9, both metabolites, 2-ME and 2-OHE, acted as anti-mitogens on EPCs. At low concentrations as low as 1 nM 2-ME and 2-OHE inhibited EPC growth from 100% to 87 ± 1.4% and 94.5 ± 1.2%, respectively (p<0.05).
**RESULTS**

![Graphs showing Estradiol metabolites 2-ME (A) and 2-OHE (B) inhibit growth (cell number) of EPCs.](image)

**DISCUSSION**

Estradiol has been shown to promote growth of ECs. In *in vivo* studies, as described, we demonstrated that estradiol promotes growth of freshly isolated CD34+/AC133+ EPCs from IVF patients. Here we provide further evidence that estradiol is mitogenic and promotes growth of culture expanded EPCs. We observed a qualitative promotion of EPC growth by observing cells under the microscope, and we confirmed this result quantitatively, by measuring the increase in cell number by ³H-thymidine incorporation. Cultured cells responding as freshly isolated ones to estradiol are of advantage regarding a future therapeutical use. In this regard, they can be isolated from patients, culture expanded, eventually treated to some specific feature required for the target therapy, and then re-infused back into the patient, so that their number will be enough to induce a beneficial effect.

In breast cancer MCF-7 cells estradiol has been shown to have a biphasic effect on cell growth, promoting at low concentrations and inhibitory at higher ones (Lippert, 2003). This effect is due to the conversion of estradiol into 2-ME and its precursor 2-OHE, which have an anti-mitogenic effect on different cells types, as MCF-7 and HASMCs. At high concentrations, the amount of metabolites produced is enough to partially counterbalance the effect of estradiol and, thus, the promoting effect is, to some extent, abrogated. To confirm this, we performed some ³H-thymidine incorporation experiment in 2-ME and 2-OHE treated EPCs. We could then observe an anti-mitogenic effect in EPCs even at low concentrations of metabolites (1 nM). When EPCs are treated *in vitro* with estradiol, the metabolism of the hormone must be taken into consideration. A clinical implication of the counteracting effects of metabolites on estradiol-promoted EPC growth may be a decreased beneficial effect of hormone replacement therapy in postmenopausal women due to genetic differences in the enzymes responsible for estradiol metabolism (CYP450s and COMT, see 1.1.3). A higher enzymatic activity and the presence of endogenous or exogenous molecules that act as inducers may negatively influence the vasculoprotective effects of estradiol mediated by EPCs. However, in presence of tumors, metabolism of estradiol may play a beneficial role. As every cell, malignant cells need nutrients and oxygen in order to grow. Since they are overproliferating cells, they need new blood vessel to sustain their growth. For this reason, inhibiting growth and vessel formation of EPCs at cancer sites could be a good approach to prevent additional blood supply, and thus tumor growth.
4.4 **ROLE OF ESTROGEN RECEPTORS (ER) IN REGULATING EPC GROWTH**

**OBJECTIVES**

Aim of these experiments was to characterize the presence of the two estrogen receptors ER\(\alpha\) and ER\(\beta\), and to investigate their relative role in mediating the growth effects of estradiol on EPCs.

**INTRODUCTION**

The biological effects of estradiol in mammalian cells are mainly mediated through the estrogen receptors (ER). To date, two ER have been characterized, ER\(\alpha\) and ER\(\beta\) (Kuiper, 1996; Walter, 1985). ERs are expressed both on the membrane and within the cytosol and the nucleus. ER in the cytosol and nucleus mediate the genomic effects of estradiol, whereas membrane ER mediate the rapid non-genomic effects of the hormone (see 1.2.1). The distribution and relative expression of the two receptors vary between different cell types; furthermore, there are studies indicating that ER\(\alpha\) and ER\(\beta\) mediate distinct and even opposite effects of estradiol (Lindberg, 2003). Therefore, in the present studies we characterized both receptors, and their role in mediating growth effects of estradiol on EPCs.

**METHODS**

To determine whether ER are present in EPCs and which role do they have in cell growth, they where first characterized using blocked antibodies. \(^3\)H-thymidine incorporation experiments were performed using ER agonists and antagonists (Fig. 10). It was also tested, by western blotting, whether estradiol modulates the expression of its own receptors.

![Diagram showing interactions of E2, agonists (PPT and DPN) and antagonists (MPP and ICI) with the ER.](image)

**FIGURE 10. Picture depicting the interactions of E2, agonists (PPT and DPN) and antagonists (MPP and ICI) with the ER.**
RESULTS

4.4.1 CHARACTERIZATION OF ER

The expression of both ERα and ERβ was detected by Western Blotting. To exclude bands due to unspecific staining, antibodies blocked with their correspondent peptide were used as negative control (Fig. 11, left side of the membranes). Bands appearing in the right side of the membranes, which were incubated with non blocked antibody, and disappearing on the left side, represent the proteins of interest. As shown in Fig. 11 both ERα (about 55kDa) and ERβ (about 60kDa) are expressed in EPCs.

![Western blotting analysis showing the expression of (A) ERα and (B) ERβ in EPCs. In both cases, left side of the membrane was incubated with blocked antibody as negative control.](image)

4.4.2 THYMIDINE INCORPORATION; ROLE OF ER IN ESTRADIOL-MEDIATED EPC GROWTH

To investigate whether the effects of estradiol on EPCs were ERα and/or ERβ mediated, pharmacological agonists for either ERα (PPT) or ERβ (DPN) and antagonist for either both receptors (ICI) or for ERα (MPP) were used. Cells were counted to determine proliferation and ³H-thymidine incorporation into the DNA of dividing cells was used to measure DNA synthesis.

![Cell counting and ³H-thymidine incorporation. ERα agonist PPT mimics the effects of estradiol on EPCs proliferation (A) and DNA synthesis (B). n=4; *p<0.05 vs control.](image)
As shown in Fig. 12, both ER agonists PPT (ERα) and DPN (ERβ) significantly mimicked the effects of estradiol on proliferation (at 100 nM 142.6 ± 3.2% and 113 ± 3.08% of untreated cells, respectively for PPT and DPN, p<0.05) and PPT also on DNA synthesis (at 100 nM 131.7 ± 3.9% of untreated cells, p<0.05).

To further look at the role of the two ERs, we used pharmacological antagonists to prevent estradiol from binding to and exert its effects via the receptors. Both cell number and DNA synthesis induction mediated by estradiol were abrogated in the presence of the antagonists after four days of treatment. ERα specific antagonist MPP (5 nM) and the ERα/ERβ antagonist ICI (10 μM) blocked the mitogenic effects of estradiol (p<0.05). The growth of untreated EPCs (control), EPCs + E2 and EPCs + E2 + MPP was 100 ± 2.7%, 148.7 ± 3.3% and 115.7 ± 2.2%, respectively for cell number and 100 ± 0.9%, 133.4 ± 1.4% and 113.5 ± 1.6%, respectively for DNA synthesis. Similarly, ICI blocked the growth effects of estradiol (109.8 ± 2.7% and 105.06 ± 0.6% of untreated control, respectively for cell number and DNA incorporation, p<0.05) (Fig. 13).

To confirm these results, ER antagonists were used to block the receptors in presence of ER agonists. As shown in Fig. 14 for both cell number (panel A) and DNA synthesis (panel B) after four days of treatment, PPT effect was reversed by ICI from 138.6 ± 4% to 105.1 ± 1.6% and from 125.9 ± 0.7% to 102 ± 1%, respectively (control growth was 100 ± 1.5% and 100 ± 1%, respectively). Similarly, co-treatment with MPP blocked the stimulatory effects of PPT from 138.6 ± 4% to 108 ± 5.08% and from 125.9 ± 0.7% to 114.3 ± 1.6%, respectively. DPN effects were reversed by ICI from 111.7 ± 2.3% to 98.5 ± 2.9% and from 108.3 ± 1.4% to 100.4 ± 2.03%
Because the mitogenic effects of estradiol were blocked by ERα antagonist and strongly mimicked by ERα agonist, this suggests that these effects are ERα mediated. However, we also observed a marginal but significant stimulatory effect of ERβ which were blocked by ICI, an ERα/ERβ antagonist, suggesting that it also may participate in inducing EPC growth.

### 4.4.3 Modulatory Effect of Estradiol on Estrogen Receptors

In the previous experiments we have shown that effects of estradiol on EPC growth are mainly ERα-mediated, whereas ERβ only has a minor role. The two ER subtypes α and β play thus a different, although not opposite, role in EPCs. Hence, we investigated whether estradiol modulates the expression of its own receptors, and, consequently, modulated its action on EPCs. As shown in Fig.15, expression of both ERα (153.8 ± 13.6% at 10 nM) and ERβ (464.8 ± 3.01% at 10 nM) is significantly upregulated by estradiol at physiological concentrations, compared to the untreated control.


**DISCUSSION**

Expression of ERα and ERβ was investigated prior to examine whether receptors are involved in estradiol-mediated growth of EPCs. Use of specific and blocked antibodies in Western Blotting technique, demonstrated the presence of both ERα and ERβ in EPCs, building the basis for the next series of experiments directed to the study of the role of ER in EPC growth promotion observed following estradiol treatment. Cell number counting and detection of the amount of synthesized DNA and using ³H-thymidine incorporation technique allowed us to determine the role of ERα and ERβ in mediating EPC growth. For both cell number increase and DNA synthesis we observed a strong increase following treatment with ERα agonist PPT. Compared to PPT, a mild but statistically significant induction of EPC growth was also observed in response to the ERβ agonist DPN. Based on these findings we hypothesized that the mitogenic effects of estradiol on EPCs are largely ERα mediated. To test this, we performed more ³H-thymidine incorporation and cell counting experiments to study the effects of estradiol in presence of ER specific antagonists. Blocking of ERα with MPP significantly reduced estradiol and PPT effects, and this reduction was a little more marked in the presence of the ERα/ERβ antagonist ICI, when also the effect mediated by ERβ was lost. These data confirm our hypothesis that estradiol acts on EPCs, predominantly, via ERα. These results are consistent with experiments that show an ERα mediation of estradiol effects on ECs (Gerald, 2002; Doshida, 2006; Chambliss, 2000). In the present study we also demonstrated that the levels of both receptors are similarly regulated by estradiol, a result that differs from those of HASMC, where ERα is downregulated, but not ERβ, and MCF-7 where both receptors are downregulated by estradiol (Barchiesi, 2003), suggesting a different regulatory effect in cardiovascular cells, leading to the hypothesis that the beneficial roles of estradiol on the cardiovascular system may also be due to the different regulation of ER in ECs and SMCs.
4.5 Estradiol Promotes EPC Growth by Regulating the Expression of Cell Cycle Proteins

**Objectives**

We demonstrated that estradiol promotes proliferation of EPCs via ERα. The objective of these experiments was to elucidate the intracellular mechanisms via which estradiol modulates EPC growth.

**Introduction**

Repair of the damaged endothelium by EPCs is an important aspect in the maintenance of the cardiovascular system. Estrogen acts as a mitogen in EPCs by promoting cell proliferation. In general, mitogens, upon binding to their receptor, activate mitogenic signal transduction pathways, which then make the cell enter the cell cycle and divide. The progression through the cell cycle is regulated by the cyclin-dependent kinases (cdk) and the cyclins, whose expression varies during the cycle. Two key proteins, which are part of mitogenic signal transduction pathways, are Akt (PKB) and MAPK (ERK 1/2). Following mitogen binding, these proteins are indirectly phosphorylated and activated and, in turn, they promote the transcription of genes encoding cell growth control proteins. Cell growth inhibitor proteins, as p21, block cell cycle progression when they are upregulated.

Here we investigated whether estradiol promotes EPC growth through the mitogenic signal transduction pathways involving the phosphorylation of Akt and MAPK. The progression through the cell cycle was also analyzed by determining the relative expression of four cyclins (D, A, E and B) and the hyperphosphorylation of Rb, which is a prerequisite for the cell to enter the S phase. As negative cell cycle regulator, the expression of cyclin-dependent kinase inhibitor p21, was also analyzed.

Since we previously have shown that proliferation of EPCs is ERα mediated, PPT was used to further confirm ERα mediation of estradiol effects on regulation of cell growth regulating proteins.

**Methods**

To determine the intracellular mechanisms via which estradiol improves EPC growth, cells were treated with physiological concentrations of estradiol, or with the pharmacological ERα agonist PPT, and the presence and relative expression of some cell cycle-regulating proteins was inspected by Western Blotting as described in 3.2.3.

**Results**

To investigate the mechanisms via which estradiol promotes EPC proliferation, cells were serum starved in order to make them enter the quiescent G0 phase of the cell cycle. We first tested the effects of physiological concentrations of estradiol (1 and 10 nM) on the activation of mitogenic signal transduction pathways proteins Akt and MAPK. Physiological concentrations were used because within this range we observed a maximal effect of estradiol on proliferation and DNA synthesis (see 4.3.2). Following 7 min of treatment an increase in phosphorylation of both proteins was observed (at 10 nM 196.8 ± 3% and 158.03 ± 9.4% compared to untreated control for MAPK and Akt, respectively), as shown in Fig. 16.
Progression from G1 to S phase was analyzed by detecting the relative amount of hyperphosphorylated Rb protein. As shown in Fig. 17, following 72 hours of treatment, estradiol increased the hyperphosphorylated band.

Subsequently, we further investigated the effects of estradiol on expression of cyclins, in order to understand more mechanisms that regulate estradiol induced EPC proliferation. As is shown in Fig. 18, following 72 hours of estradiol treatment, physiological concentrations upregulated cyclin expression. Compared to the untreated control, treatment with 10 nM estradiol increased cyclin D1 expression, which allows transition from G1 to S phase by 73%; expression of cyclin A and cyclin E, which are required for cell commitment to DNA replication, by 56% and 112%, respectively, and expression of cyclin B, which promotes transition from G2 to M phase and cell division, by 271%.

**FIGURE 16.** (A) Western blots illustrating the modulatory effect of estradiol on MAPK and Akt phosphorylation after 7 min treatment (1 and 10 nM). (B) The bar graphs represent the densitometric analysis of the western blots, and are normalized to the unphosphorylated proteins MAPK and Akt, respectively. n=3; *p<0.05 vs control.

**FIGURE 17.** (A) Western blot showing estradiol promoting hyperphosphorylation of Rb protein at physiological concentrations. (B) The bar graph represents the densitometric analysis of the blot, and is normalized to the total Rb protein. n=3; *p<0.05 vs control.
Estradiol Promotes EPCs Growth by Regulating the Expression of Cell Cycle Proteins

Results

In order to allow the progression through the cell cycle, following mitogen stimulation, the transition from G1 to S phase has to occur by mean of activation of G1/S-Cdk and S-Cdk complexes. In this regard, inhibitor protein p21 has to be downregulated. Hence, we investigated the change in p21 expression following 72 hours of estradiol treatment (10 nM); moreover, as positive control, we treated the cells with 5% FCS, in order to stimulate proliferation. As shown in Fig. 19, estradiol treatment reduces the expression of p21 by 34% compared to the untreated control. Interestingly, the inhibitory effects of estradiol were similar to the effects of FCS, which inhibited p21 by 37%.

We previously demonstrated that ERα mediates the mitogenic effects of estradiol in EPCs. Therefore, we investigated the role of this receptor in modulating the expression of the cell cycle regulating proteins. Treatment of EPCs with ERα agonist PPT (100 nM) for 7 min or 72 hours after serum
Estradiol Promotes EPCs Growth by Regulating the Expression of Cell Cycle Proteins

Results

starvation, mimicked the effects of estradiol in regulating the expression of mitogenic signal transduction proteins (Fig. 20), of cyclins (Fig. 21) and in downregulating p21 expression (Fig 22).

![Western Blots and Bar Graphs](image)

**FIGURE 20.** (A) Western blots showing PPT (100 nM) mimicking the effects of estradiol on activation of mitogenic signal transduction pathway proteins. ERα mediates the mitogenic effects of estradiol on EPCs by upregulating phosphorylation of MAPK and Akt. (B) Bar graphs represent the densitometric analysis of the blots normalized against the unphosphorylated form of the proteins. n=3; *p<0.05 vs control.

As shown in Fig. 20, PPT mimicked estradiol effect on both MAPK and Akt phosphorylation (224.1 ± 10.2% and 132.7 ± 1.6% compared to the untreated control for MAPK and Akt, respectively, p<0.05)

![Western Blots and Bar Graphs](image)

**FIGURE 21.** (A) Western blots showing PPT (100 nM) mimicking the effects of estradiol on the expression of cyclin proteins. (B) The bar graph represents the densitometric analysis of the western blots normalized to the internal control β-actin. n=3; *p<0.05 vs control.
Estradiol Promotes EPCs Growth by Regulating the Expression of Cell Cycle Proteins

Results

PPT, compared to the untreated control, significantly (p<0.05) upregulated the expression of cyclin D1 by 68%, of cyclin A by 91%, of cyclin E by 54% and of cyclin B by 200% (Fig. 21). On the other hand (Fig. 22), the effect of physiological concentrations of estradiol on downregulation of p21 expression, was mimicked by 100 nM PPT (42.1 ± 7.6%, 52.05 ± 1.3% and 41.7 ± 2.9% of the untreated control, for 1 nM E2, 10 nM E2 and 100 nM PPT respectively, p<0.05).

**FIGURE 22.** (A) Western blot showing PPT (100 nM) mimicking the effects of estradiol on EPCs. ERα mediates the mitogenic effects of estradiol on EPCs by downregulating expression of p21. (B) The bar graph represents the densitometric analysis of the blot normalized to the internal control β-actin. n=3; *p<0.05 vs control.

**DISCUSSION**

Estradiol has been shown to exert beneficial effects on the cardiovascular system by promoting survival and proliferation of ECs, therefore we hypothesized a mitogenic effect on EPCs. Earlier, we provided evidence that estradiol promotes proliferation of cultured EPCs, increases the number of circulating EPCs and enhances the CFU. Here we demonstrate that the mitogenic actions of estradiol on EPCs involve different mechanisms. Estradiol activated mitogenic signal transduction pathways and modulated the expression on different cell cycle regulator proteins. We showed that, following serum starvation, physiological concentrations of estradiol activated the cells to exit the G0 phase and start proliferating. Moreover, estradiol stimulated signal transduction pathway key protein MAPK (ERK1/2) after 7 min treatment, consistent with earlier studies using other cell types such as ECs (Russel, 2000; Klinge, 2005) and MCF-7 (Migliaccio, 1996). In contrast to EPCs and ECs, estradiol has been shown to have an opposite, i.e. inhibitory, effect on the other cardiovascular cell type HASMC, (Barchiesi, 2003). Estradiol, also activated Akt (PKB) after 7 min treatment, which leads to an inhibition of apoptosis. Furthermore, after 72 hours, estradiol promoted hyperphosphorylation of Rb, which is a substrate for cyclin D, and, in this form, supports the transcription of proteins required for the entry in the S phase. Additionally, we showed that estradiol upregulated the expression of all the four classes of cyclins (see section 1.3), comprising cyclin D, cyclin A, cyclin E and cyclin B. Furthermore, estradiol negatively modulates the expression of the G1/S-cdk and S-cdk inhibitor protein p21, which blocks the entry into the S-phase. Subsequently, we investigated the role of ERα in mediating the modulatory effects we observed in the previous experiments. Similar to our observation on EPC proliferation, the effects of estradiol on activation of mitogenic signal transduction proteins and expression of cell cycle regulating proteins was mimicked by PPT, suggesting that the growth promoting effects of estradiol on EPCs are ERα mediated. In conclusion, our findings provide evidence for the mechanisms via which estradiol can influence growth of EPCs. Additional studies are needed to further investigate the intracellular mechanisms involved in this modulation, the effects of estradiol on apoptosis-related proteins, and the role of metabolites.
4.6 Estradiol Promotes Adhesion of EPCs to ECs via ERα

**OBJECTIVES**

Adhesion of EPCs at site of injury is a prerequisite for the initiation of vascular repair. Hence, the aim of these experiments was to test the effects of estradiol on the adhesion of EPCs to endothelial cells and the role played by ERα in this process.

**INTRODUCTION**

Dysfunctional endothelium is a key factor for the onset and the progression of cardiovascular disease. If endothelial cells cannot repair the damaged site, inflammation will take place, with subsequent infiltration of leukocytes into the vessel wall and successive plaque and neointima formation (Ross, 1999; Hansson, 2005). Therefore, if ECs are aged, or if presence of risk factors impairs their ability to divide and repair damages, an external source of cells is needed in order to keep the endothelium healthy. In this context, EPCs have been shown to be able to repair damages in vessel by incorporating into the site of injury (George, 2005; Shintani, 2001; Asahara, 1999; Zhang, 2002). To initiate this, they first have to adhere to the underlying endothelial cells and then proliferate and differentiate into mature ECs. We previously demonstrated that estradiol is promoting the growth of EPCs, both of freshly isolated cells and cultured cells. Moreover, using pharmacological agents, we provided evidence that this induction is ERα mediated. We now investigated the effects of estradiol on adhesion of these cells to ECs (Fig. 23), and the role of ERα in this process.

**FIGURE 23. Microscopic picture showing EPCs (green) adhering to the underlying monolayer of EC (magnification: 10x).**

**METHODS**

Fluorescent labeled EPCs were put on ECs monolayer treated with estradiol, or different pharmacological agents, and number of adherent cells was analyzed with ELISA, as described in methods (3.2.5).
RESULTS

4.6.1 FRESHLY ISOLATED EPCs

Total MNC were isolated from peripheral blood and CD34+ cells separated using magnetic-labeled antibodies as described in methods (3.2.1.1). Directly after isolation both CD34+ and the remaining MNC fraction (i.e. CD34- cells, as negative control) were plated onto endothelial cells that had been previously treated for 24 hours with 10 nM estradiol or with the vehicle (0.1% DMSO as control). In another experiment, the two fractions of cells were treated either with vehicle or 10 nM estradiol for one hour, and then plated into fibronectin-coated wells. In both cases adhesion experiments were performed as described in methods.

As shown in Fig. 24, compared to the untreated ECs, adhesion of EPCs to ECs was significantly increased from 100 ± 3.5% to 156.3% (p<0.05) when the underlying ECs layer was pretreated with estradiol (10 nM, 24 hours) (Panel A). On the other hand, adhesion of CD34+ MNC was not affected when the underlying layer of ECs was treated with estradiol (Panel B).

![Figure 24](image1.png)

**FIGURE 24.** Bar graphs representing measurement of adherent EPCs fluorescence intensity. (A) Freshly isolated EPCs adhere more to ECs when the latter are treated with 10 nM E2, compared to untreated cells. (B) Adhesion of the CD34+ fraction (MNC) on estradiol-treated ECs does not change compared to the untreated control. n=2; *p<0.05 vs control (untreated HAEC).

Similar to EC, estradiol significantly increased the adhesion of EPCs to fibronectin from 100% to 113.6 ± 4.5% (p<0.05) (Fig. 25), however, compared to ECs, the magnitude was much less.

![Figure 25](image2.png)

**FIGURE 25.** Bar graph showing the number of fluorescent freshly isolated CD34+ EPCs adhering to fibronectin-coated dishes. Estradiol (10 nM, 1 h) increased adhesion of cells compared to the control. n=2; *p<0.05 vs control (untreated HAEC).
4.6.2 Cultured EPCs

As described for the growth experiments, circulating EPCs comprise only 0.02% of total MNC. Therefore, as with proliferation and growth studies, we performed adhesion experiments with cultured EPCs in order to investigate whether they retain the properties of freshly isolated EPCs (Fig. 26).

![Graph showing measurement of adherent EPCs fluorescence intensity.](image)

**FIGURE 26.** Bar graph showing measurement of adherent EPCs fluorescence intensity. In the case of cultured EPCs, adhesion to the underlying monolayer is enhanced only when ECs are treated with E2, but not EPCs (A). Treatment of both ECs and EPCs does not influence cell adhesion (B). n=6; *p<0.05 vs control.

Cultured EPCs were treated for 24 hours either with vehicle (0.1% DMSO) or 10 nM estradiol and layered over endothelial cells that had been also treated for 24 hours with vehicle or 10 nM estradiol. Adhesion experiments were performed as described in methods (3.2.5). Adhesion of cultured EPCs was enhanced by 21% compared to the control (p<0.05), when the underlying ECs monolayer was treated with estradiol. On the contrary, treatment of either EPCs (Fig 26A), or both EPCs and ECs (Fig. 26B) did not increase cell adhesion.

4.6.3 Role of ERα in Mediating Adhesion

Using selective pharmacological agents to mimic estradiol effects or to block ER, we provided evidence for an ERα mediated adhesion of EPCs to ECs. As shown in Fig. 27, PPT (100 nM) treatment of ECs mimicked estradiol adhesion promotion (139.7 ± 8.2% of untreated control), whereas ERβ agonist DPN (100 nM) did not have any effect on EPCs adhesion (101.6 ± 23% of untreated control). These findings suggest that estradiol induces EPCs adhesion via an ERα mediated process.
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Indeed, blocking of either ERα with MPP or both ERα and ERβ with ICI abrogated the effects of PPT (from 139.7 ± 9.2% to 110.6 ± 9.4% and 106.5 ± 7.02% for PPT+MPP and PPT+ICI, respectively, p<0.05) on EPC adhesion (treatment with MPP or ICI alone did not significantly influence the basal EPC adhesion), suggesting that estradiol induced EPC adhesion to ECs is ERα mediated (Fig. 28).

Indeed, blocking of ERα with the MPP antagonist (1 μM) abrogated the effects of estradiol (from 141.1 ± 7.1% to 117.01 ± 7.2%, p<0.05), suggesting that ERβ is not involved in promoting the adhesion of EPCs to ECs despite estradiol binding (Fig. 29).
4.6.4 2-ME MODULATION OF ADHESION

Our data provide evidence that the adhesion promoting effects of estradiol are ERα mediated. However, in vivo, estradiol is metabolized to biologically active molecules, i.e. 2-ME, which act via an ER-independent mechanism. Therefore we investigated whether an ER-independent mechanism is also involved in adhesion modulation. As shown in Fig. 30, both estradiol and 2-ME modulated the adhesion of EPCs in a biphasic manner, with induction of adhesion at low, physiological, concentrations and no effects at high concentrations (1μM). At 10 nM estradiol and 2-ME induced EPC adhesion by 41% and 18%, respectively, compared to the untreated control.

FIGURE 29. Bar graph representing the amount of fluorescent EPCs adhering to treated ECs. Blocking of ERα with 1 μM MPP prior to E2 treatment abrogated its effects. n=4; p<0.05: *vs control, **vs 10 nM E2.

FIGURE 30. Bar graph showing the number of fluorescent adherent EPCs to ECs. Low doses of both estradiol and 2-ME (10 nM) promote adhesion, whereas at higher doses adhesion is not influenced. n=3; p<0.05: *vs control, **vs 10 nM E2, § vs 10 nM 2ME.
4.6.5 TNF-α MODULATION OF ADHESION

Under steady state, EPCs are mainly retained within the bone marrow, and are mobilized into the circulation when there is need for repair. Damage of the endothelium leads to inflammation, and one of the most important inflammatory cytokines is TNF-α. Therefore, we wanted to elucidate whether TNF-α promotes adhesion of EPCs to ECs and whether estradiol modulates TNF-α action. To test this, we ECs were treated with TNF-α (10 ng/ml) and E2 (10 nM).

![Graph showing the number of fluorescent adherent EPCs. Promotion of adhesion by TNF-α (10 ng/ml) is partially inhibited by estradiol (10 nM), and E2 pretreatment prevents adhesion induction by TNF-α.](image)

**FIGURE 31.** Bar graph showing the number of fluorescent adherent EPCs. Promotion of adhesion by TNF-α (10 ng/ml) is partially inhibited by estradiol (10 nM), and E2 pretreatment prevents adhesion induction by TNF-α. *p<0.05: * vs control, ** vs E2; § vs TNF-α; n=4.

As shown in Fig. 31, TNF-α promoted EPCs adhesion to the underlying monolayer (156.4 ± 13.6% of untreated control). Subsequent treatment with estradiol (10 nM) reduced this induction by 40%. We hypothesize that TNF-α is promoting overexpression of adhesion molecules, while estradiol is modulating this effect to a basal level, by preventing adhesion molecules upregulation induced by TNF-α.

**DISCUSSION**

Estradiol has modulatory effects on adhesion molecules involved into leukocytes and neutrophils adhesion to the vessel walls, downregulating their expression, and preventing thus atherosclerosis development (Caulin-Glaser, 1996; Geraldes, 2006). In these experiments, we showed that estradiol increased adhesion of EPCs to ECs, and this may be related to an upregulation of adhesion molecules expression. Therefore, we can hypothesize, that estradiol selectively modulates expression of different adhesion molecules in ECs. In this regard, further investigations are needed to evaluate this possibility and to characterize these molecules. We provided evidence that estradiol treatment of ECs improved adhesion of both freshly isolated CD34+ and cultured EPCs. EPCs in both stages respond the same way to estradiol primed ECs, building thus the basis for a clinical employment of these cells, since their low number in circulation makes it preferable to isolate them from the blood of the patient and expand them in culture, prior therapeutical employment. In addition to these findings, we also showed that treatment of EPCs with estradiol did not further enhance adhesion (either to untreated or estradiol-treated ECs). Interestingly, treatment of freshly isolated EPCs increased their adhesion on fibronectin. Therefore, in this case, we observed a divergence in the behavior of freshly isolated CD34+ and cultured...
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EPCs. However, it has not been tested, whether treatment of freshly isolated EPCs also promotes adhesion to ECs as it does to fibronectin. More studies are required in order to elucidate whether in the case of EPCs treatment there is a difference between freshly isolated and cultured cells.

In summary, treatment of ECs with estradiol promotes adhesion of EPCs. This effect may reflect an in vivo situation following injury. Within the body, ECs, but not EPCs, are in constant contact with blood and with everything that is circulating within it, included estradiol. On the other side EPCs are principally retained in the bone marrow, and mobilized only when there is a need for endothelium repair or neovascularization. Thus, ECs are primed with estradiol, whereas EPCs are not. This is important as far as postmenopausal women are concerned. After menopause, ovaries stop to produce hormones, and subsequently the priming of ECs is lost. This implies that, beside a lower number of circulating EPCs and a lower growing ability (as we demonstrated in section 4.2), the adhesion potential of EPCs is also impaired in postmenopausal women, and this may also consequently contribute to a higher risk of cardiovascular disease, if they are not taking HRT.

We also provided evidence that adhesion, as it occurs for proliferation, is ERα mediated. ERα agonist PPT mimicked estradiol effects, promoting EPCs adhesion to the treated ECs. Blocking of ERα with pharmacological antagonist MPP confirmed this result. Because downstream metabolites of estradiol are biologically active, we also hypothesized that metabolism of estradiol may also play a role in EPC adhesion. Indeed, a biphasic effect in modulation of adhesion was observed by both estradiol and 2-ME, with promotion of adhesion at 10 nM and no effect at higher concentrations (1 μM) In this regard, 2-ME, which is an anti-mitogenic substance has been shown to induce MCF-7 growth at low doses (Liu, 2004).

Endothelial dysfunction, one of the first steps in the onset of atherosclerosis, leads to vascular inflammation (Morita, 2005), which, in turns, leads to cytokine production. Cytokines promote the homing of EPCs at the sites of injury in the vasculature (Takahashi, 1999). Therefore we investigated the role of TNF-α in modulating EPCs adhesion to ECs. TNF-α is an inflammatory cytokine which induces expression of adhesion molecules for leukocytes during an inflammatory process. We provided evidence that adhesion of EPCs is enhanced by TNF-α. Estradiol diluted the effects of TNF-α by modulating EPC adhesion, and lowering their number observed when the cells were treated with physiological concentrations of estradiol. Estradiol has been shown to inhibit adhesion of leukocytes and monocytes to ECs (Geraldes, 2006; Ling, 2006). Priming of ECs with estradiol prior TNF-α treatment also reduced EPCs adhesion (data not shown). As far as postmenopausal women are concerned this priming is lost and, as a consequence, during the inflammatory CVD adhesion of leukocytes is not prevented, leading to a progression of the disease. It is thus feasible that estradiol mediates its beneficial effect on the cardiovascular system by promoting adhesion of EPCs to the vessel walls and by inhibiting adhesion during inflammatory processes.
4.7 Estradiol Promotes Microvessel Formation by EPCs via ERα and Tyrosine Kinases

OBJECTIVES

The main aim of these experiments was to investigate the effects of estradiol on microvessel formation by EPCs, and to delineate the role of estrogen receptors in mediating these effects. Furthermore, we elucidated the intracellular mechanisms via which estradiol modulates microvessel formation.

INTRODUCTION

EPCs have been shown to participate in both angiogenesis and neovasculogenesis processes. Moreover, besides forming vessels in vivo, a characteristic of EPCs is to spontaneously form them in vitro (Asahara, 1997). Neovasculogenesis is a process taking place in the developing embryo as well as in the adult. In CVD, neointima formation reduces the blood supply to the surrounding tissues, moreover, atherosclerotic plaques may eventually break with subsequent thrombus blocking the vessel and causing ischemia in the nearby organ. In this regard, the development of new vessels will be of crucial importance to restore the correct blood flow to and through the involved tissue or organ. Because protein tyrosine kinases are known to play a role in angiogenic processes, it is of interest to investigate whether estradiol is also affecting microvessel formation via this pathway. To verify this hypothesis, the tyrosine kinase inhibitor SU5416 has been used to block microvessel formation via tyrosine kinase activation (Mologni, 2006; Zhong, 2004). To test the efficacy of SU5416 inhibition in our microvessel formation model, we used it along with factors that are known to induce vessel formation, such as VEGF, HGF and SDF-1, via tyrosine kinase receptors. Vascular Endothelial Growth Factor (VEGF), a member of the platelet-derived growth factor family, has been shown to be involved in angiogenesis and vasculogenesis. It mediates its effects by binding VEGF-Receptor, a tyrosine kinase, causing it to dimerize and autophosphorylate, thus further traducing the signal. Hepatocyte Growth Factor (HGF), is secreted by mesenchymal cells and has been shown to be a regulator of cell growth and motility. It binds to c-Met receptor, activating a tyrosine-kinase signaling cascade. Stromal cell-derived factor-1 (SDF-1, also known as CXCL12) is a chemokine, which has been shown to play a role in angiogenesis, by recruiting EPCs from the bone marrow. Moreover, at the protein level, we evaluated the expression of HO-1 protein. HO-1 has been shown to induce vessel formation via the Akt and MAPK pathways (Fig. 32) (Deshane, 2007) and, moreover, we have previously shown that in EPCs estradiol activates Akt and MAPK (section 4.5). Based on these findings we hypothesized that estradiol may also influence EPC vessel formation via HO-1. Hence, as another aim we tested whether estradiol induces the expression of HO-1 in EPCs.
METHODS

EPCs were treated for 30 minutes with the substance to test and then plated onto Matrigel as described in methods (see 3.2.8).

RESULTS

4.7.1 Estradiol Promotion of Microvessel Formation

EPCs are able to spontaneously form microvessels in vitro when plated onto Matrigel matrix. Matrigel is composed of a mixture of proteins secreted by mouse tumor cells, which resembles the extracellular environment found in many tissues. Treatment of cells with 10 nM estradiol promoted microvessel formation. Compared to the untreated control the number of junctions formed in response to 10 nM estradiol were 138.5 ± 4.6% (p<0.05). Moreover, the effects of estradiol were mimicked by the ERα agonist PPT which increased microvessel junction formation by 156.9 ± 5.3% (p<0.05 vs untreated control). Pretreatment of EPCs with the ERα antagonist MPP abrogated the stimulatory effects of estradiol on junction formation by 80%, indicating that estradiol stimulates microvessel formation via activation of ERα (Fig. 33).
On the other hand, effects of estradiol were not mimicked by ERβ agonist DPN, which had no effect on microvessel formation by EPCs. As shown in Fig. 34, compared to the untreated control, the number of junctions formed by cells treated with 10 nM DPN were not significantly different compared to the untreated control ( ), whereas junctions formed by PPT (100 nM) treated cells were increased by 62.6 ± 26.5% (p<0.05 vs control). These results support previous findings indicating that promotion by estradiol of microvessel formation by EPCs is ERα mediated.

FIGURE 33. (A) Bar graph showing number of junctions formed by EPCs. Estradiol (10 nM) promotes microvessel formation, and use of the pharmacological agonist PPT (100 nM) and antagonist MPP (1 μM) provide evidence that the effects are ERα mediated. (B) Photomicrographs showing microvessel formation by EPCs on Matrigel under the different conditions (magnification: 4x). n=2; * vs control, ** vs E2, § vs MPP.

FIGURE 34. (A) Bar graph showing the number of junctions by EPCs. ERβ agonist DPN (10 nM) had no effect on microvessel formation, whereas ERα agonist PPT (100 nM) increased it. (B) Photomicrographs showing microvessel formation by EPCs under the different conditions (magnification 4x). n=2; * vs control.
4.7.2 HO-1 EXPRESSION

HO-1 protein has been shown to promote microvessel formation, mainly via the products formed during conversion of heme into biliverdin and CO (see section 1.5.4). Moreover, this induction is mediated via activation of the Akt pathway. Since our previous studies demonstrate that estradiol stimulates Akt phosphorylation, we investigated whether the induction of microvessel formation by estradiol was due to HO-1 upregulation.

![Figure 35](image)

*FIGURE 35. (A) Western blot showing the regulation of HO-1 expression by estradiol (10 nM). (B) Bar graph showing the densitometric analysis of the western blot, and normalized to the internal control β-actin. n=3; *p<0.05 vs control.*

As shown in Fig. 35, expression of HO-1 was significantly upregulated by estradiol. At a concentration of 10 nM estradiol increased HO-1 expression by 2-fold (from 100 ± 0.3% to 193.9 ± 2.8%, p<0.05 vs control).

4.7.3 THE TYROSINE KINASE INHIBITOR SU5416 IMPAIRS MICROVESSEL FORMATION

We previously provided evidence that estradiol upregulates the expression of HO-1 in EPCs. Since HO-1 is activated via a tyrosine kinase-induced pathway (Akt), we investigated whether blocking of this pathway inhibited microvessel formation induced by estradiol. To block tyrosine kinase receptors, which are activated by growth factors, we used the tyrosine kinase inhibitor SU5416 (Fig. 36). To test whether SU5416 worked in our system, first we used it to block the effect of VEGF, HGF and SDF-1, which are promoting vessel formation via a tyrosine kinase pathway.
As shown in Fig. 37, 30 min pretreatment with SU5416 (5 μM) significantly inhibited the effects of VEGF, HGF and SDF-1 (each 100 ng/ml, by 98%, 74% and 178% respectively).

Consequently, we tested whether induction of microvessel formation by estradiol (10 nM) was abrogated by blocking tyrosine kinase pathway by means of SU5416 (30 min pretreatment, 5 μM), observing a reduction of 93% of junctions in the presence of SU5416, compared to estradiol (Fig. 38).
To further investigate one of the mechanisms regulating microvessel formation, we evaluated the expression of HO-1 in the presence of estradiol (10 nM) and SU5416 (30 min pretreatment, 1 and 5 μM). As shown in Fig. 39, inhibition of tyrosine kinase activity by SU5416 downregulated the expression of HO-1 by 63.6% and 72.6% (from 100 ± 4.8% to 33.4 ± 2.4% and to 27.4 ± 14.9% at 1 μM and 5 μM, respectively, p<0.05 vs control).

![Figure 38](image1.png) **FIGURE 38.** (A) Bar graph showing the number of junctions formed by EPCs, and the inhibition by SU5416 (30 min pretreatment, 5 μM) estradiol-induced (10nM) microvessel formation. (B) Microscopic pictures showing microvessel formed by EPCs on Matrigel under the different conditions (magnification: 4x). n=2; *p<0.05: *vs control, **vs E2.

![Figure 39](image2.png) **FIGURE 39.** (A) Western blot showing SU5416 (1 and 5 μM) abrogating the modulatory effect of estradiol (10 nM) on HO-1 expression. (B) The bar graph represents the densitometric analysis of the western blot, and is normalized to the internal control β-actin. n=3; *p<0.05 vs control.
4.7.4 MICROVESSEL FORMATION PROMOTION VIA TYROSINE KINASE-ACTIVATED PATHWAYS

To confirm that SU5416 blocks the effects of estradiol by inhibiting the mitogenic transduction signaling pathways, we studied the modulation of phosphorylation of MAPK and Akt in the presence of SU5416. As shown in Fig.40, 10 nM (7 min) estradiol significantly induced MAPK phosphorylation by 55% (from 100 ± 17.4% to 154.8 ± 2.6%) and Akt by 98% (from 100 ± 1.3% to 198.1 ± 3.3%), compared to the untreated control, and this effect was abrogated by SU5416 pretreatment (from 154.8 ± 2.6% to 68.5 ± 1.8% and from 198.1 ± 3.3% to 55.6 ± 26.4% compared to estradiol for MAPK and Akt respectively; 30 min, 5 μM, p<0.05).

![Western blot showing expression of P-MAPK and P-Akt](image)

**FIGURE 40.** (A) Western blot showing the expression of P-MAPK and P-Akt after 7 min treatment of EPCs with 10 nM estradiol, and the inhibition of phosphorylation by 30 min pretreatment with 5 μM SU5416, because of blocking of protein tyrosine kinase activity. (B) The bar graphs represent the densitometric analysis of western blot normalized to total MAPK or total Akt. n=3; p<0.05: *vs control, **vs E2.

4.7.5 INHIBITION OF AKT PHOSPHORYLATION REDUCES MICROVESSEL FORMATION

In the previous experiments we have shown that blocking of tyrosine kinase activity by SU5416 inhibits microvessel formation and downregulates HO-1 expression, which, in turn, is involved in angiogenesis and vasculogenesis (4.7.3). Moreover, the presence of SU5416 prevents phosphorylation of Akt protein (4.7.4), which is involved in HO-1 activation. Therefore, we performed some microvessel formation
experiments using the upstream Akt activator PI3K inhibitor LY294002 (Vlahos, 1994) to further investigate the intracellular mechanisms involved in vasculogenesis by EPCs.

As shown in Fig. 41, the microvessel formation inducing effect of estradiol was inhibited by LY294002 from 218.09 ± 15.9% to 152.4 ± 9.2% (p<0.05 vs 10 nM E2).

**FIGURE 41.** (A) Bar graph showing the number of junctions formed by EPCs treated with E2 (10 nM) or pretreated with LY294002 (10 μM). (B) Microscopic pictures showing microvessels formed by EPCs on Matrigel under the different conditions (magnification: 4x). n=2; p<0.05: *vs control, **vs 10 nM E2.

4.7.6 **Effects of 2-ME on Microvessel Formation**

As we previously have shown (see section 4.3.1), at pharmacological concentrations (>100 nM), the mitogenic effects of estradiol are reduced because of the conversion of estradiol to 2-ME, a potent inhibitor which can counterbalance this effect. The stimulatory effects of 2-ME at lower concentrations are likely due to its reconversion to 2-OHE, which has a low ER affinity. Indeed, a similar effect has been shown for MCF-7 cells (Dawling, 2003). Therefore, we performed some microvessel formation experiments in order to study the effects of 2-ME on EPCs, which has already been shown to inhibit angiogenesis in tumors (Fotsis, 1994). As shown in Fig. 42, 2-ME had a biphasic effect on microvessel formation, promoting it at low concentrations (10 and 100 nM) and inhibiting it at higher doses (1 and 10 μM).
Estradiol Promotes Microvessel Formation by EPCs via ERα and Tyrosine Kinases

**Results**

![Graph showing dose-dependent number of junctions formed by EPCs treated with 2-ME (10 nM-10 μM).](image)

**FIGURE 42.** (A) Bar graph showing the dose-dependent number of junctions formed by EPCs treated with 2-ME (10 nM-10 μM). (B) Microscopic pictures showing microvessels formed by EPCs on Matrigel under the different conditions (magnification: 4x). n=3; p<0.05: *vs control, **us 100 nM 2-ME.

**DISCUSSION**

EPCs are characterized by their ability to spontaneously build microvessels. In the presence of cardiovascular disease, where vessel occlusion by neointima and thrombus formation may eventually lead to ischemia (Libby, 2002), formation of new vessels in order to restore the correct blood flow through the affected organ, is of crucial importance. In the present study we showed that estradiol enhanced the ability of EPCs to form microvessels. Moreover, using pharmacological agents, we provided evidence that estradiol action is ERα mediated. To look at some mechanisms that are involved in this modulation of microvessel formation, we investigated the changes in the expression of HO-1, which is a protein activated via MAPK and Akt pathways, and promotes vessel formation. Previously, we provided evidence for increased MAPK and Akt phosphorylation by estradiol in EPCs, and thus we supposed an increase in HO-1 expression by estradiol via these pathways. Our results confirmed this hypothesis, and showed that estradiol at physiological concentrations upregulated HO-1 expression. Subsequently, we used a pharmacological agent, SU5416, to block protein tyrosine kinase activity, in order to prevent microvessel formation via this pathway. We first tested SU5416 efficiency in our system utilizing it to inhibit the effect of factors that are promoting vessel formation via protein tyrosine kinases: VEGF, HGF, and SDF-1. We could thus demonstrate that SU5416 worked in our system, and we then investigated the effects on estradiol-induced microvessel formation. Our experiments demonstrate that blockade of protein tyrosine kinase activity by SU5416 modulates estradiol-enhanced microvessel formation. This may suggest that there is an interaction between the signaling coming from the estradiol/ER complex and from tyrosine kinase receptors, as it has been shown in MCF-7 cells [rev. in (Arpino, 2008)]. However, more experiments have to be conducted to further investigate this aspect. Moreover, HO-1 expression was also downregulated in the presence of SU5416. Therefore, we confirmed that estradiol upregulates HO-1 expression via activation of MAPK and Akt pathways. Additional evidence for this hypothesis comes from the studies on the modulatory effects of SU5416 on MAPK and Akt phosphorylation. Estradiol induced phosphorylation of both MAPK and Akt was abrogated by pretreatment of EPCs with SU5416. Moreover, inhibition of Akt phosphorylation by mean of the PI3K inhibitor LY294002 partially abrogated the estradiol induced
microvessel formation by EPCs (Fig. 43). In addition to the above findings, we also provided evidence for a biphasic effect of 2-ME on microvessel formation by EPCs. Low concentrations of 2-ME have been shown to promote MCF-7 growth because of reconversion into 2-OHE by CYP (Dawling, 2003), and as we showed for adhesion, we found an increase in microvessel formation at 2-ME concentrations of 10 and 100 nM, whereas at higher doses (1 and 10 μM) the reconversion of 2-ME into estradiol was not enough to counterbalance its anti-mitogenic effect, therefore we observed a decrease in microvessel formation. It is thus feasible that the beneficial effects shown by estradiol on the cardiovascular system are, in part, due to its microvessel formation promoting effects. However, microvessel formation is not only important as far as cardiovascular disease is concerned. Tumor growth is closely connected to blood supply and formation of new vessels in order to support it, and EPCs have been shown to be involved in tumor vasculogenesis (Rafii, 2002). In this regard, inhibition of microvessel formation by EPCs mediated by SU5416 may play a crucial role to prevent tumor growth. In this regard, 2-ME has been shown to inhibit cancer cell growth and angiogenesis in tumors, suggesting it may be used as an antitumor drug. However, this contention should be viewed with caution, as we also provide evidence that at low concentrations 2-ME does not inhibit, but rather induces, microvessel formation, and this may have deleterious consequences for antitumor therapy. Further studies are necessary to confirm these findings, and to further investigate the mechanisms involved in estradiol-promoted microvessel formation by EPCs.

**FIGURE 43**. Schematic picture depicting the intracellular mechanisms involved in microvessel formation by EPCs. Growth factors, via tyrosine kinase receptor, and E2, via ERα, activate mitogenic signal pathways upregulating thus HO-1 expression. HO-1 is then involved in angiogenesis and neovasculogenesis promotion. This process is inhibited by SU5416 and LY294002. Modified from Deshane et al, 2007, JEM.
**OBJECTIVES**

Endothelial derived NO plays a key role in protecting the cardiovascular system. Because EPCs are like ECs, the goal of the following experiments was to determine the production of NO by EPCs and its regulation.

**INTRODUCTION**

Nitric oxide is an important molecule for the CV system. It is produced by ECs and induces protective effects on the cardiovascular system (see section 1.4.3.1) by promoting SMCs relaxation, which results in widening of the vessel lumen. Moreover, NO inhibits growth of SMCs, which are responsible for neointima formation. It also inhibits adhesion of leukocytes to vessel walls and platelet aggregation, both factors that participate in progression of atherosclerosis. NO has also been shown to promote vessel formation. Furthermore, in ECs, estradiol has been shown to regulate NO production. Therefore, we investigated the effects of estradiol and the role of ERα on NO production and on the phosphorylation of eNOS, the protein responsible for its synthesis. Furthermore, since eNOS is known to be activated via tyrosine kinase receptor (Haynes, 2000), we tested whether SU5416 abrogates estradiol effects on its activation.

**METHODS**

NO production was measured using Griess reagent (3.2.6) and eNOS expression was analyzed by western blotting, as described in 3.2.6.

**RESULTS**

**4.8.1 NO PRODUCTION**

We first investigated the effects of estradiol on production of NO by measuring its concentration in the culture medium, and, using the pharmacological ERα antagonist MPP, the role of the receptor in mediating estradiol action. Treatment of EPCs with 10 nM estradiol increased production of NO (106.7 ± 3.06% compared to the untreated control), and pretreatment with 1 μM of the ERα antagonist MPP reduced this effect by 10%, as shown in Fig. 44.
4.8.2 eNOS EXPRESSION IS MODULATED BY ESTRADIOL VIA ERα

Because the protein responsible for NO synthesis is eNOS, which is activated via the Akt pathway, we investigated phosphorylation modulation of eNOS by estradiol, as well the role of ERα in mediating estradiol effects. As shown in Fig. 45, 7 min of treatment of EPCs with 10 nM estradiol lead to a significant increase in eNOS phosphorylation by 56% compared to the untreated control. Moreover, the ERα agonist PPT (100 nM) mimicked the estradiol effect (166.8 ± 13.01% compared to the untreated control). EPC pretreatment with the specific ERα antagonist MPP (30 min, 1 μM) abrogated estradiol effects from 156.3 ± 3.5% to 103.4 ± 1.6% (p<0.05). Pretreatment with the unspecific ER antagonist ICI (30 min, 1 μM), also reduced the effects of estradiol by 64% (from 156.3 ± 3.5% to 102.3 ± 1.3%), providing thus evidence for an ERα mediated estradiol effect on eNOS activation.

FIGURE 44. Analysis of NO production by EPCs by mean of Griess reagent. The bar graph shows the amount of NO produced, compared to the untreated control. n=3; p<0.05: *vs control, **vs E2.

FIGURE 45. (A) Western blot showing the modulation of eNOS phosphorylation by estradiol (10 nM), ERα agonist PPT (100 nM) and antagonists MPP and ICI (1 μM each) after 7 min treatment. (B) The bar graph represents the densitometric analysis of western blot, normalized to the unphosphorylated eNOS. n=3; p<0.05: *vs control, **vs E2.
NO Production by EPCs is Enhanced by Estradiol via ERα

Results

Since eNOS is activated via the Akt pathway, we also investigated whether blocking of protein tyrosine kinase activity by SU5416 inhibited the stimulatory effects of estradiol. Pretreatment of EPCs with 5 μM SU5416 for 30 min, was enough to abrogate estradiol (10 nM) effects on eNOS phosphorylation by 73% (7 min treatment), as shown in Fig. 46.

![Figure 46](image)

**FIGURE 46.** (A) Western blot showing the inhibition of estradiol induction of eNOS phosphorylation by 5 μM SU5416 pretreatment (30 min). (B) The bar graph represents the densitometric analysis of the western blot, normalized to the internal control β-actin. n=3; p<0.05. *vs control, **vs E2.

Discussion

NO is a molecule which has been shown to have beneficial effects on the cardiovascular system (see section 1.4.3.1). Moreover, in ECs, estradiol modulates NO production, promoting thus, among the others, vasodilatation. In the previous experiments, we provided evidence that estradiol promotes EPC proliferation, adhesion and microvessel formation, thus contributing to cardiovascular protection. To further look at this aspect, we investigated the changes in NO production in EPCs following estradiol treatment. Our experiments indicated that physiological concentrations of estradiol significantly increased NO production by EPCs. Moreover, use of a pharmacological ERα antagonist blocked estradiol action, indicating an ERα involvement in NO induction. However, the stimulation of NO by estradiol was only marginal, i.e. 6.7%, compared to the untreated control. This may be due to the fact that the enzyme responsible for NO synthesis, eNOS, is a constitutive enzyme, which constantly produces NO. Therefore, the basal NO level in EPCs may be already near the maximal level, and further induction will thus give only a slight increase. To confirm our findings at the molecular level, we investigated the modulation of eNOS phosphorylation by estradiol, which we hypothesized to be increased, and by agonist and antagonists of ERα. We showed that, as hypothesized, estradiol was significantly upregulating the phosphorylation of eNOS, and, as shown by treatment with the ERα agonist PPT and the specific and unspecific antagonists MPP and ICI, this effect was ERα mediated. In addition to the above findings, we also provided evidence that blocking of protein tyrosine kinase activity by SU5416 inhibited estradiol-induced eNOS activation, which occurs via the Akt pathway. Additional studies are needed to further study the intracellular mechanisms regulating NO production and also the effects of estradiol metabolites in eNOS phosphorylation and NO synthesis (Fig. 47). Our findings provide evidence that the beneficial effects of estradiol on the cardiovascular system are also due to the ERα–mediated increase in NO production in EPCs.
FIGURE 47. Schematic picture depicting the intracellular mechanisms involved in NO production by EPCs. E2, via ERα, activates Akt signal transduction pathway. Akt-P promotes eNOS phosphorylation, increasing thus NO production, which is a CV protective molecule. This process is blocked by SU5416.
4.9 SMOOTH MUSCLE-LIKE CELLS

OBJECTIVES

The aim of this series of experiments was to characterize SM-like progenitor cells and to investigate the effect and the mechanisms of estradiol action on their growth.

INTRODUCTION

In CVD the cells composing the vessel walls, ECs and SMC, have opposite roles. While ECs are damaged and are not able to repair injuries of the endothelium anymore, SMC overproliferate and build neointima, thereby occluding the vessel and restricting blood flow. In this regard, estradiol has beneficial effects, because it promotes EC growth and inhibits growth of SMCs (see section 1.4.3). Moreover, it has been shown that more than 60% of the cells building neointima after vessel damage are bone marrow-derived EPCs which are c-kit+ (Wang, 2006). During the normal routine of EPC isolation, in few cases, despite the use of the same protocol and same media as usual, cells did not differentiate into an EC-like type at all, but they instead had an elongated shape and composed a hill and valley pattern (Chamley-Campbell, 1979) (Fig. 48). These characteristics are not typical for ECs, rather they are distinctive for SMCs. Therefore, before proceeding with experiments, we characterized these cells, in order to elucidate whether they were SM-like progenitor cells. Moreover, we investigated the effects of estradiol on their growth, and some intracellular mechanisms involved in it.

![FIGURE 48. Photomicrographs of (A) EPCs, (B-C) SM-like progenitor cells and (D) HASMC (Magnification: A-B: 4x, C-D: 10x).](image)

METHODS

Total MNC were isolated and cultured as described for EPCs, but despite the use of endothelial basal medium (EBM) they did not develop as endothelial-like cells. The phenotype they showed was comparable to that of smooth muscle cells. Experiments were conducted as for EPCs, but cells were not starved prior to treatment.

RESULTS

4.9.1 CHARACTERIZATION

To test whether the new cell type that differentiated from umbilical vein blood MNC was actually a smooth muscle progenitor cell, we characterized them using immunofluorescence techniques, using antibodies against a combination of smooth muscle specific markers (Tang, 2008), including α-smooth
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muscle actin, vimentin (member of intermediate filaments, plays a role in supporting and anchoring the position of the organelles in the cytosol, it also controls the transport of LDL) and desmin (an intermediate filament protein found in the sarcomer, present in muscle cells, important in muscle cell structure and architecture connecting parts of the cytoplasm). As shown in Fig. 49, cells were positive for all the three markers, confirming their SM-like character.

**FIGURE 49.** Microscopic pictures of isolated umbilical vein blood cells showing the expression of SMCs markers. (A) Desmin, (B) α-smooth muscle actin and (C) vimentin were used to characterize the cells. (D) EPCs were used as negative control (SM-actin shown).

After providing evidence that the cells developed into a SM-like cell type, we characterized them for the expression of CD34 (has for EPCs) and c-kit (also known as CD117, is present on hematopoietic stem cells. Through the binding with its ligand, stem cell factor, it promotes survival, proliferation and differentiation of cells [rev. in (Edling, 2007)]) to confirm their progenitor character. As shown in Fig. 50, both markers were expressed, indicating that these cells were progenitor cell-derived SM cells.

**FIGURE 50.** Microscopic picture of SM-like cells expressing progenitor markers CD34 (green) and c-kit (red).
4.9.2 FACS; ESTRADIOL REDUCES THE NUMBER OF CIRCULATING C-KIT\(^+\) CELLS

It has been shown that after vascular injury, up to 60% of the smooth muscle cells that participate in neointima formation are bone marrow-derived c-kit (CD117\(^+\)) cells (Wang, 2006), and it has been shown that estradiol inhibits growth of SMCs (Barchiesi, 2003). Therefore, we hypothesized an inhibitory effect of estradiol on SM-like cell proliferation. We first performed some in vivo experiments, using the same samples as for 4.2.2, to investigate the effects of estradiol on the number of circulating c-kit\(^+\) cells. Here, number of circulating c-kit\(^+\)/AC133\(^+\) cells was compared for each IVF patient between high and low estradiol level samples, by FACS. As shown in Fig. 51, as increase in estradiol plasma concentration, corresponded to a decrease in the number of circulating c-kit\(^+\)/AC133\(^+\) cells by 36% (from 100% to 64.3 ± 17.2%) compared to the low estradiol control.

![Figure 51](chart.png)  
**FIGURE 51.** Bar graph representing FACS analysis of circulating SM-like cells in IVF patients’ blood. The number of SM-like cells is decreasing in high level E2 samples compared to the low level E2 samples. n=45; *p<0.05 vs low.

4.9.3 ESTRADIOL INHIBITS GROWTH OF SM-LIKE CELLS

To look at the effects of estradiol on SM-like cells, cell number and \(^3\)H-thymidine incorporation into DNA of growing cells were evaluated after four days of treatment with estradiol. Moreover, expression of cell cycle regulating proteins was analyzed by Western Blotting.

4.9.3.1 Thymidine incorporation

Cell proliferation was quantitatively measured by detecting the amount of incorporation of \(^3\)H-thymidine into the DNA of dividing cells following four days of estradiol treatment of growing cells (2.5% FCS). As shown in Fig. 52, estradiol significantly inhibited proliferation (cell number) and DNA synthesis (\(^3\)H-thymidine incorporation), of SM-like cells already at physiological concentrations (at 10 nM by 23% and 16% compared to the untreated control, respectively).
There are studies indicating that ERα contributes to vasoprotection by estradiol, by mediating its effects on SMC growth inhibition (Pare, 2002). Therefore, we investigated whether ERα also mediated estradiol effects in SM-like cells. We used pharmacological agonists for either ERα (PPT) or ERβ (DPN) and either unspecific ER antagonist (ICI) or specific ERα antagonist (MPP). Cells were counted to determine proliferation and ³H-thymidine incorporation into the DNA of dividing cells was used to measure DNA synthesis.

As shown in Fig. 53, both ER agonists PPT (ERα) and DPN (ERβ) mimicked the effects of estradiol on both proliferation (at 100 nM 67.4 ± 1.6% and 84.4 ± 2.2% of untreated cells, respectively for PPT and DPN, p<0.05) and DNA synthesis (at 100 nM 74.3 ± 1.04% and 89.4 ± 3.06% of untreated cells, respectively for PPT and DPN, p<0.05).

To further look at the role of the two ERs, we used pharmacological antagonists to prevent estradiol from binding to and exerting its effects via the receptors. Both cell number and DNA synthesis inhibition mediated by estradiol were reverted in presence of the antagonists after four days of treatment. The ERα specific antagonist MPP (5 μM) reduced estradiol effect by 19% and 11% for cell number and DNA synthesis, respectively, and ERα/ERβ antagonist ICI by 22% and 18%, respectively (Fig. 54).
To confirm these results, ER antagonists were used to block the receptors in the presence of ER agonists. As shown in Fig. 55, for both cell number (panel A) and DNA synthesis (panel B) after four days treatment, PPT (100 nM) effect was reverted by 10 μM ICI by 10% and 9% respectively, and by 5 μM MPP by 11% in both cases. DPN (10 nM) effect was reverted by 10 μM ICI by 3.2% and 0.5% respectively.

4.9.3.2 Cell cycle

To investigate the mechanisms through which estradiol inhibits SM-like cells proliferation, as for EPCs (see section 4.5), modulation of the expression of mitogenic signal transduction pathway proteins, cell cycle regulator proteins, and inhibitors of cell cycle proteins was analyzed. We first tested the effects of physiological concentrations of estradiol (1 and 10 nM) on the activation of mitogenic signal transduction pathways protein Akt. Following 7 min of treatment a significant decrease in phosphorylation of this protein was observed (at 10 nM 81.82%±0.82 compared to untreated control), as shown in Fig. 56.
Smooth Muscle-Like Cells

Results

**FIGURE 56.** (A) Western blot showing the inhibition of Akt phosphorylation by 10 nM estradiol treatment for 7 min. (B) The bar graph represents the densitometric analysis of the western blot normalized to the unphosphorylated form of the protein. n=3; *p<0.05

Furthermore, the ERα agonist PPT (100 nM) and specific and unspecific antagonists MPP (5 μM) and ICI (10 μM) were used to confirm the role of ERα in mediating inhibition of SM-like cells growth. As shown in Fig. 57, PPT mimicked estradiol effect (39.3 ± 3.6% of untreated control), and MPP pretreatment (30 min) abrogated this effect (104.7 ± 5.7% of untreated control). Moreover, ICI reverted the effect of estradiol by 24%, providing evidence for an ERα mediation of mitogenic signal transduction pathways inhibition.

**FIGURE 57.** (A) Western blots showing the reversion of estradiol (10 nM) and PPT (100 nM) effects on Akt phosphorylation inhibition by MPP (5 μM) and ICI (10 μM) pretreatment (30 min) of SM-like cells. (B) The bar graphs represent the densitometric analysis of the western blots normalized to unphosphorylated Akt. n=3; *p<0.05; * vs PPT, * vs E2.

To further delineate some intracellular mechanisms involved in mediating inhibitory effects of estradiol on SM-like cells, we analyzed the regulation of Rb hyperphosphorylation and cyclin expression. As shown in Fig. 58, hyperphosphorylation of Rb protein, which allows the progression from G1 to S phase,
was also inhibited by 48 hours of treatment with the ERα agonist PPT (100 nM) from 100 ± 0.2% to 87.9 ± 1.8%.

**FIGURE 58.** (A) Western blot showing the downregulation of the hyperphosphorylated form of Rb protein by 100 nM PPT, indicating an ERα mediation of estradiol effects. (B) The bar graph represents the densitometric analysis of the blot, and is normalized to total Rb. n=3; *p<0.05 vs control.

Furthermore, as shown in Fig. 59, 72 hours of estradiol treatment (10 nM) upregulated the expression of the cell cycle inhibitor protein p21 (from 100 ± 1.24% to 144.9 ± 0.2%, p<0.05), further confirming the inhibitory effect of estradiol on SM-like cell proliferation.

**FIGURE 59.** (A) Western blot showing the upregulation of the expression of the cell cycle inhibitory protein p21 by 10 nM E2. (B) The bar graph represents the densitometric analysis of the blot, and is normalized to the internal control β-actin. n=3; *p<0.05 vs control.

Moreover, as shown in Fig. 60, after 48 hours treatment, cyclin D expression was significantly downregulated by 100 nM PPT by 25% (from 100 ± 3.9% to 85.4 ± 2.1%) compared to the untreated control. Cyclin A and cyclin E were significantly downregulated by 100 nM PPT by 42% (from 100 ± 2.8% to 58.2 ± 4.3%) and 28% (from 100 ± 1.6% to 72.3 ± 3%) compared to the untreated control, respectively. Expression of cyclin B was downregulated by 10 nM estradiol by 25% (from 100 ± 10% to 85.2 ± 3.4%) and by 100 nM PPT by 34% (from 100 ± 10% to 66.5 ± 4.5%), further confirming ERα mediation of estradiol effects.
DISCUSSION

Some of the cells that we isolated from umbilical cord vein blood did not develop into an EC-like type, but rather into a SM-like cell type. Previous studies done in our lab (Barchiesi, 2003) showed that some of the beneficial effects of estradiol on the cardiovascular system are due to SMCs growth inhibition. Moreover, it has been shown, that more than 60% of smooth muscle cells building neointima after vessel injury are bone marrow-derived (Wang, 2006), as the cells we isolated form blood. Therefore, we decided to perform some experiments in order to investigate whether these cord blood-isolated cells were actually SM-like, and whether similar to SMC they responded to estradiol treatment. We could demonstrate with immunofluorescence that our new cell type was actually a SM-like progenitor cell type. We expected estradiol having similar effects on SM-like progenitors as on SMC, i.e. acting on them as an antimitogen. FACS analysis of cells isolated from IVF patients showed that the number of circulating c-kit+/AC133+ cells was lower in the high estradiol samples compared to the low estradiol levels ones, indicating estradiol inhibited mobilization of SM-like cells from bone marrow, a result that
is contrary to that of EPCs, where estradiol increases their circulating number (see section 4.2.2). Moreover, ^3H^-thymidine incorporation experiments and cell counting confirmed an inhibition of SM-like cell growth by estradiol. Pharmacological ERα agonist PPT mimicked estradiol effects. Although the effects of estradiol and PPT were reversed by both ERα antagonist MPP and ERα/ERβ agonist ICI, they were not completely abolished. Hence, it is possible that the inhibitory effects of estradiol are mediated via an ERα-dependent as well as an ERα-independent mechanism. In this context, experiments done in our lab have previously shown that the antimitogenic effects of estradiol in SMCs are mediated via an ER-independent mechanism involving the conversion of estradiol to 2-ME. However, whether this mechanism is also involved in mediating the antimitogenic effects in progenitor cell derived SM cells, remains to be investigated. In addition to the above experiments, we also analyzed the modulatory effects of estradiol on the expression of cell cycle regulatory proteins in order to elucidate the intracellular mechanisms via which estradiol inhibits proliferation of these cells. Western blot analysis confirmed previous results, showing an inhibition of mitogenic signal transduction pathways (Akt phosphorylation inhibition) by estradiol. The effects of estradiol were mimicked by ERα agonist and blocked by ERα antagonist, suggesting that the effects are ERα mediated. Similar to the effects on proliferation, the expression of positive cell cycle regulator proteins was downregulated by estradiol and by PPT, accordingly to the ERα mediation model. Moreover, the expression of the negative cell cycle regulator protein p21 was upregulated by estradiol. Our experiments provide evidence for a double and opposing action of estradiol on bone marrow-derived cardiovascular progenitor cells. On one side it promotes mobilization, proliferation, microvessel formation, NO production and adhesion of EPCs, which promote endothelial repair, neovasculogenesis and hence cardiovascular health and atherosclerosis prevention. On the other hand, estradiol inhibits bone marrow mobilization and proliferation of SM-like progenitors, which are participating into neointima formation and thus atherosclerosis progression and pathogenesis. These two aspects together are contributing to the beneficial effects of estradiol on the cardiovascular system. However, more experiments should be done to further study the intracellular mechanisms leading to growth inhibition of SM-like cells and to investigate the effects of estradiol on neointima formation.

4.9.4 Phenotype Adaptation of SM-like Cells in the Presence of Estradiol

Objective

The purpose of these experiments was to investigate whether SM-like progenitor cells are modifying their phenotype in the presence of estradiol.

Introduction

SM-like progenitor cells and EPCs are both bone marrow-derived cells. We have previously shown that, in IVF patients, higher levels of estradiol in plasma are increasing the number of circulating EPCs and decreasing that of SM-like progenitors. Moreover, both types of cells have been shown to influence atherosclerotic disease progression, with EPCs promoting health of the endothelium positively, and SM-like progenitor inducing negative effects, by participating in neointima formation. Thus, it is feasible, that the amount of estradiol circulating within the blood influences the fate of cardiovascular disease, by altering the balance between the two cell types, their bone marrow mobilization and their growth ability. It has been shown that ECs are changing their phenotype into that of SMC when VEGFR is blocked (Sakao, 2007). Thus, since our two cell types are progenitor cells, and retain the ability to
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differentiate into different cell types, we investigated whether in the presence of estradiol a change in phenotype occurred.

**METHODS**

Protein expression was detected by immunofluorescence as described in methods (3.2.4).

**RESULTS**

We qualitatively analyzed the modulation of endothelial marker expression in SM-like cells, following estradiol treatment. As shown in Fig. 61, treatment of cultured SM-like progenitor cells with 10 nM estradiol promoted the expression of endothelial markers. After four days, an increase in the expression of vWF and CD146 could be detected, and VEGF-R expression was increased after 15 days. The expression of SMC markers was not significantly altered (data not shown).

![Microscopic pictures showing the expression of endothelial markers in control (upper row) and 10nM estradiol-treated (lower row) SM-like cells.](Image)

**DISCUSSION**

Beneficial actions of estradiol on the cardiovascular system are due to its mitogenic effect on ECs and its anti-mitogenic effects on SMCs. In previous experiments we provided evidence that estradiol increases the number of circulating CD34+ EPCs and decreases the number of circulating c-kit+ SM-like cells (see sections 4.2.2 and 4.9.2), therefore promoting endothelium and cardiovascular system health. It has been shown that ECs can change their phenotype into that of SMCs when VEGF-R is blocked (Sakao, 2007). Here we provided evidence that expression of ECs characteristic proteins was increased in presence of estradiol in SM-like progenitor cells. This suggests that estradiol may protect the cardiovascular system also by promoting the differentiation of SM-like progenitor cells, which are responsible for neointima formation following vascular injury, into ECs.
5. CONCLUSIONS AND PERSPECTIVES

Cardiovascular disease is the leading cause for mortality in both men and women in the world. Estradiol induces beneficial effects on the cardiovascular system, in part, by preventing endothelial damage and by promoting endothelial cell growth. Because endothelial health is crucial for preventing vaso-occlusive disorders, together with the fact that EPCs have been shown to actively participate in angiogenesis and neovascularogenesis, we hypothesized that estradiol may protect against cardiovascular disease by promoting EPC growth. In this study we provide evidence in support of this hypothesis.

We demonstrate that estradiol promotes mobilization of EPCs into the circulation and induces proliferation of both freshly isolated and cultured EPCs. Moreover, we provide evidence that the mitogenic effects of estradiol are ERα mediated. With regard to the intracellular mechanisms mediating the growth promoting effects of estradiol in EPCs, we demonstrated that, via ERα, estradiol activates the mitogenic signal transduction pathways MAPK and Akt, promoting hyperphosphorylation of the Rb protein, upregulates the expression of cyclin D1, cyclin A, cyclin E and cyclin B, and downregulates the expression of the cell cycle inhibitor protein p21.

Importantly, we provided evidence that estradiol increases the adhesion of EPCs to endothelial cell monolayers and that, via ERα, estradiol promotes EPC-induced microvessel formation, and that these effects are mediated via activation of the mitogenic signal transduction pathway Akt and upregulation of HO-1 protein expression.

Because endogenous metabolites of estradiol can be biologically active, we investigated the effects of 2-OHE and 2-ME on EPC growth. In contrast to estradiol, its metabolites inhibit EPC growth. We also showed that the estradiol metabolite 2-ME has a biphasic effect on microvessel formation, by promoting it at concentrations <100 nM and inhibiting it at higher concentrations.

Moreover, we demonstrated that production of the cardiovascular protective molecule NO by EPCs is enhanced by estradiol via an ERα-mediated activation of the Akt pathway and phosphorylation of eNOS.

Apart from endothelial dysfunction/damage, the abnormal growth of SMCs contributes to the vascular remodeling process associated with atherosclerosis and injury-induced neointima formation. Because bone marrow-derived c-kit+ cells are similar to SMCs and contribute to neointima formation, we investigated the effects of estradiol on their growth. Our results provide evidence that, via ERα, estradiol inhibits the proliferation of c-kit+ cells, and these inhibitory effects are mediated via the downregulation of MAPK and Akt phosphorylation, cyclin D, A, E and B expression and upregulation of p21.

Taken together, our findings provide evidence that estradiol may protect against vaso-occlusive disorders associated with cardiovascular disease by inducing endothelial repair by stimulating EPC growth, and by inhibiting the growth of c-kit+ SM-like progenitor cells.


PRESENTATIONS

ANNUAL REPORTS AND ORAL PRESENTATIONS

2006  “Progenitor stem cells: biological role, practical approach and pathophysiological importance”, at Gynecology and Endocrinology Colloquium, University Hospital Zurich.

2008  “Estradiol induces mitogenesis in human progenitor endothelial cells by upregulating cyclins and downregulating p21”, at 4th Symposium of the Zurich Center for Integrative Human Physiology (ZIHP).

2008  “Role of endothelial progenitor cells in estradiol-mediated vascular protection: potential intracellular mechanisms”, at Gynecology and Endocrinology Colloquium, University Hospital Zurich.

2009  “Regulation of Endothelial Progenitor Cells Growth by Estradiol: Role of Estrogen Receptors and Intracellular Mechanisms” at Institute of Cell Biology, ETH Zurich

2009  “Estradiol stimulates capillary formation by human endothelial progenitor cells: role of estrogen receptors, heme oxygenase-1, and tyrosine kinase” at 63rd High Blood Pressure Research Conference, Chicago, USA

POSTER PRESENTATIONS

2007  “Estradiol induces mitogenesis in human progenitor endothelial cells by upregulating cyclins and downregulating p21” at 61st Annual High Blood Pressure Research Conference, Tucson, USA

2008  “Estradiol induces mitogenesis in human progenitor endothelial cells by upregulating cyclins and downregulating p21” at 7th Day of Clinical Research, University Hospital Zurich

2009  “Estradiol stimulates human endothelial progenitor cells (EPCs) mediated capillary formation via estrogen receptor-α and activation of heme oxygenase-1 and protein kinase” at 8th Day of Clinical Research, University Hospital Zurich
“Estradiol (βE) promotes adhesion and mitogenesis in CD34+ human progenitor endothelial cells (PECs)” at Experimental Biology 2009, New Orleans, USA

“Estradiol (βE) stimulates human endothelial progenitor cells (EPCs) mediated capillary formation via estrogen receptor (ER)-α linked activation of heme oxygenase-(HO)1 and tyrosine kinase” at 5th Symposium of the Zurich Center for Integrative Human Physiology (ZIHP).