Doctoral Thesis

Defining the Role of Androgens in Vascular Remodeling Associated with Cardiovascular Disease

Author(s):
Plutino, Yuliya

Publication Date:
2014

Permanent Link:
https://doi.org/10.3929/ethz-a-010344811

Rights / License:
In Copyright - Non-Commercial Use Permitted
DEFINING THE ROLE OF ANDROGENS IN VASCULAR REMODELING ASSOCIATED WITH CARDIOVASCULAR DISEASE

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zürich)

presented by

YULIYA PLUTINO

Master of Science UZH

born on 14.12.1985

citizen of
Belarus

accepted on the recommendation of

Prof. Dr. Sabine Werner, examiner
Prof. Dr. Wilhelm Krek, co-examiner
Prof. Dr. Christian Grimm, co-examiner
Prof. Dr. Raghvendra K. Dubey, co-examiner

2014
# Table of Contents

1 INTRODUCTION .................................................................................................................. 13

1.1 Androgens and Their Physiology .................................................................................. 13
   1.1.1 Biological Effects ................................................................................................. 13
   1.1.2 Synthesis and Metabolism .................................................................................. 13
   1.1.3 Androgen Receptor ............................................................................................. 15
   1.1.4 Mechanisms of Androgen Actions (Genomic vs. Non-genomic Actions) .......... 17

1.2 Androgens and Cardiovascular Disease ....................................................................... 19

1.3 Endothelial and Endothelial Progenitor Cells ............................................................... 22
   1.3.1 The Role of EPCs in Vascular Repair .................................................................. 22
   1.3.2 EPC Function in Cardiovascular Disease and in Testosterone Deficient Men ...... 24
   1.3.3 Endothelial Barrier Function .............................................................................. 25

1.4 The Molecular Mechanisms Regulating EC Function .................................................. 27
   1.4.1 The Akt/mTOR/VEGF Signaling in Angiogenesis .............................................. 27
   1.4.2 TGFβ/BMP Signaling and Its Role in Endothelial Cell Function ....................... 28

1.5 The Role of Androgens in SMC Function and Their Contribution to Cardiovascular Disease ...................................................................................................................... 32

2 GENERAL HYPOTHESIS AND OBJECTIVES ................................................................... 33

3 MATERIALS ......................................................................................................................... 35

3.1 Cell Culture .................................................................................................................. 35

3.2 Antibodies and Peptides ............................................................................................. 36

3.3 Chemicals and Buffers ............................................................................................... 37

3.4 Instruments and Software ........................................................................................... 39

4 METHODS ........................................................................................................................ 41
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Cell Culture</td>
<td>41</td>
</tr>
<tr>
<td>4.2</td>
<td>Growth Studies</td>
<td>42</td>
</tr>
<tr>
<td>4.3</td>
<td>RT-PCR</td>
<td>42</td>
</tr>
<tr>
<td>4.4</td>
<td>Western Blotting Analysis</td>
<td>43</td>
</tr>
<tr>
<td>4.5</td>
<td>Vasculogenesis Assay</td>
<td>44</td>
</tr>
<tr>
<td>4.6</td>
<td>Bead Sprouting Angiogenesis assay</td>
<td>44</td>
</tr>
<tr>
<td>4.7</td>
<td>Co-culture Assay</td>
<td>45</td>
</tr>
<tr>
<td>4.8</td>
<td>Permeability Assay</td>
<td>45</td>
</tr>
<tr>
<td>4.9</td>
<td>SiRNA Transfection</td>
<td>46</td>
</tr>
<tr>
<td>4.10</td>
<td>Calcium Measurements</td>
<td>46</td>
</tr>
<tr>
<td>4.11</td>
<td>Statistical Analysis</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>RESULTS AND DISCUSSION</td>
<td>49</td>
</tr>
<tr>
<td>5.1</td>
<td>DHT Improves EPC Function</td>
<td>49</td>
</tr>
<tr>
<td>5.1.1</td>
<td>DHT Stimulates Vasculogenesis in EPCs</td>
<td>50</td>
</tr>
<tr>
<td>5.1.2</td>
<td>AR Mediates the Capillary Formation by EPCs</td>
<td>51</td>
</tr>
<tr>
<td>5.2</td>
<td>The Akt-mTOR-VEGF Signaling Pathway Mediates DHT-Stimulated Vasculogenesis by EPCs</td>
<td>56</td>
</tr>
<tr>
<td>5.2.1</td>
<td>The Akt/mTOR/VEGF Signaling Pathway in Vasculogenesis</td>
<td>57</td>
</tr>
<tr>
<td>5.2.2</td>
<td>DHT Induces Akt and mTOR Phosphorylation in EPCs</td>
<td>59</td>
</tr>
<tr>
<td>5.2.3</td>
<td>A Cross-Talk between AR and the Akt/mTOR/VEGF Axis</td>
<td>60</td>
</tr>
<tr>
<td>5.3</td>
<td>ALK1/Smad1/5/8 Signaling Mediates DHT-Induced Barrier Function but Not Vasculogenesis in EPCs</td>
<td>64</td>
</tr>
<tr>
<td>5.3.1</td>
<td>DHT Triggers the Activation of the ALK1/Smad1/5/8 Signaling</td>
<td>65</td>
</tr>
<tr>
<td>5.3.2</td>
<td>DHT-Induced Activation of Smad1/5/8 Signaling Is Mediated by Intracellular Calcium Influx</td>
<td>67</td>
</tr>
<tr>
<td>5.3.3</td>
<td>The Role of Smad1/5/8 in Vasculogenesis</td>
<td>70</td>
</tr>
</tbody>
</table>
5.3.4 The Role of the ALK1-Smad1/5/8 Pathway in Regulating EPC Barrier Function.. 71

5.4 DHT Up-Regulates AR Expression in EPCs and AoSMCs but Induces Differential AR-Mediated Effects on EPC and AoSMC Growth .................................................. 80
  5.4.1 DHT Regulates AR by Stabilizing AR Protein in EPCs ................................ 81
  5.4.2 DHT Up-regulates AR Expression by Stabilizing AR Protein in AoSMCs ...... 85
  5.4.3 The Mitogenic Effects of DHT on Male AoSMCs .................................... 86
  5.4.4 DHT Modulates the Cell Cycle Regulators in AoSMCs ............................ 86

6 CONCLUSIONS AND PERSPECTIVES ................................................................. 91

7 REFERENCES ...................................................................................................... 95
Acknowledgments

I would like to sincerely thank Prof. Dr. Raghvendra Dubey for his guidance, patience and encouragement during this project. His enthusiasm and valuable ideas helped me to stay motivated at the moments of difficulties and his experience in academic research and especially in Reproductive Endocrinology helped me to expand my scientific knowledge.

I would like to express my gratitude to Prof. Sabine Werner for supervising my project, providing helpful suggestions and advices and time for evaluating my thesis. I am grateful to the members of my PhD committee, Prof. Wilhelm Krek and Prof. Dr. Christian Grimm, for their valuable scientific feedback during progress reports.

I am very thankful to current and former members of the Dubey group: Lisa Unterleutner, Lisa Rigassi, Federica Barchiesi, Doris Müller, Jasmine Wiget, Bea Woodtli, Nikiana Simigdala and Marta Szutkowska for their support, scientific discussions, daily life advice and nice atmosphere in the lab.

I would like to thank other groups in Wagiareal in Schlieren from Institute of Clinical Chemistry and Institute of Pharmacology and Toxicology for their technical assistance and help. I am also thankful to other people on the floor for a good company outside of Schlieren.

I would like to thank Prof. Bruno Imthurn for opportunity to participate in ATVB 2014 conference and the team of Clinic of Reproductive Endocrinology for nice time during summer and Christmas events.

I would like to thank Luca, my family and friends for supporting and understanding me during these years.

I gratefully acknowledge Swiss National Foundation (31003A-138067) and Swiss – Romanian Cooperation Program (IZERO 142213/1) for the financial support of this project.
Summary

Growing evidence suggests a central role of androgens in cardiovascular health of men. In spite of numerous studies conducted to decipher the precise roles of testosterone (T) in cardiovascular disease (CVD), no agreement has been achieved regarding the protective or deleterious effects of T on the cardiovascular system (CVS). Therefore, it is essential to clarify how T affects the CVS in order to identify the potential risks and benefits of testosterone replacement therapy (TRT), increasingly used by elderly men.

We hypothesize that androgens modulate vascular remodeling processes associated with CVD. Because endothelial dysfunction and abnormal smooth muscle cell growth are the main determinants of vascular remodeling processes, we investigated the effects of androgens on these processes. Proper EC function is required for tissue repair and wound healing by building a network of new blood vessels. Interestingly, besides residual ECs, circulating endothelial progenitor cells (EPCs), derived from bone marrow and spleen, have been found to actively participate in vascular repair. Therefore, our main focus was to study the effects of androgens on the function of EPCs. To address this question, we utilized dehydrotestosterone (DHT), a T metabolite, which mimics the effects of T and is not converted to estradiol. Hence, the use of DHT ascertains that the observed effects are solely androgenic and not compromised by conversion to estradiol.

In the present study, we demonstrated that DHT induces capillary formation by EPCs using three in vitro angiogenesis assays (two-dimensional matrigel-based assay, co-culture of EPCs on the confluent monolayer of human cardiac fibroblasts (HCF) and bead sprouting assay). Moreover, we demonstrated that the actions of DHT in EPCs were mediated via androgen receptor (AR). Indeed, the stimulatory effects of DHT were abrogated by the AR antagonist flutamide and by AR siRNA. Furthermore, our results provide the evidence that DHT promotes vasculogenesis in EPCs by activating the Akt/mTOR/VEGF pathway. In this regard, the PI3K/Akt inhibitor LY294002, the mTOR inhibitor rapamycin and VEGF neutralizing antibodies abolished the effects of DHT on EPC function. Interestingly, we report that the angiogenic effects of DHT on EPCs are mediated by a cross-talk between AR signaling and the Akt pathway. In this context, inhibition of both signaling pathways reduced vasculogenesis in EPCs. Moreover, AR blockade prevented DHT-induced phosphorylation of Akt and abolished the activation of the Akt/mTOR/VEGF signaling.

Another key finding of this research suggests a protective role of DHT on endothelial cell function. We found that DHT activates TGFβ signaling implicated in the regulation of
endothelial function and vascular physiology. DHT induced the phosphorylation of the transcription factors Smad1/5/8 via ALK1 but not ALK5, resulting in the up-regulation of the downstream target Id-1 and also Endoglin. Additionally, the BMP inhibitor Noggin prevented DHT-stimulated Smad1/5/8 phosphorylation, indicating a role of BMP2 and BMP4 in mediating the effects of DHT on the ALK1/Smad1/5/8 signaling. Furthermore, we demonstrated that DHT triggered an increase in intracellular calcium which leads to the activation of Smad1/5/8 signaling. In this regard, DHT stimulated a rapid flux of calcium, measured by loading EPCs with the calcium sensitive agent fluo4. Furthermore, inhibition of calcium signaling with the G protein coupled receptor inhibitor pertussis toxin, the phospholipase C inhibitor U73122 and the calcium chelator BAPTA-AM abrogated Smad1/5/8 phosphorylation by DHT. Surprisingly, our findings suggest that the ALK1/Smad1/5/8 signaling has no effect on vasculogenesis in EPCs, since Smad1 siRNA did not affect DHT-induced capillary formation. More importantly, our results provide evidence that the ALK1/Smad1/5/8 pathway is responsible for DHT-stimulated barrier function in EPCs, which is confirmed by the up-regulation of the tight junction protein occludin. Moreover, DHT abrogated thrombin-induced barrier disruption.

Since the biological actions of androgens are mediated by AR, we investigated how DHT regulates AR expression in EPCs. Here, we demonstrated that DHT increased AR expression in EPCs by promoting its stability upon ligand binding and by modulating its translation, but not transcription. We speculate that these findings might be of importance for developing therapeutic approaches targeting AR signaling. For example, priming EPCs with DHT may enhance its therapeutic potential in men receiving stem cell therapy following MI or cardiovascular events.

To fully examine the action of DHT on vascular remodeling, we also studied its effects on proliferation of aortic smooth muscle cells, which contribute to intima-media formation and vaso-occlusion. In this regard, we found that DHT induced the proliferation of AoSMCs and up-regulated the expression of CyclinA and CyclinD1 and down-regulated the cell cycle inhibitors p21 and p27. Surprisingly, these results suggest a deleterious role of androgens in vascular remodeling processes. However, it is possible that the mitogenic actions only occur in the absence of ECs.

Taken together, our findings support a protective role of DHT on EPC function by stimulating vasculogenesis, a key process in tissue regeneration, and by promoting barrier function, essential for preserving vascular integrity in CVD. However, the mitogenic effect of DHT on AoSMCs indicates a potential risk of androgen application for the cardiovascular system.
Based on these findings, we hypothesize that androgens would induce their protective actions by improving endothelial function and repair. However, under pathological conditions with severe vascular damage or remodeling associated with CVD, androgens may promote the deleterious effects by stimulating SMC growth and vaso-occlusion. The exact mechanisms and factors, which determine the outcome of androgen treatment and regulate the balance between the beneficial and deleterious effects of androgens have to be further investigated.
Zusammenfassung


Ein weiteres wichtiges Ergebnis dieser Forschung unterstützt eine schützende Rolle von DHT auf die Endothelfunktion. DHT aktivierte die TGF-Signalisierung, die an der Regulation der endothelialen Funktion und an Gefäßstörungen beteiligt ist. Zudem wurde gezeigt, dass DHT die Phosphorylierung der Transkriptionsfaktoren Smad1/5/8 durch ALK1, aber nicht durch ALK5, induzierte und so zur Erhöhung von Id-1 und Endoglin führte. Zusätzlich hemmte der BMP Inhibitor Noggin die DHT-stimulierte Phosphorylierung von Smad1/5/8. Dies lässt auf eine Rolle von BMP-2 und BMP-4 bei der Vermittlung der Effekte von DHT auf ALK1/Smad1/5/8 Signalisierung schließen. Weiterhin zeigten wir, dass DHT einen intrazellulären Calciumanstieg, gemessen über calciumsensitives Fluo4, auslöste. Das führte zur Aktivierung der Smad1/5/8 Signalisierung, welche durch G-Protein Inhibitor Pertussis Toxin, Phospholipase Inhibitor U73122 und Calcium Chelator Bapta-AM gehemmt wird. Außerdem zeigte sich, dass ALK1/Smad1/5/8 Signalisierung keinen Einfluss auf die Vaskulogenese in EVZs hat, da Smad1 siRNA DHT-stimulierte Kapillarbildung nicht beeinflusste. Schließlich belegen unsere Ergebnisse, dass der ALK1/Smad1/5/8 Signalweg für die DHT-stimulierte Barrierefunktion in EVZ verantwortlich ist, indem er reguliert das Tight Junction Proteins Ocludin, welches ein Schlüsselregulator der endothelialen Integrität ist.


Zusammenfassend weisen unsere Resultate auf eine schützende Rolle von DHT auf EVZ-Funktion hin: die Behandlung mit DHT führt zur Stimulierung der Vaskulogenese, einem Schlüsselprozess bei der Gewebe-Regeneration, und zur Förderung der Barrierefunktion, die eine zentrale Rolle in der endothelialen Integrität spielt. Der proliferative Effekt von DHT auf glatte Muskelzellen deutet jedoch auf ein potenzielles Risiko der Anwendung von
Zusammenfassung
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-HSD</td>
<td>17β-Hydroxysteroid Dehydrogenase</td>
</tr>
<tr>
<td>ActD</td>
<td>ActinomycinD</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin Receptor-like Kinase</td>
</tr>
<tr>
<td>AoSMC</td>
<td>Aortic Smooth Muscle Cells</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain-Barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bichinonic Acid</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic Monophosphate</td>
</tr>
<tr>
<td>CMFDA</td>
<td>5-Chloromethylfluorescein Diacetate</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>CY</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DHT</td>
<td>Dehydrotioestosterone</td>
</tr>
<tr>
<td>DMEM-F12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotheritol</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial Basal Medium</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>EPCs</td>
<td>Endothelial Progenitor Cells</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-regulated Kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-Releasing Hormone</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HCF</td>
<td>Human Cardiac Fibroblasts</td>
</tr>
<tr>
<td>hEGF</td>
<td>Human Epidermal Growth Factor</td>
</tr>
<tr>
<td>hFGF</td>
<td>Human Fibroblast Growth Factor</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 Kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl Fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex Hormone Binding Globulin</td>
</tr>
<tr>
<td>SmBM</td>
<td>Smooth muscle Basal Medium</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Tumor Growth Factor Beta</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junctions</td>
</tr>
<tr>
<td>TRT</td>
<td>Testosterone Replacement Therapy</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
</tbody>
</table>
Introduction

1 Introduction

1.1 Androgens and Their Physiology

1.1.1 Biological Effects

Androgens belong to a group of endogenous sex hormones and play a primary role in maintaining male health [1]. During puberty, androgen levels are increasing and reach a peak in adolescence, thus regulating age-dependent development of male secondary sexual characteristics and gametogenesis. Moreover, androgens exert their effects on various organ systems in the human body. During the lifespan, androgens have an impact on physical (increase skeletal muscle mass), sexual (libido) and bone health (prevent osteoporosis) as well as on the mental and emotional state of men. Moreover, male sex hormones have been shown to influence cardiovascular health, affecting hypertension, atherosclerosis and vascular tone.

1.1.2 Synthesis and Metabolism

The androgen synthesis is regulated by the hypothalamic-pituitary-gonadal axis. Hypothalamus produces gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH) [2][3]. These hormones in turn regulate production and release of gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) and adrenocortico-tropic hormones (ACTH) from the anterior pituitary gland. ACTH stimulates the synthesis of precursors of testosterone (T) – dehydroepiandrosterone (DHEA) from cholesterol in the adrenal glands [2][4]. LH initiates the synthesis of T from cholesterol in Leydig cells of the testis [5][6]. T production requires a series of enzymatic reactions and can be mediated via several routes. One of them begins in the mitochondria, where cholesterol molecules are cleaved by long side chain enzymes to pregnenolone, which is processed by 17-α-hydroxylase to 17-α-hydroxypregnenolone and the latter is converted into DHEA by 17,20-lyase (Figure 1). Subsequent reactions of 17-β-hydroxysteroid dehydrogenase (17β-HSD) lead to the conversion of DHEA to testosterone. The levels of T in the plasma are maintained constant due to a negative feedback of the hypothalamic-pituitary-gonadal axis, which involves the inhibition of the production of GnRH and thereby gonadotropins by elevated serum T levels [7]. Approximately 95% of the total plasma T synthesis occurs in Leydig cells,
whereas the adrenal gland produces most of the plasma DHEA, DHEA sulfate, androstenedione and less than 1% of T derived from adrenal DHEA.

![Diagram of androgen biosynthesis](image-url)

**Figure 1:** A simplified model of androgen biosynthesis from cholesterol. *CYP11A1*, cholesterol desmolase; *DHEA-S*, dehydroepiandrosterone sulfate; *17β-HSD*, 17-β-hydroxysteroid dehydrogenase.

Free T enters the cell by passive diffusion and initiates its signaling. In the cell, T can be converted by 5α-reductase into dehydrotestosterone (DHT) in the testis as well as in other androgen target tissues (adipose, brain, muscle, skin, adrenal cortex) (Figure 2) [8][9]. T and DHT represent the most active androgens in the body. However, the biological activity of DHT is much higher than that of T, since its binding affinity to the androgen receptor (AR) is 2-folds greater than that of T [10]. Therefore, DHT amplifies the signal in the peripheral tissues where T availability is lower [11]. Interestingly, 5α-reductase inhibitor decreased T effects in rat aortic endothelial and smooth muscle cells, suggesting the presence of 5α-reductase in vascular cells and a prevalent role of DHT and not of T in these cells [12][13][14]. T can be also converted into estrogen by P450 aromatase [15], expressed abundantly in women, whereas DHT is a non-aromatizable androgen. Low levels of aromatase in men prevent the conversion of T into estrogens.
In the circulation T is bound to either albumin (ca. 55%) or to sex hormone binding globulin (SHBG) (ca. 45%) and only around 1-2% of circulating androgens accounts for free or bioavailable T [16]. Increase in SHBG levels can mimic a decrease in free T, resulting in unaltered total T levels. Plasma T concentration ranges between 10-35nM in male and 0.7-2nM in females [17][18].

1.1.3 Androgen Receptor

Androgens mediate their biological activity via intracellular androgen receptors (AR), which are abundantly expressed in androgen-dependent tissues, but also in vascular tissue (endothelial cells, vascular smooth muscle cells) [19]. The AR gene is located on X chromosome at Xq11.2-q12 position [9]. AR was first cloned in 1988 [20][21] and in 1990 was identified as a ligand-inducible transcription factor, belonging to the superfamily of nuclear receptors [22]. AR is a 110 kDa cytoplasmic protein with 918 amino acids, consisting of a NH$_2$- terminal domain (NTD), a DNA-binding domain (DBD), a hinge domain, and a ligand-binding domain (LBD) (Figure 3) [23][24].

The NH$_2$- terminal domain plays an important role in mediating AR transcriptional activity and represents the recruitment surface for co-regulator interaction. Variations between individual polyglutamine and polyglycine repeats in NTD result in low homology of AR NTD with those of other steroid receptors. An inverse correlation between the length of polyglutamine repeats (normal range 8-31) and AR trans-activation has been observed in in vitro studies [25][26]. For example, the pathogenesis of Kennedy’s disease is linked to the length of over 40 polyglutamine repeats [27] and is associated with reduced AR transcriptional activity. The range of polyglycine repeats lies around 10-30, and their complete deletion leads to decreased trans-activity of AR in vitro [28]. The activation function-1 domain of AR (AF-1) is involved in ligand-independent activation of AR upon an
Introduction

artificial separation from LBD, allowing a formation of constitutively active AR [29]. Moreover, NTD interacts with LBD, resulting in the stabilization of the ligand-receptor complex [9][30][31].

In contrast to NTD, LBD and DBD are highly conserved among steroid receptors. DBD contains two zinc fingers required for DNA recognition and the stabilization of interacting DNA-AR complex [25].

The hinge region is located between DBD and LBD of AR. The hinge region contains a nuclear localization signal and a PEST sequence (proline-, glutamate-, serine-, threonine-rich), the latter is a feature of proteins undergoing ubiquitin-mediated proteasomal degradation [32].

The AR LBD consists of 12 α-helixes forming a ligand-binding pocket. Ligand binding triggers a conformational change in LBD following a fold of helix 12 across the ligand-binding pocket, thereby exposing the surface for interacting partners [9][23].

![AR protein structure](image)

**Figure 3: Androgen receptor structure. Adapted from A. Galani et al.[24]**

The regulation of AR protein levels is controlled by degradation processes. Ligand-independent degradation of AR is mediated by the ubiquitin-proteasome system or PTEN/caspase3-pathway [25]. The ubiquitin-proteasome system involves the phosphorylation of target proteins and further ligation by ubiquitin ligase serving as recognition signal for the 26S proteasome. The phosphoinositol-3kinase (PI3K)/Akt pathway has been demonstrated to mediate the phosphorylation of AR for consequent ligation by Mdm2, the identified ligase for AR degradation [25][33]. Another mechanism for AR degradation in a prostatic cancer cell line results from PTEN binding to AR DBD, thereby preventing AR trans-activity and inducing AR degradation [25].
1.1.4 Mechanisms of Androgen Actions (Genomic vs. Non-genomic Actions)

The binding of the ligand to the AR initiates the conformational changes and subsequently the dissociation from heat shock proteins, dimerization and nuclear translocation of AR. In the nucleus, a recruitment of co-activators and general transcription factors (GTFs) enhances the androgen-induced transcriptional activity of AR [34]. Androgen actions that require the transcriptional activity of AR are characterized as genomic (Figure 4). However, some biological effects of androgens have been observed within a very short time, suggesting the non-genomic actions of androgens (Figure 5). These effects are characterized by a rapid induction occurring within seconds to minutes, independent of transcription/translation inhibition and mediated via membrane-associated receptors. Some studies have demonstrated an induction of intracellular androgen signaling using T bound to bovine serum albumin (BSA), which is unable to penetrate the cell membrane. Therefore, it has been suggested that G-coupled protein receptor, SHBG receptor or not yet identified membrane AR may be responsible for these rapid actions of androgens [35][36]. Hence, androgens can exert their biological effects via both genomic and non-genomic mechanisms [18].

**Genomic signaling**

![Genomic signaling diagram](image)

**Figure 4: A hypothetical model for the genomic mechanism of androgen actions.** Upon ligand binding, androgen receptor acts as a transcription factor to mediate androgen effects. T, testosterone; DHT, dehydrotestosterone; AR, androgen receptor; hsp90, heat shock protein 90.
The non-genomic actions of androgens have been shown in various cell types. T regulates vascular tone by triggering nitric oxide (NO) release or by modulating calcium and potassium channels [37][38]. Dilatory effects of androgens in porcine coronary arteries [39] and in rat aorta are mediated via activation of K⁺ channels [40]. However, a major role of the non-genomic actions of androgens is attributed to calcium signaling. Androgen-induced changes in intracellular calcium levels have been observed in prostate cells, Sertoli cells, macrophages, cardiomyocytes, and skeletal muscle cells [41][42][43][44][45]. The non-genomic effects are known to be mediated by second messengers. Indeed, AR-independent effects of male sex hormones are regulated by cAMP, protein kinase A (PKA), MAPK/ERK1/2, and c-Src/MAPK [46][47][48].

Figure 5: A hypothetical model for the non-genomic mechanisms of androgen actions. Rapid non-genomic actions of androgens are mediated by second messengers via membrane-associated receptors. T, testosterone; GPCR, G-protein coupled receptor; SHBG, sex hormone binding globulin; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PLC, phospholipase C; PI3K, phosphoinositol-3-kinase; MAPK, mitogen-activated protein kinase.
1.2 Androgens and Cardiovascular Disease

The decrease in androgen levels in aging men is accompanied by increased incidence of cardiovascular disease (CVD). This observation has led to the suggestion that androgens may be protective in cardiovascular health of men [49][50][51]. A decline in testosterone (T) levels with age represents a normal physiological process (Figure 6), characterized by a drop in total T concentration from 35nmol/L (a normal range) to <10-12nmol/L [52]. T deficiency affects 7% of men in ages between 40-50, moreover, it increases to 30% and 50% in men over 70 and 80 years, respectively [52][53]. Symptoms of hypogonadism include deterioration in bone health, osteoporosis, muscle atrophy, increased visceral obesity, prostate dysfunction, loss of libido, reduced spermatogenesis and increased fatigue [54]. Moreover, decline in T levels is associated with increased incidence of CVD and morbidities linked to CVD. With the increase in life expectancy and longevity, there is a need to clarify the cause and effect relationship between testosterone deficiency and CVD and to better define the role of testosterone replacement therapy (TRT) in elderly men.

Figure 6: Age-dependent distribution of men with low testosterone levels. Adapted from M. Kaushik et al. [50].

The findings from clinical studies provide disparate results, which support as well as negate the notion for the protective actions of androgens on the cardiovascular system [55][56][57]. Several studies in elderly hypogonadal men have shown an association between low serum testosterone and enhanced mortality from CVD independent of other risk factors [58][59][60]. Furthermore, the incidence of heart disease was increased in men undergoing testosterone
deprivation therapy to treat prostate cancer [61]. Increased carotid intima-media thickness (IMT) has been observed in middle-age hypogonadal men [62]. In men with coronary heart disease, TRT for 8 week modestly improved myocardial perfusion and reduced arterial stiffness [63]. The beneficial effects of TRT on cardiovascular health might be due to improved hypercholesteremia, dyslipedemia [64][65], decreased obesity, insulin resistance and hypertension [66][67]. However, in recent clinical studies it has been observed that testosterone supplementation had a negative effect in men with hypogonadism and led to increased incidence of CVD [68][69]. Interestingly, one cohort study points out the deleterious effects of TRT, which were associated with an increased risk for myocardial infarction (MI) in older men and also in younger men with pre-existing cardiovascular defects [70]. In other studies weak or no associations between hypogonadism and CVD in men was observed [71][72].

![Figure 7: A schematic representation of vascular remodeling processes associated with cardiovascular disease. EC, endothelial cell; SMC, smooth muscle cell.](image)

Contradictory findings have also been noted in *in vivo* studies. Testosterone treatment reduced aortic atherosclerosis in castrated rabbits, thereby protecting against the development of CVD [73]. Interestingly, in castrated male swine, testosterone treatment but not vehicle inhibited
coronary neointima formation in restenosis following angioplasty [74]. On the contrary, androgens increased the mean arterial pressure in spontaneously hypertensive male rats compared to female and castrated male rats [75]. Hence, both deleterious and protective roles have been attributed to the effects of testosterone on the cardiovascular system.

Abnormal growth of vascular smooth muscle cells contribute to the vascular remodeling associated with vaso-occlusive disorders in CVD. Several studies have reported the proliferative effects of androgens on vascular smooth muscle cells suggesting a link between androgen actions and CVD (Figure 7) [76][77][78][79]. Moreover, the negative effects of androgens on cardiac function have also been shown. For example, following MI, androgens induced myocardial hypertrophy and promoted cardiac rupture, which was prevented by castration [80]. These findings suggest a deleterious role of androgens in the cardiovascular system.

Another important vascular target of androgens, which affects cardiovascular health, is the modulation of endothelial function. One clinical study demonstrated that flow-mediated-vasodilation of the brachial artery was decreased in men with low T levels independently of other risk factors, indicating that T deficiency is associated with endothelial dysfunction [81]. Numerous studies have shown that testosterone induces NO production in endothelial cells (EC) and hence leads to vasodilation and protects the endothelium by NO-mediated mechanisms [82][83][84]. Moreover, the effects of testosterone on EC function were further investigated in in vivo and in vitro studies. In AR knockout mice neovascularization of ischemic hind limb was impaired compared to WT mice [85]. Sieveking et al. studied the effects of DHT on angiogenesis using different models and demonstrated that DHT induced angiogenesis in male ECs and these effects were AR-mediated. Moreover, they confirmed these findings using in vivo experiments. In this context, DHT treatment following orchidectomy increased neovascularization of matrigel plugs and improved hind limb ischemia in castrated mice [86]. This study suggests a potential role of angiogenic progenitor cells in the recovery of ischemic tissue due to their increased mobilization from bone marrow stimulated by DHT.
1.3 Endothelial and Endothelial Progenitor Cells

1.3.1 The Role of EPCs in Vascular Repair

Angiogenesis is a process of new blood vessel formation, which plays an important role in physiology and pathophysiology. In contrast to the essential role of angiogenesis during embryonic development, the beneficial effects of new blood vessel formation in adult tissues is limited to certain events such as menstrual cycle, wound healing and organ regeneration/vascular repair. Importantly, angiogenesis contributes to cardiovascular regeneration due to neovascularization of an injured tissue (following myocardial infarction, ischemia). However, excessive angiogenesis is associated with pathologies such as tumors, atherosclerotic plaque vascularization and several ocular/retinal diseases (proliferative diabetic retinopathy, choroidal neovascularization [87][88][89]).

Formation of new blood vessels from the pre-existing capillaries is referred as angiogenesis, whereas vasculogenesis underlies de novo capillary formation involving circulating progenitor cells [77]. Previously, only mature EC surrounding the injury site have been shown to contribute to the repair of damaged tissue. However, the importance of endothelial progenitor cells (EPCs) in postnatal neovascularization is now well established and confirmed by numerous in vivo studies [90][91][92][93]. EPCs are circulating bone-marrow and spleen derived cells [93][95] and were first described by Asahara et al., where EPCs were isolated based on the presence of cell surface antigens [94]. Mononuclear cells (MNC) from the peripheral and the umbilical cord blood are the main EPC sources [96][97]. Interestingly, EPCs isolated from the umbilical cord formed vessels better than peripheral blood EPCs [92]. Disparity has been noticed regarding EPC characterization and origin, since different isolation methods are used, resulting in different populations of EPCs with distinct functions in vasculogenesis [98]. MNCs differentiated into endothelial cell lineage express the endothelial cell (EC) markers CD31, CD105, CD144, CD146, KDR (VEGF receptor2) and eNOS [99] and are capable of acetylated low density lipoproteins (AcLDL) uptake [100][101]. However, often EPCs are identified by antigens CD34 and CD133, which are expressed also on hematopoietic stem cells (HSC), hence introducing heterogeneity into putative EPC population [94][102].

Recent findings revealed two types of EPCs: early (expressing myeloid antigens CD14, CD45, typical for monocyte/macrophages) and late outgrowth EPCs (OEC) with high proliferative potential described by several groups [96][100][103][104]. However, Sieveking
et al. demonstrated that only late OECs participate in vasculogenesis and incorporate into the vessel, while early EPCs contribute to the blood vessel formation in a paracrine manner [101]. Therefore, the effect of EPCs on neovascularization may vary depending on the EPC subpopulation used.

Mobilization of EPCs from the bone marrow and their homing and recruitment at the site of injury is regulated by various angiogenic factors (Figure 8) [105][106][107]. Ischemia-induced hypoxia triggers hypoxia inducible factor 1(HIF-1) production [108], which in turn mediates the release of VEGF and certain chemokines (SDF-1) to stimulate EPC migration [101][109]. In order to participate in neovascularization of ischemic tissue, EPCs have to penetrate the endothelium. This requires an increased expression of adhesion molecules and an enhanced permeability of the endothelium, which might be induced by VEGF. E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular endothelial adhesion molecule-1 (VCAM-1) are important regulators of successful recruitment of EPCs to the injury site [110][111].

**Figure 8: A schematic representation of EPC participation in new blood vessels formation.** *Adapted from Kawamoto and Asahara* [90].

Angiogenesis occurs in two steps, including the activation and the resolution phases. The activation phase is characterized by disruption of basement membrane, differentiation of EC into tip and stalk cells and elongation, migration and proliferation of EC. The resolution phase
is described as a maturation of the vessel wall, involving ceasing of proliferation of EC, recruitment of pericytes and vascular smooth muscle cells [112].

### 1.3.2 EPC Function in Cardiovascular Disease and in Testosterone Deficient Men

EPC numbers are an important marker of vascular health. Since these cells actively participate in the repair of vascular injury, it is essential to maintain sufficient numbers of functional EPCs. Hill et al. demonstrated that the number of EPCs in the peripheral blood was inversely correlated with a degree of cardiovascular risk factors in men. Moreover, EPCs isolated from subjects with higher risk for CVD underwent senescence more rapidly than those from men with low risk factors [113]. Another study showed that patients with coronary artery disease (CAD) had lower number of circulating EPCs compared to healthy subjects. In addition, the migratory capacity of EPCs was impaired in men with CAD, indicating an association between the function of EPCs and cardiovascular health [114]. Hence, the preservation of a healthy pool of EPCs plays an important role in preventing CVD [115].

Men with Klinefelter’s syndrome (47, XXY) are characterized by low testosterone levels, cardiac valve alterations, obesity, and they display increased mortality from CVD [116]. Recently, Di Mambro et al. investigated the levels of circulating EPCs in men with Klinefelter’s syndrome (KS). Interestingly, KS patients had decreased numbers of EPCs compared to healthy subjects [117]. However, it is not evident that lower levels of EPCs are solely due to hypogonadism in men with KS. A correlation between low circulating EPCs and hypogonadism has been demonstrated in young hypogonadotrophic hypogonadal (HH) men without any cardiovascular risk factors [118]. Moreover, a beneficial role of testosterone in EPC maintenance was observed after T replacement therapy in HH patients, resulting in increased numbers of EPCs, most probably through augmented EPC mobilization from bone marrow [118][119]. These findings support the idea that low testosterone levels have a deleterious impact on men’s health.

Promising outcomes of in vivo studies using EPCs in cardiovascular regeneration has permitted their potential use for clinical applications. Indeed, EPC infusion into patients with cardiovascular disease has been shown to significantly reduce symptoms associated with CVD. For example, intramyocardial injection of bone marrow BM-AC133+ into patients with chronic MI and chronic CAD resulted in better tissue perfusion [120][121]. Similar results were obtained in patients with recent or acute MI following intracoronary injection with EPCs [122][123].
Taken together, these findings highlight the importance of EPCs in cardiovascular repair, particularly in hypogonadal men, and support the therapeutic role of EPCs in treating CVD in future.

### 1.3.3 Endothelial Barrier Function

The maintenance of vascular homeostasis is indispensable to prevent vascular pathologies (diabetic retinopathy, pulmonary disorders, bowel inflammation, and stroke). One of the functions of the endothelium is a serving as a barrier between circulating blood and underlying tissue. Complications associated with the loss of endothelial integrity represent a serious danger to organ vitality, which may subsequently be life-threatening. For example, development of edema during cerebral stroke is due to an altered integrity and functionality of the endothelium in the brain [124]. Leaky blood vessels, impaired tissue perfusion and hemorrhages are other consequences of a defective endothelial monolayer [125][126].

Selective vascular permeability in epithelial and endothelial cells is regulated by paracellular and transcellular mechanisms. Transcellular mechanisms allow the transport of molecules across the cytoplasm through channels, vesicles and pumps [127]. In contrast, paracellular permeability allows the selective flux of ions, molecules, solutes and water passing between the neighboring cells. The permeability is mediated by intercellular junction complexes, which include adherens junctions (AJ), tight junctions (TJs), gap junctions and desmosomes [128]. In the present work, we focused on the role of tight junctions in EPC monolayers [125][129][130].

TJ structure resembles fibril-like strands as documented by electron microscopy [131]. TJs are present throughout the vascular tree, but highly concentrated in the blood-brain-barrier (BBB) and the blood-retinal-barrier (BRB), where the permeability is tightly regulated [125]. TJs consist of occludin, the claudin family and junctional adhesion molecule-A (JAM-A) [127][129][130].

The claudin family accounts for more than 20 proteins (claudin1-24), of which claudin3/5 appear to have the most prominent role in EC, particularly in the brain microcirculation [124][132][133]. Claudins are small proteins with a molecular mass of 20-25 kDa, containing four transmembrane domains, two extracellular loops and cytoplasmic C- and N- termini. Contact between extracellular loops of the neighboring cells provides cell-cell interactions, whereas an association of the carboxyl terminal region with the cytoplasmic proteins Zone Occludens (ZO-1, ZO-2 and ZO-3) stabilizes TJ strands [134][135]. The role of claudins in
the barrier function has been extensively investigated. Many members of the claudin family have been found to contribute to TJ assembly and to the regulation of the permeability in the epithelial tissues [132]. However, the important role in regulating the permeability of endothelial cells is attributed to claudin5. An in vivo study showed that claudin5 knockout mice elicit an increased leakage of the BBB, suggesting a key role of claudin5 in regulating the barrier function in the microcirculation of the BBB [136].

Occludin was the first TJ protein discovered by Furuse et al. in 1993 [137]. Occludin is a 550 amino acid protein, detected by antibodies at approximately 65 kDa. Similar to the structure of claudins, occludin contains four transmembrane domains, two extracellular loops forming zipper-like structure and serving to seal adjacent cells, and cytoplasmic C- and N-terminal regions. The C-terminal end of occludin is anchored to ZO-1 in the cytoplasm [138], necessary for occludin localization in TJ and its stability. ZO-1 mediates the binding of occludin to cytoskeleton F-actin [139]. In spite of the similar structures of occludin and claudins, they do not resemble in sequence, which might be a possible explanation for their functional differences. As various in vitro studies point out, reduction in occludin expression in endothelial cells has been linked to increased permeability [140][141][142][143][144]. Interestingly, in occludin knockout mice, TJ assembly was morphologically unaltered, however, numerous pathologies have been observed in these mice such as male infertility, testis atrophy, chronic gastritis and other epithelial abnormalities, suggesting a role of occludin in the regulation of the barrier function instead of its role in TJ assembly [145].

TJs are dynamic structures associated with the cytoskeleton and have a capacity of quick adaptation to a changing environment such as shear stress or other alterations in the circulation [124]. Therefore, TJ proteins undergo constant rearrangements to provide structural flexibility, mediated by internalization or changing localization within TJ [146][147]. Important regulators of TJ assembly, maintenance and degradation are posttranslational modifications [126][148]. Proteolytic degradation by matrix metalloproteinases (MMP) has been shown to modulate the occludin level in the endothelium. Several in vivo studies demonstrated MMP 2/9-mediated proteolysis of occludin in the brain during the BBB disruption [149][150][151][2][152][153]. The phosphorylation of the C-terminal region of TJ proteins results in enhanced permeability due to the cellular localization of TJ, reduced interaction with ZO-1 or TJ degradation. Numerous kinases have been described to regulate TJ function: protein kinase C [154], MAPK [155], Rho Kinase [156][157], CK1 and CK2 [158][159] and cAMP [160][161][162]. Finally, VEGF is a key
regulator of the endothelial function and increases vascular permeability by disrupting the TJs [163][164][165].

1.4 The Molecular Mechanisms Regulating EC Function

1.4.1 The Akt/mTOR/VEGF Signaling in Angiogenesis

Mammalian target of rapamycin (mTOR) regulates important cellular processes. mTOR belongs to the phosphoinositide 3-kinase related kinase (PIKK) family of serine/threonine kinases [166]. There are two complexes of mTOR: mTOR complex1 (mTORC1) and mTORC2. mTORC1 consists of mTOR interacting with a protein Raptor and the inhibitory proteins FKBP38 and PRAS40 [167][168][169]. The N-terminus of mTOR contains HEAT repeats (Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A, TOR1), which mediate binding to the Raptor [170], whereas the FRB (FKBP12-rapamycin binding) domain is responsible for binding its inhibitors rapamycin [171] and FKBP38 [172].

Upstream regulation of mTOR involves the modulation of the inhibitory Tuberous Sclerosis Complex (TSC) TSC1-TSC2 complex [173][174] and mTOR activator Ras homolog enriched in brain (Rheb) [175][176][177]. TSC2 has a GTPase activating protein (GAP) activity towards the small GTPase Rheb. Moreover, Akt phosphorylates TSC2, leading to the dissociation of the TSC1-TSC2 complex and to the activation of Rheb (through its binding to GTP), ultimately resulting in the activation of mTOR [178].

Downstream substrates of mTORC1 are 4E-binding protein1 (4E-BP1) [179], a translation repressor, and p70 S6 kinase (S6K), an activator of protein synthesis [180][181]. The phosphorylation of 4E-BP1 by mTOR results in the inactivation of a translation inhibitor and therefore promotes protein synthesis and cell growth. Hypoxia, mitogenic stimuli, cellular nutritional and energetic states have been shown to regulate the mTOR activity [182].

mTORC2 consists of the proteins Rictor [183][184], mSin1 and PRR5 and regulates the cytoskeleton organization [185]. mTORC2 has been shown to be insensitive to rapamycin upon short exposure [185]. However, a persisting presence of rapamycin inactivates mTORC2 [186][187]. An interaction of mTORC2 with TSC1-TSC2 has been also observed [188]. Despite certain similarities between mTOR complexes, the exact role of mTORC2 is still unclear and under investigation.

An important regulator of mTOR signaling is the phosphoinositol-3kinase (PI3K)/Akt pathway. The PI3K/Akt mechanism is a key regulator of numerous cellular processes such as
cell proliferation, survival, migration, and neovascularization. PI3K belongs to a class of enzymes phosphorylating the inositol ring of phosphatidylinositol, thereby converting phosphatidylinositol-4,5-biphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3) [189][190]. In turn, PIP3 induces the recruitment of serine/threonine kinase Akt to the plasma membrane and its subsequent activation by phosphorylating at S473 and T308 by phosphoinositide dependent kinase-1 (PDK1) [191][192]. Many studies have confirmed the central role of the Akt/mTOR/VEGF pathway in regulating the essential function of EC -angiogenesis [193][194][195][196][197]. The anti-angiogenic activity of the new PI3K/Akt inhibitors revealed new possibilities for their clinical applications in cancer. Several studies demonstrated a decrease in tumor-associated angiogenesis in tumor xenograft models using a pan PI3K inhibitor (against all classes of PI3K, improved LY294002) PI103 [198], PI3K class I inhibitor SF1126 [199] and ZSTK474 [200]. Treatment with a combined inhibitor for Akt and mTOR, developed by Novartis (NVP-BEZ235), blocked VEGF-induced angiogenesis in vivo (in agar chambers implanted in mice) [201] and showed anti-carcinogenic properties [202]. Additionally, specific inhibition of mTOR with rapamycin reduced VEGF production in human prostate cancer cells [194] and abrogated the angiogenic effects of VEGF and the tumor growth in vivo [196]. Moreover, in mice which constantly express Akt (myrAKT1), rapamycin inhibited angiogenesis [195]. Interestingly, OSI-021 and OXA-01, the dual inhibitors for mTORC1 and mTORC2, had greater inhibitory effects on angiogenesis and tumor growth as compared to rapamycin alone [193].

1.4.2 TGFβ/BMP Signaling and Its Role in Endothelial Cell Function

The transforming growth factor β (TGFβ) superfamily of cytokines includes TGFβ1,-2,-3, bone morphogenetic proteins (BMPs) subfamily, growth differentiation factors (GDF) and activins. The members of the TGFβ family have an impact on various cellular processes by regulating cell growth, apoptosis, differentiation and migration [203][204]. There is an increasing evidence which suggests an important role of the TGFβ family in angiogenesis [205][206].

Two types of receptors can be distinguished, which mediate the TGFβ signaling: type I and type II receptors, both owing serine/threonine kinase activity. Upon ligand binding, type II receptors form a heteromeric complex with type I receptors. This results in the phosphorylation and the activation of type I receptors (Figure 9), which in turn phosphorylate
the transcription factor receptors-Smads (R-Smads). R-Smads bind to the common Smad4 and translocate to the nucleus to trigger the transcription of the target genes [207][208]. R-Smads include Smad1/5/8 and Smad2/3, which are phosphorylated by different type I receptors and lead to distinct signaling pathways. Activity of R-Smads as well as TGFβ family receptors are also regulated by the inhibitory Smad6 and Smad7 (I-Smad), which induce the proteasomal degradation of R-Smads and hence prevent R-Smad signaling [209][210].

![Diagram of TGFβ signaling](image)

**Figure 9:** The activation of TGFβ signaling and its downstream effectors. TGFβ, Transforming growth factor β; BMP, bone morphogenetic protein; TβRI, TGFβ receptor I; TβRII, TGFβ receptor II, BMPR2, BMP receptor 2; ALK, activin receptor like kinase; Id-1, inhibitor of differentiation-1; PAI-1, plasminogen activator inhibitor type-1.

TGFβ requires to be cleaved in order to become active [212]. It binds to the TGFβ type II receptor (TβRII) and to the TGFβ type I receptors called activin receptor like kinase 1 and 5 (ALK1 and ALK5). ALK1 and ALK5 activation results in the phosphorylation of the downstream Smad1/5/8 and Smad2/3, respectively [203][204]. In contrast, BMPs are secreted
in an active form [211][213] and bind to the BMP type II receptors (BMPRII) [214] and to the activin type II receptors (ACVRII). This binding allows the complex formation with type I receptors such as ALK2, ALK3 (BMPRIA) and ALK6 (BMPRIB) [215][216]. In addition, recent findings show that BMP9 and BMP10 activate ALK1, although it is known as a receptor for TGFβ [217].

ALK1 phosphorylates Smad1/5/8, which up-regulate the expression of the target gene inhibitor of differentiation-1 (Id-1). In contrast, ALK5 specifically activates Smad2/3 resulting in the stimulation of the downstream plasminogen activator inhibitor type 1 (PAI-1) [207]. Accessory receptors (also named type III receptors) such as Endoglin have been shown to potentiate the activation of ALK1 signaling [217][205][218][206]. Interestingly, TGFβ family members were found to regulate the cellular processes independently of recruiting Smads. Non-Smad signaling is mediated by MAPK, ERK1/2, JNK/p38, or PI3K/Akt [219][220][221].

TGFβ signaling is required for the embryonic development, since numerous in vivo studies have shown an early lethality in embryos which were defective in the components of the TGFβ pathway. The importance of the ALK1/Smad1/5/8 pathway in vascular development has been reported. Smad1 mutant mice die during the development due to allantois defects and lack of the placenta formation [222]. Similar findings were obtained with Smad5 knockout mice, which died during embryogenesis [223]. Smad4 mutant mouse embryos died due to cardiovascular defects [224].

TGFβ signaling has been intensively investigated with regard to its function in the endothelial cells in adult tissues. Therefore, many studies describe the effects of the TGFβ family members on angiogenesis. The role of the accessory receptor Endoglin in regulating in vivo angiogenesis has been well documented [205][206][217][218][225]. Additionally, the pro-angiogenic effects of the Endoglin/ALK1/Smad1/5/8 pathway have been observed in response to the hypoxia following myocardial infarction (MI) in a mouse model [226]. Moreover, the downstream targets of Smad1/5/8 the Id1 and Id3 have been implicated in tumor-associated angiogenesis [227]. Also, patients with hereditary hemorrhagic telangiectasia (HHT), characterized by the loss-of-function mutation in ALK1, have dilated leaky vessels with impaired endothelial integrity. Approximately 85% of the patients with HHT have mutations in Endoglin and ALK1 genes [206][229][230][231][232][233]. Smad4 mutation was identified in patients with juvenile polyposis-HHT syndrome and with HHT [234][235]. Interestingly, pulmonary arterial hypertension (PAH), which is also characterized by endothelial dysfunction, is associated with the mutations in ALK1 signaling. For example,
ALK1+/- mice developed pulmonary hypertension (PH) [236]. Moreover, mutations in ALK1-BMPRII were found in patients with PAH [237]. Smad8 mutation was observed in one patient with PAH, and the functional relevance was demonstrated in an in vivo mouse study [238][239].

Contradictory findings regarding ALK1-mediated signaling in angiogenesis also exist. For example, Larrivée et al. demonstrated that ALK1 inhibition results in the retinal hypervascularization in vivo, indicating the anti-angiogenic effects of ALK1 signaling [228]. However, most of the studies emphasize a pivotal role of ALK1 signaling in maintaining EC function and vascular health.

Growing evidence suggests an important role of BMPs in regulating EC function and in vascular pathologies [240][241][242]. BMPs were discovered as regulators of bone formation [243]. The effects of BMP2/4 and BMP9/10 have been mostly demonstrated in endothelial cells [244]. ALK3, ALK6 are the primary type I receptors for BMP2 and BMP4, whereas BMP9 and BMP10 bind to the same receptor as TGFβ – ALK1.

In this regard, numerous studies showed the role of ALK1 in tumor-associated angiogenesis upon BMP treatment [205][245][246][247]. Inhibition of the BMP9/10/ALK1/Id1 axis results in reduced angiogenesis [248] and BMP2 stimulates angiogenesis in HUVECs [249]. However, the inhibitory effects of BMP-activated ALK1 signaling have also been reported. For example, the overexpression of BMP4 reduced the neovascularization in the eye [250]. The anti-angiogenic effects of BMP9 have been demonstrated in vivo in chicken chorioallantoic membrane assay and in mouse sponge angiogenesis assay [251] and in vitro in bovine artery endothelial cells (BAEC) [252].

The complexity of TGFβ signaling arises from the diversity of ligands and receptors and their various possible combinations as well as the context- and cell type-specific signaling outcomes.

Additionally, the TGFβ/BMP signaling has also been shown to regulate the endothelial barrier function. Indeed, the TGFβ1/ALK5/Smad4 axis is implicated in increased permeability of pulmonary endothelial cells [256]. However, another study demonstrated an increased leakage in the pulmonary endothelium in the absence of BMPRII in human pulmonary artery endothelial cells and in a mouse BMPRII knockout model [257]. Interestingly, the BMP2/Smad1/5/Id-1 pathway has been shown to negatively affect the barrier function of the lung epithelium [258].
1.5 The Role of Androgens in SMC Function and Their Contribution to Cardiovascular Disease

As discussed above, the role of androgens in the cardiovascular system of men is not completely unveiled and currently under debate. The deleterious effects of androgens on the cardiovascular system may arise from their actions on vascular smooth muscle cells. Abnormal growth of smooth muscle cells plays a critical role in the etiology of vaso-occlusive disorders associated with coronary artery disease, atherosclerosis and hypertension. In this regard, several in vitro studies have demonstrated that T and DHT augmented migration and proliferation of rat and human vascular smooth muscle cells [76][77][78][79]. These findings suggest that androgens might promote media-intima thickness of the vascular wall and exert atherogenic effects.

The effects of androgens on cardiac function and morphology have also been studied. Androgens induced cardiac hypertrophy and fibrosis, associated with heart failure in guanylyl cyclase-A deficient mice [259]. In another study in mice, the effects of estrogens and testosterone on cardiac function following MI were compared. In contrast to estrogens, androgens stimulated myocardial hypertrophy, deteriorated cardiac recovery and promoted cardiac rupture after MI. All these effects were reversed by castration [80]. These results provide the evidence for a negative role of androgens in the cardiovascular homeostasis.
2 General Hypothesis and Objectives

Numerous studies (clinical and observational) have reported the contradictory effects of androgens in cardiovascular health of men. Therefore, the main aim of this study was to elucidate the role of androgens in the cardiovascular system. To address this question, we investigated the effects of DHT on vascular remodeling processes associated with cardiovascular disease (CVD), which are characterized by pathological growth of vascular smooth muscle cells and endothelial dysfunction.

The link between testosterone deficiency and decreased numbers of circulating EPCs, a hallmark of cardiovascular disorders, has been demonstrated in hypogonadal patients [118][119]. Since the contribution of endothelial progenitor cells (EPCs) to cardiovascular repair has been well documented, we examined the role of androgens in regulating EPC function. In our study, we used dehydrotestosterone (DHT) instead of testosterone (T) due to its inability to be converted to estrogens. We hypothesize that DHT might affect the function of EPCs and thereby contribute to the tissue regeneration following vascular injury. Hence, our first goal was to investigate the effects of DHT on vasculogenesis in EPCs. Because most of the biological actions of DHT are mediated by androgen receptor (AR), we assessed the role of AR in the modulatory effects of DHT on EPC function.

The second goal of our study was to investigate the mechanisms mediating DHT-induced effects on EPC function. Because many studies reported a central role of the Akt/mTOR/VEGF pathway in regulating endothelial function, we examined whether activation of this signaling pathway is also responsible for the actions of DHT in EPCs. Furthermore, we assessed the interplay between AR and the Akt signaling in EPCs upon DHT treatment.

TGFβ signaling regulates various aspects of endothelial cells (EC), however, not much is known about its role in EPCs. Hence, we examined whether DHT regulates EPC function by activating TGFβ signaling. We hypothesize that the non-genomic actions of DHT are involved in this mechanism. Moreover, with regard to TGFβ signaling, the barrier function of EPCs was examined. The barrier dysfunction is characterized by increased vascular permeability and is associated with impaired endothelial integrity and several vascular disorders. Therefore, we postulate that DHT might modulate vascular health by regulating the barrier function of EPCs.
The third aim of the present study was to evaluate AR regulation by DHT in EPCs. Because AR activation is involved in the regulation of EPC function by DHT, we investigated whether transcription, translation or ubiquitin-mediated proteasomal degradation are implicated in AR regulation by DHT. Since vascular remodeling processes involve excessive smooth muscle cell growth, hence, to establish whether DHT has the positive or deleterious effects on the cardiovascular system, we studied how DHT regulates the proliferation of male aortic smooth muscle cells. Moreover, we examined the DHT effects on the expression of cell cycle regulators. Taken together, our study was designed to clarify the role of androgens in vascular remodeling processes associated with cardiovascular disease. Our findings will help us understand the benefits and drawbacks of testosterone replacement therapy in elderly men.
3 Materials

3.1 Cell Culture

Antibioticum-Antimycotium Gibco BRL, Paisley, UK (15240-096)
Collagen (rat tail) Roche Diagnostics, Mannheim, DE (11179179001)
DMEM/F12 Gibco BRL, Paisley, UK (21331-020)
EBM-2 Basal Medium Lonza, Walkersville, USA (CC-3156)
EGM-2 SingleQuots Lonza, Walkersville, USA (CC-4176)
FCS Charcoal Stripped Thermoscientific, Waltham, MA, USA (SH30068.03)
FCS Thermoscientific, Waltham, MA, USA (SH30070)
HBSS (without Ca²⁺ and Mg²⁺) Biocentec, Allschwill, CH (3-02K34-I)
L-Glutamine Life Technologies, Carlsbad, CA, USA (25030-024)
LSGS Gibco BRL, Paisley, UK (S003-10)
PBS tablets Gibco BRL, Paisley, UK (18912-014)
SMGS Cascade Biologics, Inc, USA (S-007-25)
Trypsin Sigma Aldrich, Buchs, CH (T-3924)

AoSMC (male) Lonza, Walkersville, USA (CC-2571)
AoSMC (female) Cascade Biologics, Inc, USA (C-007-5C)
ECFC (female) Lonza, Walkersville, USA (00189423)
HCF Cell Applications, Inc., San Diego, CA, USA (306-05f)
HUVEC Lonza, Walkersville, USA (CC-2517)

Cell culture flasks, dishes, plates TPP, Trasadingen, CH
Cryotubes Nalge Nunc International, USA (377224)
Reaction tubes (1.5ml), tips Eppendorf, Hamburg, DE
Transwell-COL permeable inserts Corning, Amsterdam, NLD (3496)
### Materials

#### 3.2 Antibodies and Peptides

**Primary Antibodies**

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Vendor/Location</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β-actin</td>
<td>Sigma, St. Louis, USA</td>
<td>A5441</td>
</tr>
<tr>
<td>Anti-AKL5</td>
<td>GeneTex, Taiwan, R.O.C.</td>
<td>GTX102784</td>
</tr>
<tr>
<td>Anti-Akt</td>
<td>Cell Signaling Technology, MA, USA</td>
<td>9272</td>
</tr>
<tr>
<td>Anti-ALK1</td>
<td>Santa Cruz, Dallas, Texas, USA</td>
<td>sc-28976</td>
</tr>
<tr>
<td>Anti-AR</td>
<td>Cell Signaling Technology, MA, USA</td>
<td>3202</td>
</tr>
<tr>
<td>Anti-Claudin-5</td>
<td>Millipore, Temecula, CA</td>
<td>ABT45</td>
</tr>
<tr>
<td>Anti-Cyclin D1</td>
<td>Millipore, Temecula, CA</td>
<td>06-137</td>
</tr>
<tr>
<td>Anti-Endoglin</td>
<td>Millipore, Temecula, CA</td>
<td>05-1424</td>
</tr>
<tr>
<td>Anti-Id1</td>
<td>Abcam, Cambridge, UK</td>
<td>ab66495</td>
</tr>
<tr>
<td>Anti-mTOR</td>
<td>Cell Signaling Technology, MA, USA</td>
<td>2972S</td>
</tr>
<tr>
<td>Anti-Occludin</td>
<td>Millipore, Temecula, CA</td>
<td>ABT146</td>
</tr>
<tr>
<td>Anti-Phospho-Akt (Ser473)</td>
<td>Cell Signaling Technology, MA, USA</td>
<td>9271</td>
</tr>
<tr>
<td>Anti-Phospho-mTOR (Ser2481)</td>
<td>Cell Signaling Technology, MA, USA</td>
<td>2974</td>
</tr>
<tr>
<td>Anti-Phospho-Smad1/5/8</td>
<td>Millipore, Temecula, CA</td>
<td>AB3848</td>
</tr>
<tr>
<td>Anti-Phospho-Smad2</td>
<td>Millipore, Temecula, CA</td>
<td>AB3849</td>
</tr>
<tr>
<td>Anti-Phospho-Smad3</td>
<td>Millipore, Temecula, CA</td>
<td>07-1389</td>
</tr>
<tr>
<td>Anti-Raf1</td>
<td>Cell Signaling, Technology, MA, USA</td>
<td>9422</td>
</tr>
<tr>
<td>Anti-Smad1/5/8</td>
<td>Abcam, Cambridge, UK</td>
<td>ab13723</td>
</tr>
<tr>
<td>Anti-Smad2/3</td>
<td>Millipore, Temecula, CA</td>
<td>07-408</td>
</tr>
</tbody>
</table>

**Secondary Antibodies**

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Vendor/Location</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-Mouse IGF-Peroxidase Conj.</td>
<td>Pierce, Rockford, USA</td>
<td>31430</td>
</tr>
<tr>
<td>Goat anti-Rabbit IGF-Peroxidase Conj.</td>
<td>Pierce, Rockford, USA</td>
<td>31460</td>
</tr>
<tr>
<td>IRDye 680 Conj. Goat anti-Mouse IgG</td>
<td>LI-COR, Nebraska, USA</td>
<td>926-32220</td>
</tr>
<tr>
<td>IRDye 680 Conj. Goat anti-Rabbit IgG</td>
<td>LI-COR, Nebraska, USA</td>
<td>926-32221</td>
</tr>
<tr>
<td>IRDye 800 Conj. Goat anti-Mouse IgG</td>
<td>LI-COR, Nebraska, USA</td>
<td>926-32210</td>
</tr>
<tr>
<td>IRDye 800 Conj. Goat anti-Rabbit IgG</td>
<td>LI-COR, Nebraska, USA</td>
<td>926-32211</td>
</tr>
</tbody>
</table>
Peptides

- Human VEGF antibody
  - R&D Systems, Minneapolis, USA (MAB293)
- Recombinant Human ALK1 Fc Chimera
  - R&D Systems, Minneapolis, USA (370-AL-100)
- Recombinant Human BMP9
  - R&D Systems, Minneapolis, USA (3209-BP)
- Recombinant Human TGFβ
  - R&D Systems, Minneapolis, USA (240-B)

3.3 Chemicals and Buffers

<table>
<thead>
<tr>
<th>Chemical/Buffer</th>
<th>Supplier, City, Country (Catalog)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M Tris/HCl pH 6.8</td>
<td>Biorad, Reinach, CH (161-0799)</td>
</tr>
<tr>
<td>1.5M Tris/HCl pH 8.8</td>
<td>Biorad, Reinach, CH (161-0799)</td>
</tr>
<tr>
<td>10x Tris/Glycine Buffer</td>
<td>Bio-Rad, Reinach, CH (161-0771)</td>
</tr>
<tr>
<td>10x Tris/Glycine/SDS Buffer</td>
<td>Bio-Rad, Reinach, CH (161-0772)</td>
</tr>
<tr>
<td>5x siRNA Buffer</td>
<td>Dharmacon, Lafayette, USA (B-002000-UB-100)</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>Calbiochem, San Diego, USA (114666)</td>
</tr>
<tr>
<td>Ammonium Persulfate (APS)</td>
<td>Sigma, St. Louis, USA (A6761)</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Sigma, St. Louis, USA (A-1153)</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>Sigma Aldrich, Buchs, CH (A1076)</td>
</tr>
<tr>
<td>BCA Protein Assay Kit</td>
<td>Pierce, USA (23227)</td>
</tr>
<tr>
<td>BD Matrigel</td>
<td>BD Biosciences, Franklin Lakes, USA (356237)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma, St. Louis, USA (A-3059)</td>
</tr>
<tr>
<td>Cell Lysis Buffer</td>
<td>Cell Signaling, Danvers, USA (9803)</td>
</tr>
<tr>
<td>CellTracker™ Green CMFDA</td>
<td>Molecular Probes, Eugene, OR, USA (C7025)</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Sigma, St. Louis, USA (PS1002)</td>
</tr>
<tr>
<td>Cytodex beads 3</td>
<td>GE Healthcare, Uppsala, Sweden (17-0485-01)</td>
</tr>
<tr>
<td>Dehydrotestosterone</td>
<td>Sigma Aldrich, Buchs, CH (10300)</td>
</tr>
<tr>
<td>DharmaFECT 4</td>
<td>Fischer Scientific, Wohlen, CH (T-2004-03)</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>Sigma Aldrich, Buchs, CH (41640)</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>Fermantas, Hanover, MD, USA (R0891)</td>
</tr>
<tr>
<td>EGTA</td>
<td>Sigma Aldrich, Buchs, CH (E3889)</td>
</tr>
<tr>
<td>Ethanol 70%</td>
<td>Kantonspothekie Zurich</td>
</tr>
<tr>
<td>Fibrinogen Type I</td>
<td>Sigma Aldrich, Buchs, CH (F0895)</td>
</tr>
<tr>
<td>Fluo-4, AM</td>
<td>Molecular Probes, Eugene, OR, USA (F14201)</td>
</tr>
</tbody>
</table>
Materials

Flutamide Sigma, St. Louis, USA (F9397)
Hyperfilm ECL Amersham, Dübendorf, CH (RPN2103K)
Ionomycin Sigma Aldrich, Buchs, CH (19657)
Loading Butter (5x) Fermentas, Burlington, Canada (R0891)
LY0294002 Calbiochem, Merck, Darmstadt, D (440202)
Methanol Sigma Aldrich, Buchs, CH (32213)
MG132 Calbiochem, San Diego, USA (1474790)
Noggin R&D Systems, Minneapolis, MN, USA (6057-NG-025)
Pertussis Toxin Sigma Aldrich, Buchs, CH (P7208)
PMSF Sigma, St. Louis, USA (P-7626)
Ponceau S Solution (2%) Sigma, St. Louis, USA (P-7767)
Precision Plus Dual Color Standard Biorad, Reinach CH (161-0374)
Rapamycin Millipore, Temecula, CA (553210)
Rotiphorese Gel 30 Carl Roth GmbH, Karlsruhe, D (3029.1)
SB525334 Tocris, Ballwin, MO, USA (3211)
SJN2511 Tocris, Ballwin, MO, USA (3211)
Sodium Chloride (NaCl) Sigma Aldrich, Buchs, CH (71381)
Sodium Dodecyl Sulfate (SDS) Sigma, St. Louis, USA (L-5750)
Sodium Hydroxyde (NaOH) Sigma, St. Louis, USA (S-8045)
SuperSignal West Dura Pierce, Rockford, USA (34075)
SuperSignal West Pico Pierce, Rockford, USA (34080)
Temed Sigma, St. Louis, USA (T-9281)
Thrombin Sigma, St. Louis, USA (T-4648)
Tween 20 Sigma, St. Louis, USA (P-7949)
U73122 Sigma, Buchs, CH (U6756)

siRNA transfection

On-TARGETplus non-targeting Pool Thermo Scientific Dharmacon, Lafayette, USA (D-001810-10-20)
(UGGUUUACUGUGACUA,
UGGUUUACUGUGUGUGA,
UGGUUUACUGUUUCUGA,
UGGUUUACUGUUUCCUA)
Materials

On-TARGETplus SMARTpool Smad1 Thermo Scientific Dharmaco, Lafayette, USA
(L-012723-00-0005)
(GCUCAUGUCUCUACUAUGA,
GCGGUUGCUUAUGAGA,
CAACAAUCGUGUGGGUGAA,
CAAAUGGGUUCACCUA)

On-TARGETplus SMARTpool AR Thermo Scientific Dharmaco, Lafayette, USA
(L-003400-00-0005)

3.4 Instruments and Software

Western Blotting:

Mini-PROTEAN 3 Electrophoresis Cell Biorad, Reinach, CH
Mini-Trans-Blot Electrophoretic Transfer Cell Biorad, Reinach, CH
Power Pac 200, Power Supplier Biorad, Reinach, CH

Instruments:

Coulter Z1, Cell Counter Coulter Electronics, Luton, UK
LI-COR Odyssey Infrared Imaging System LI-COR, Nebraska, USA
Olympus CKX41 Microscope Olympus, Volketswil, CH
Olympus IX81 Olympus, Volketswil, CH
7500 Fast Real-Time PCR system Life Technologies, Darmstadt, D
Tecan Infinite series M200 Tecan, Salzburg, Austria

Software:

Magellan 6 Tecan, Salzburg, Austria
Odyssey 3.0 LI-COR, Nebraska, USA
Scion Image Scion Corporation, Frederick, USA
StatView, Version 5.0.1 SAS Institute, Cary, USA
Xcellence pro Olympus, Volketswil, CH
4 Methods

4.1 Cell Culture

**EPCs (ECFC):** Human Endothelial Progenitor Cells (Endothelial Colony Forming Cells) of female origin from umbilical cord blood were obtained from Lonza (Walkersville, USA). EPCs at 4-8 passages were used for the experiments.

Sub-culturing: Prior to seeding the cells, culture flasks were coated with rat tail collagen (Roche; Mannheim, Germany) at 50 μg/ml diluted in 0.02N acetic acid. After 1 hour of incubation at RT, collagen was removed and the surface washed with PBS before placing a fresh medium. EPCs were grown in 5% CO₂ in humidified air at 37°C in 75cm² (T75) flasks (Techno Plastic Products, Trasadingen, Switzerland) and were cultured in Endothelial Basal Medium-2 (EBM-2) (Lonza), supplemented with 10% FCS (HyClone, ThermoFischer) and EGM-2 SingleQuots (Lonza, CC-4176), containing hydrocortisone, human epidermal growth factor (hEGF), fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF-B), human recombinant insulin-like growth factor (R3-IGF-1), ascorbic acid, heparin and gentamicin/amphotericin-B. For treatments, EBM (Lonza) without phenol red and growth factors was used. When required, EPCs were starved for 24 hours in EBM containing 0.4% FCS and with the growth factors. Upon 80% confluence, the cells were washed with HBSS (without Ca²⁺ and Mg²⁺) and detached using 0.25% trypsin-HBSS for 5 minutes at 37°C. After neutralization in an equal amount of the complete medium, the cells were centrifuged at 1200rpm for 5 minutes.

Defrosting: A vial was kept in a water bath at 37°C for several seconds, and the cells were thawed completely by adding warm complete medium into the vial and then were transferred into T75 flask.

Cryopreservation: The cells were trypsinized and centrifuged at 1200 rcf for 5 minutes, following resuspension of the pellet in the solution consisting of 80% complete medium, 10% DMSO and 10% FCS. The suspension was aliquoted in the cryopreservation vials and stored at -80°C for several days and then transferred into liquid nitrogen for a long term storage.

**HUVECs:** Human Umbilical Vein Endothelial Cells were obtained from Lonza (Walkersville, USA). The cells were cultured in DMEM/F12 (Sigma) supplemented with Low Serum Growth Supplements, containing fetal bovine serum, hydrocortisone, hEGF, hFGF,
heparin and gentamicin/amphotericin solution (Cascade Biologics, Gibco, Carlsbad, USA) and additional 10% FCS. HUVECs were used at 4-9 passages for the experiments. For starvation conditions and treatments, DMEM/F12 containing 1% BSA without supplements was used.

**HCFs:** Human Cardiac Fibroblasts (Cell Applications, Inc., San Diego, USA) were cultured in DMEM/F12 containing 10% FCS. HCFs were used for the co-culture experiments with EPCs and for this purpose were plated in 24-well plates and grown till confluence.

**AoSMCs:** Aortic Smooth Muscle Cells were acquired from male donor (Lonza, Walkersville, USA). AoSMCs were grown in Smooth muscle Basal Medium (SmBM™) containing SmGM™-2 SingleQuots (hEGF, insulin, hFGF-B, FBS and gentamicin/amphotericin-B).

### 4.2 Growth Studies

Growth studies were conducted in AoSMCs starved for 24 hours. AoSMCs were seeded in 24-well plates in the basal medium containing 0.4% FCS and without supplements and exposed to various agents. To study the inhibitory effects of some treatments on the growth of AoSMCs, 20 ng/ml platelet-derived growth factor (PDGF) was also added to the cells. After 3-4 days the cells were loosen by trypsinization and counted with Coulter Counter.

### 4.3 RT-PCR

To perform quantitative PCR total RNA was extracted from EPCs, which were grown under different conditions in 6 cm dishes. For this purpose, the RNeasy Mini Kit (Qiagen) was used. First, cell membranes and content were dissolved in 350 μl lysis buffer. Then the collected lysates were passed through the columns to filter the cell debris; the resulting solutions were mixed with 70% ethanol and applied to RNA-binding columns. Next, DNA was digested with DNases followed by thorough washing steps. Finally the purified RNA was eluted twice to increase the total concentration. Obtained RNA could be stored at -80°C or immediately processed to the reverse transcription (RT).
The reverse transcription of RNA into cDNA was performed using High Capacity cDNA Reverse Transcription kit (Life Technologies) consisting of 10x RT Buffer, 10x RT Random Primers, 25x dNTP Mix and MultiScribe Reverse Transcriptase and RNase inhibitor, which was applied to 10 μl of RNA, followed by incubation at RT for 10 minutes, then at 37°C during 2 hours and finally for 5 minutes at 85°C. Transcribed cDNA was then diluted accordingly to the necessary amount for experiments (1:4 in sterile water). cDNA was stored at -20°C.

Taqman gene expression assays were used, which are composed of appropriate primers diluted in fast master mix (Life Technologies). The following primers were used: eukaryotic GAPDH, AR, β-actin. Master Mix (5 μl/well) and sample 2 μl/well) were added in triplicates into the 384-well MicroAmp optical microplate (Applied Biosystems). RT-PCR was performed with the 7500 Fast system Instrument (Applied Biosystems). Gene expression values were determined by cycle threshold levels and normalized to GAPDH RNA.

4.4 Western Blotting Analysis

The cells were grown in 6 cm dishes and when necessary starved prior to treatment for 24 hours. For lysis the cells were kept on ice and washed with ice-cold PBS, followed by adding 60 μl of lysis buffer, containing 20 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1mM EDTA, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1 mM sodium vanadate (Na3VO4), 1 μg/ml leupeptin (Cell Signaling 9803), 0.5% PMSF and 0.2% sodium dodecyl sulfate (SDS). Extracted proteins were sonicated for 15 seconds and were stored at -20°C. 10 μl of the sample was used to quantify the total protein amount with the bichinonic acid (BCA) Protein assay kit (Pierce, Rockford, USA), where the proteins were diluted in H2O at 1:4 and incubated in BCA solution for 30 minutes at 37°C in a 96-well plate (Nunc, Roskilde, Denmark).

Equivalent amounts of proteins were combined with 4x Laemmli sample buffer (Biorad) containing 77.8 mM Tris-HCl, pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue, and 20x DTT (Invitrogen). The samples were denaturated at 95°C for 5 minutes, loaded and separated on 1.0 mm 8-12.5% gels at 90V during 90 minutes. Precision Plus dual colors Standards (Bio-Rad, Reinach, Switzerland) were used as protein standards. Proteins were transferred to nitrocellulose membranes at 32A during 60 minutes. Ponceau S staining was used to control the transfer. To prevent unspecific bindings, the blots were incubated in
Methods

blocking solution (5% dry milk, dissolved in PBS/1% Tween (PBS-T)) for 1 hour. The proteins of interest were detected by incubation with primary antibodies in 5% BSA overnight at 4°C. The subsequent incubations with secondary LICOR or horseradish peroxidase-conjugated antibodies were performed for 1 hour at room temperature in 5% milk in PBS/T. After washing for 30 minutes with PBS-T, the blots were analyzed with LI-COR system or were covered with Lumi-Light substrate (Pierce) for 5 minutes and then exposed to Hyperfilm ECL films.

4.5 Vasculogenesis Assay

Vasculogenesis assay was performed in EPCs or HUVECs [260][261][262]. Matrigel (BD Biosciences) was used to cover the bottom of the wells of μ-slide, following one hour polymerization of the gel at 37°C. Meanwhile, the cells were trypsinized and centrifuged as described above. Cell suspensions at different concentration were prepared in 1ml of the basal medium containing 0.4% BSA, and various testing agents were added to the cells for 30 minutes. Afterwards, 50 μl of cell suspension was placed into the well of μ-slides and incubated at 37°C for 16 hours. Pictures from random spots of each well were acquired with Olympus inverted microscope and the length of microvessels was analyzed using Xcellence Pro software.

4.6 Bead Sprouting Angiogenesis assay

EPCs were mixed with Cytodex 3 beads (Sigma) at concentration of 2’500 beads per 1*10^6 cells in the complete medium and incubated in a FACS tube at 37°C for 4 hours while gently mixing the tube every 20 minutes. Afterwards, the beads were transferred into 6 cm in vitro fertilization dish (Falcon) for overnight incubation at 37°C. On the next day, the beads coated with the cells were placed into 10 ml falcon tube and slowly washed three times with 1ml of the complete medium. After counting the beads in 5 μl of the suspension on a coverslip, the beads concentration was adjusted to 250 beads/ml in a solution containing 2 mg/ml fibrinogen (Sigma) and 0.15 units/ml aprotinin (Sigma). A total of 500 μl of embedding solution was mixed with 0.625 units/ml of thrombin (Sigma) per well in 24-well plate. Fibrin gels were incubated for 5 minutes at RT and 15-20 minutes at 37°C to form a clot. The complete medium was added to the gel for 30 minutes and then replaced with the basal medium.
containing endothelial cell supplements and different treatments. Sprouting could be observed after two-three days of incubation the gels at 37°C and 5% CO₂.

4.7 Co-culture Assay

Human Cardiac Fibroblasts (HCFs) were seeded in 24-well plate and grown until confluence in the complete medium (DMEM/F12 containing 10% FCS). On the day of the experiment, EPCs were stained with 2μM CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes, Invitrogen) for 45 minutes in a serum-free medium, following incubation for another 30 minutes in a fresh complete medium in order to eliminate byproducts (chloromethyl groups) formed during cytosolic cleavage of the dye. Thereafter, endothelial cells were plated on the monolayer of HCFs at 5’000-10’000 cells/well in an appropriate basal medium containing 0.4% BSA and without supplements. The capillary formation was observed after 72 hours, and the pictures were acquired from different areas of each well using Olympus fluorescent microscope (at 492 and 517nm, excitation and emission wave length, accordingly) (Figure 10).

![Figure 10: A schematic representation of the co-culture angiogenesis assay](image)

4.8 Permeability Assay

Endothelial cell barrier function was studied in the permeability assay using 10kDa FITC-Dextran (Sigma). EPCs (100’000cells/insert) were grown on 3μm pore-sized collagen-coated Transwell-COL inserts (Corning) in 24-well plate. The cells were left for two-three days to establish a mature monolayer. On the day of the experiment, 100nM DHT or other treatments were added to the upper and the lower chambers in the basal medium containing 0.4% FCS,
Methods

whereas 100 mg/ml FITC-Dextran was added only to the upper chamber. The plate was protected from light and the samples were taken from the lower chamber after 20, 40, 60, 120 and 180 minutes of treatment. Noggin (250ng/ml) and ALK1 fc (100ng/ml) were added to the cells 1 hour prior to treatment. Fluorescence was measured by the excitation at 490nm and the emission at 520nm using Tecan Infinite series M200 (Figure 11).

![Figure 11: A schematic representation of the permeability assay](image)

4.9 SiRNA Transfection

EPCs were grown in 6 cm dishes till 60-70% confluence. The cells were starved overnight prior to the transfection. AR, Smad1 and non-targeting On-Target Plus Smart Pool siRNA (ThermoFischer) were applied at 50 nM using 5 μM Dharmafect. SiRNA stock solutions (100μM) were diluted in 1x Buffer to 5 μM. Antibiotics- and serum-free medium was used to prepare siRNA and Dharmafect solutions and incubated for 5 minutes at RT, followed by combining both solutions and incubating them for 20 minutes at RT. The solutions then were added to antibiotics-derived complete medium and placed onto the cells. After 24 hours of incubation with the transfecting agents, the medium was replaced to the complete one containing antibiotics. Silencing of the target genes was observed after 72 hours of transfection and confirmed by Western blotting.

4.10 Calcium Measurements

EPCs were grown in 24-well plates till confluence. Intracellular calcium influx was measured with the calcium-sensitive dye fluo4 (Molecular Probes, Invitrogen). The cells were loaded with 2μM fluo4 in the basal medium containing 0.4% FCS and without supplements for 50 minutes at RT, then EPCs were washed three times with HBSS to remove any dye bound to the cell surface and incubated in indicator-free basal medium for another 30 minutes. First,
the basal fluorescence was measured (the excitation and the emission at 485 and 520nm, accordingly), followed by adding the treatments to the cells and detecting stimulated fluorescence intensities during 20 minutes with image acquisition at 10 seconds intervals. Fluctuations in calcium levels were calibrated relative to the basal concentration of Ca\(^{2+}\).

4.11 Statistical Analysis

Data was analyzed using ANOVA and statistical significance (p<0.05) was calculated according to Fisher’s Least Significant Difference test.
5 Results and Discussion

5.1 DHT Improves EPC Function

OBJECTIVE

Endothelial dysfunction/damage is associated with cardiovascular disease (CVD), moreover, endothelial repair by local endothelial cells or by circulating EPCs is protective against CVD. Since the role of androgens in cardiovascular disease remains largely unknown, the aim of the present study was to investigate the role of DHT in regulating EPC function, an important marker of cardiovascular health. We examined the effects of DHT on EPC-induced vasculogenesis and the role of AR in mediating these effects.

INTRODUCTION

EPCs are important biological marker in cardiovascular health. Clinical studies have demonstrated an association between low levels of circulating EPCs and cardiovascular disease [113][114]. The degree of risk factors is also correlated with the number of circulating EPCs. Furthermore, decreased number of EPCs have been found in men with hypogonadism and independent of other risk factors [118]. The protective role of EPCs in the cardiovascular system arises from their role in new blood vessel formation, an important process in tissue regeneration and wound healing. Upon tissue injury, the pro-angiogenic factors stimulate mobilization of EPCs from the bone marrow and their recruitment at the site of injury as well as subsequent participation in neovascularization of the damaged area [90]. Numerous in vivo studies have confirmed the role of EPCs in ischemia-induced vascularization [90][91][92][93].

The role of androgens in CVD is currently under debate, since both deleterious and protective effects of androgens have been observed in clinical, in vivo and in vitro studies. As EPCs represent an indicator of vascular health, we assessed the impact of DHT on EPC function. Additionally, using the pharmacological (AR antagonist) and molecular approaches (siRNA), we examined the role of AR in mediating the cellular effects of DHT in EPCs.
RESULTS AND DISCUSSION

METHODS

As described in section 4.

RESULTS

5.1.1 DHT Stimulates Vasculogenesis in EPCs

In the present study, we assessed the effects of DHT on EPC function. We addressed this question using several in vitro vasculogenesis techniques. In the two-dimensional matrigel-based assay, we observed the stimulatory effect of DHT (100nM) on the capillary formation in EPCs from 100±3.2% in unstimulated cells to 179±4.8% in the presence of DHT (p<0.05) (Figure 12). Similar results were obtained from the co-culture of EPCs on the monolayer of human cardiac fibroblasts, where the tube formation was increased from 100±8.2% to 161±3% in control and DHT-treated cells, respectively (Figure 13). Moreover, we demonstrated that DHT stimulated vasculogenesis in EPCs in the sprouting assay, performed using cytodex beads covered with EPCs and embedded in fibrin gel (Figure 14). Taken together, our findings provide evidence that androgens stimulate capillary formation/function by EPCs and suggest that androgens may positively influence the cardiovascular repair processes.

Figure 12: The stimulatory effects of DHT on vasculogenesis in EPCs. EPCs were placed on the top of solubilized matrigel in the basal medium containing 0.4%BSA without supplements and in the presence or absence of 100nM DHT. After 16 hours of treatment, tube lengths were measured microscopically using Excellence Pro software. All data are expressed as means ± SEM. Representative of five experiments. P< 0.05 versus control.
Results and Discussion

Figure 13: DHT stimulates the tube formation in EPCs in the co-culture vasculogenesis assay. HCFs were grown in the complete medium till confluent monolayers were formed. EPCs were labeled with fluorescent CellTracker™ Green CMFDA (2μM) and were placed on the top of HCFs monolayer in the basal medium containing 0.4%BSA. Following 2-3 days of the co-culture the tube length was measured microscopically using Excellence Pro software. All data are expressed as means ± SEM. Representative of three experiments. P< 0.05 versus control.

Figure 14: DHT increases sprouting in EPCs. Cytodex beads were incubated with EPCs in the complete medium for 4 hours at 37°C, following overnight incubation in non-adherent dishes. EPC-coated beads were mixed with fibrinogen/aprotinin solution and upon adding to thrombin in 24-well plate were left to form a clot. After 30 minutes, fibrin gels were equilibrated in the basal medium for 1 hour, following treatment with DMSO or 100nM DHT in the basal medium containing endothelial supplements and 1%FCS. 10%FCS was used as a positive control. Sprouting was observed after 2-3 days of incubation at 37°C and 5%CO₂. Representative of three experiments.

5.1.2 AR Mediates the Capillary Formation by EPCs

As biological actions of androgens are mediated by AR, we studied its role in mediating the stimulatory effects of DHT on EPC function. To address this question, we first used AR
siRNA to down-regulate its expression. After having confirmed the silencing of AR with Western blotting (Figure 15B), we used AR siRNA (50nM) in the matrigel vasculogenesis assay. We observed that DHT was no longer able to induce capillary formation by EPCs in the presence of AR siRNA (42±8.2% and 36±7.8% in the vehicle and in the DHT-treated cells, respectively) (Figure 15A).

To further confirm our observation that the stimulatory effects of DHT on capillary formation are mediated by AR, we employed flutamide, the pharmacological inhibitor of AR. As shown in Figure 16, DHT-stimulated tubulogenesis by EPCs was significantly abrogated in the presence of AR antagonist flutamide (1μM).

Figure 15: AR siRNA abrogated DHT-stimulated increase in the capillary formation by EPCs. (A) EPCs were transfected with 50nM AR siRNA for 72 hours and subsequently utilized for the capillary assay in matrigel. Treatment with 100nM DHT for 16 hours increased the capillary formation by EPCs, which was abrogated by AR siRNA. (B) Silencing of AR was confirmed by Western blotting. All the data are expressed as means ±SEM. Representative of two experiments. P < 0.05 versus control.
Results and Discussion

Figure 16: AR antagonist blocked the angiogenic effects of DHT in EPCs. The tube formation by EPCs was studied using two-dimensional matrigel assay both in the presence or absence of 100nM DHT and/or 1μM AR antagonist flutamide. All the data are expressed as means ±SEM. Representative of three experiments. P< 0.05 versus control, DHT+FLU versus DHT.

DISCUSSION

The role of androgens in the cardiovascular system is not yet clear, however, testosterone replacement therapy (TRT) is used in patients with low testosterone (T) levels. Since an increase in life expectancy and longevity is accompanied by a natural age-related decline in T levels, there is an enhanced demand for TRT. Hence, it is essential to elucidate the risks and benefits of testosterone treatment to maintain men’s health.

CVD is one of the major age-related diseases. Cardiovascular risk factors lead to the vascular remodeling including endothelial dysfunction. This also results in impaired tissue regeneration following injury. EPCs, known to promote vascular repair by contributing to neovascularization, play an important role in vascular homeostasis. The numbers and function of circulating EPCs are dramatically reduced in patients with cardiovascular risk factors or CVD and low testosterone levels. Hence, we investigated the role of DHT on the vascular remodeling processes and on EPC function contributing to vascular repair.

In our study, the ability of EPCs to form capillaries in the presence of DHT has been tested in three in vitro models. In the matrigel-based vasculogenesis assay DHT (100nM) stimulated the capillary formation by EPCs after 16 hours of treatment. Some studies have shown that the capillary formation in matrigel is not unique to endothelial cells alone, and other cell types (fibroblasts, smooth muscle cells, Leydig cells, baby mouse kidney cells, and monocytes) can also form tube-like structures on the matrigel [263][264][265][266]. Therefore, we utilized
another in vitro vasculogenesis model, which was demonstrated by Sieveking et al. to be specific only for endothelial cells, as other cell types were not able to support tubulogenesis in this model [100]. This model is based on the co-culture of EPCs on the confluent monolayer of fibroblasts. EPC-labeled with CellTracker™ Green CMFDA dye were grown on human cardiac fibroblasts for 2-3 days, the time necessary for the capillary formation. We observed that DHT increased vasculogenesis in the co-culture model similar to the matrigel assay (61% and 75%, respectively). Hence, for practical reasons, we used the matrigel-based model for further studies.

Formation of new blood vessels is initiated by sprouting and differentiation of EC into tip and stalk cells with migratory and proliferative activities, respectively [267]. Therefore, we assessed the effects of DHT on sprouting in EPCs. We found that sprouting was more pronounced in the presence of DHT compared to control. Taken together, our observations provide evidence that DHT treatment exerts a beneficial effect by stimulating the function of EPCs. Our findings are consistent with the other recent studies demonstrating the pro-angiogenic effects of androgens. An in vivo study revealed that tissue regeneration following hind limb ischemia was impaired in orchedectomized mice. These effects were reversed by T treatment, in part by promoting mobilization of circulating EPCs from the bone marrow [86]. Another study demonstrated poor neovascularization of ischemic tissue and lower CD34+ cell number in the peripheral blood following acute MI in castrated rats in comparison to the non-castrated ones [268]. These effects, however, were reversed by testosterone treatment, indicating the protective role of androgens. Foresta et al. reported that synthetic androgen R1881 (not able to be converted to E2) increased proliferation, migration and colony-formation in EPCs via AR from healthy men [269].

Most effects of androgens are mediated via AR. Upon activation, AR is translocated into the nucleus, where it induces transcription of target genes. These effects are referred to as genomic actions of androgens. We examined whether DHT induces vasculogenesis by EPCs via AR. Our finding that AR antagonist flutamide significantly reduced DHT-stimulated tubulogenesis, together with the fact that AR siRNA inhibited DHT-induced vessel formation by EPCs suggests that the stimulatory effects of DHT on EPC function are AR-dependent and genomic in nature. Sieveking et al. reported that DHT exerts the stimulatory effects on angiogenesis via AR in endothelial cells (EC), derived from male, whereas there was no effect on female EC. Interestingly, overexpression of AR in female EC resulted in augmented angiogenesis in their model [86]. In our study, we used EPCs of female origin, moreover, we observed the pro-angiogenic effects of DHT also on female HUVECs (data not shown).
Importantly, both female EPCs and HUVECs express AR and DHT was able to up-regulate AR expression, implying that activation of AR signaling occurs in a sex-independent manner. This contention is supported by the finding that compared to control mice both male and female AR knockout (ARKO) mice have impaired recovery following hind limb ischemia [85]. Interestingly, in this study, bone marrow transplantation from ARKO into wild type (WT) mice did not worsen reperfusion and transplantation of bone marrow from WT into ARKO mice did not improve tissue neovascularization, indicating that progenitor cells didn’t contribute to the ischemic recovery in this model. However, these results might also point out to a defective mobilization and recruitment of EPCs to the injury site in ARKO mice, which could explain no effect of WT bone marrow transplantation into ARKO mice. The mechanisms for androgen-mediated mobilization of progenitor cells from the bone marrow need to be further explored.

In conclusion, our findings provide evidence that androgens (DHT) stimulate capillary formation by EPCs. More importantly, these effects are genomic and AR-mediated. Since EPCs contribute to endothelial repair and EPC numbers decrease with hypogonadism, EPC culture in the presence of androgens may serve as a therapeutic approach for CV repair in older men.
5.2 The Akt-mTOR-VEGF Signaling Pathway Mediates DHT-Stimulated Vasculogenesis by EPCs

OBJECTIVE

Our previous findings demonstrated pro-angiogenic effects of DHT in EPCs, hence, we further investigated the underlying mechanisms. We examined the role of the Akt/mTOR/VEGF signaling, a key angiogenic pathway, in mediating DHT-induced vasculogenesis in EPCs. Additionally, we studied the relationship between AR and the Akt/mTOR signaling in EPCs.

INTRODUCTION

The role of the Akt/mTOR/VEGF signaling pathway in mediating angiogenesis has been supported by numerous studies [193][194][195][196]. The PI3K/Akt inhibitors are used as anti-angiogenic agents in cancer therapy [199][200]. Similarly, inhibition of mTOR and VEGF reduces angiogenesis [195][197]. VEGF plays a key role in tissue regeneration following injury. Hypoxia upon tissue damage triggers the VEGF production and release. In addition to the stimulation of adjacent endothelial cells to restore impaired vascularization, VEGF together with other angiogenic factors promotes mobilization of EPCs from the bone marrow and their recruitment to the injury site and participation in new blood vessel formation [101][109]. Interestingly, DHT treatment has been shown to modulate VEGF production. Sieveking et al. demonstrated that DHT increases VEGF mRNA in endothelial cells [86]. The direct link between androgens and VEGF via AR has been provided by Yoshida et al., showing an interaction between VEGF receptor2, AR and PI3K in HUVECs [85]. However, whether the same mechanism(s) are modulated by androgens in EPCs, remains unknown.

In the present study, we assessed the effects of DHT in the matrigel-based vasculogenesis assay in EPCs in the presence or absence of the PI3K/Akt, mTOR or VEGF inhibitors. Moreover, we examined the activation of the Akt/mTOR signaling pathway following DHT treatment. Additionally, we studied an association between AR and Akt pathway in EPCs.
RESULTS

5.2.1 The Akt/mTOR/VEGF Signaling Pathway in Vasculogenesis

To further investigate the mechanisms of DHT-induced vasculogenesis in EPCs, we examined the role of the Akt/mTOR/VEGF signaling in our model. We assessed the effects of 100nM DHT in the presence of the Akt, mTOR or VEGF inhibitors in the matrigel-based vasculogenesis assay. Treatment with the PI3K/Akt inhibitor LY0294002 (1μM) significantly abrogated DHT-induced capillary formation from 119±0.2% to 28±0.6% (Figure 17). Moreover, the mTOR inhibitor rapamycin (10nM) also blocked DHT-stimulated vasculogenesis in EPCs from 123±0.2% to 60±0.99% (Figure 18).

![Figure 17](image17.png)

**Figure 17: Inhibition of PI3K/Akt prevents DHT-mediated increase in tubulogenesis.** Matrigel assay was used to assess EPC function in the presence of the Akt inhibitor LY0294002 (1μM) and 100nM DHT. All the data are expressed as means ±SEM. Representative of three experiments. P< 0.05 versus control, LY+DHT versus DHT.

The activation of the Akt/mTOR signaling pathway leads to the VEGF production. VEGF is well known to stimulate the formation of new blood vessels and plays an important role in regulating endothelial cell function. Moreover, VEGF is a key angiogenic mediator for many pro-angiogenic molecules. Therefore, we examined the role of VEGF in DHT-induced vasculogenesis.

METHODS

Described in section 4.
vasculogenesis in EPCs by using VEGF neutralizing antibodies (AB). As shown in Figure 19, VEGF AB (250ng/ml) abrogated DHT-stimulated capillary formation by EPCs from 141±0.3% to 45±18%.

Figure 18: The role of mTOR in DHT-mediated capillary formation by EPCs. EPCs were layered on the matrigel and incubated in the basal medium supplemented with 0.4%BSA in the presence of the mTOR inhibitor rapamycin (10nM) and 100nM DHT for 16 hours. Rapamycin significantly reduced DHT-induced tubulogenesis in EPCs. All the data are expressed as means ±SEM. Representative of three experiments. P< 0.05 versus DHT.

Figure 19: VEGF inhibition reduced DHT-stimulated microvessel formation. Vasculogenesis was evaluated in the matrigel assay in the presence of VEGF neutralizing antibodies (250 ng/ml) and 100nM DHT. All the data are expressed as means ±SEM. Representative of three experiments.
To further verify the role of the Akt/mTOR/VEGF pathway in regulating the function of EPCs, we performed the sprouting assay. Inhibition of the Akt/mTOR/VEGF signaling attenuated the number of sprouts, implying that this pathway plays a prominent role in mediating angiogenic effects of DHT on EPCs (Figure 20).

**Figure 20:** Inhibition of the Akt/mTOR/VEGF axis prevented DHT-induced sprouting in EPCs. EPC-coated beads were embedded in fibrin gels in the basal medium containing supplements and 1% FCS in the presence of LY294002 (1μM), rapamycin (10nM) and VEGF neutralizing antibodies (250 ng/ml) – the PI3K/Akt, mTOR and VEGF inhibitors, respectively, and 100nM DHT. Sprouting was evaluated after 2 days of treatment. Representative of two experiments.

**5.2.2 DHT Induces Akt and mTOR Phosphorylation in EPCs**

As demonstrated previously, DHT promotes microvessel formation by EPCs via the Akt/mTOR/VEGF pathway. To further ascertain the role of this mechanism, we examined the effects of DHT on the phosphorylation of Akt and mTOR. As shown in Figure 21A, treatment with 100nM DHT increased the levels of phospho-Akt (Ser 473) in a time-dependent fashion with maximal induction after 30 minutes. Similarly, DHT induced the phosphorylation of mTOR within 60 minutes (Figure 21B). In addition, we observed that inhibition of Akt with 1μM LY294002 prevented DHT-induced phosphorylation of mTOR (Figure 22). These findings suggest that phosphorylation of mTOR is downstream from Akt.
Results and Discussion

5.2.3 A Cross-Talk between AR and the Akt/mTOR/VEGF Axis

In our vasculogenesis studies with EPCs, we observed that DHT effects are mediated by AR and the Akt/mTOR/VEGF signaling pathways. Therefore, we next investigated the relation between AR and the Akt/mTOR pathway. We studied the effects of the AR antagonist flutamide and AR siRNA on Akt phosphorylation. As shown in Figure 23, pre-treatment of...
EPCs with 1µM flutamide for 1 hour, followed by stimulation with 100nM DHT for 30 minutes reduced the phosphorylation of Akt from 131.5±11.7% to 92.5±6.9%. Similar results were obtained using AR siRNA, which decreased DHT-induced phospho-Akt levels (Figure 24). Taken together, these results indicate that there is a cross-talk between AR and Akt, which subsequently activates the downstream mTOR/VEGF signaling.

Figure 23: AR inhibition blocks DHT-induced Akt phosphorylation. The AR antagonist flutamide (1µM) prevented the phosphorylation of Akt by DHT treatment for 30 minutes. EPCs were serum-deprived for 24 hours before the treatment. All the data are expressed as means ±SEM. Representative of three experiments. P< 0.05 versus DHT.

Figure 24: AR siRNA abrogate DHT-stimulated Akt phosphorylation. EPCs were transfected with AR siRNA for 72 hours and stimulated with 100nM DHT for 30 and 45 minutes in the basal medium containing 0.4% FCS and without supplements. Representative of two experiments.
DISCUSSION

In the present study, we examined the possible mechanisms responsible for the stimulatory actions of DHT on EPC function. Multiple studies have reported that the PI3K/Akt signaling plays an essential role in regulating postnatal new blood vessel formation and angiogenesis-related processes. In fact, hind limb ischemia in the Akt1/-/- mice resulted in impaired tissue revascularization [270][271]. With regard to EPC role in the tissue repair, Ackah et al. reported that EPC levels were decreased in the peripheral blood in the Akt1/-/- mice compared to WT following ischemia, suggesting the link between a defective mobilization of EPCs and Akt deficiency [270]. An injection of the spleen-derived EPCs into WT mice following ischemia improved limb perfusion, but only by EPCs from WT and not from Akt1/-/- mice. Therefore, we hypothesized that DHT may also induce PI3K/Akt to modulate EPC function. Indeed, in our study, the PI3K/Akt inhibitor LY2944002 blocked the vasculogenic effects of DHT in EPCs. Akt has been shown to phosphorylate endothelial nitric oxide synthase (eNOS), leading to the nitric oxide (NO) production [272]. Moreover, eNOS/-/- mice elicit impaired neovascularization of ischemic hind limb and fail to respond to the VEGF treatment, suggesting a key role of NO in modulating a postnatal angiogenesis [273]. Interestingly, there is also evidence that androgens promote vasorelaxation by inducing NO synthesis. For example, in human aortic endothelial cells (HAEC), treatment with T stimulates eNOS phosphorylation and NO synthesis via a cross-talk between AR and the PI3K/Akt pathway [274]. Based on these findings and our observations, it is feasible that the angiogenic effects of DHT involve a cross-talk between AR-NO and PI3K/Akt.

Another important regulator in angiogenesis is the mTOR pathway. Its activation has been observed in various tumors, and mTOR inhibition reduced tumor growth. Rapamycin, known as the mTOR complex1 (mTORC1) inhibitor, has been shown to block tumor-associated angiogenesis in mice (by blocking VEGF production) [196]. However, some studies revealed that a longer exposure to rapamycin leads also to mTORC2 inhibition and to the anti-angiogenic effects in tumor xenografts [186]. In fact, the dual inhibitor of mTOR yielded better results than mTORC1 inhibitor alone in reducing angiogenesis in a tumor xenograft model in vivo [193]. In our study, we found that rapamycin abolished DHT-induced capillary formation in EPCs. Hence, our findings support the role of the PI3K/Akt/mTOR axis in mediating DHT-stimulated EPC function. Our results are consistent with other studies, where a dual inhibition of PI3K and mTOR with the novel drug NVP-BEZ235 blocked VEGF-stimulated angiogenesis in vivo [201].
Finally, we demonstrated that DHT failed to stimulate EPC function (vasculogenesis) in the presence of VEGF neutralizing antibodies. Our findings are in concert with the concept of Y. Chen, who demonstrated that the pro-angiogenic effects of T treatment in castrated rats with induced MI were due to an increased VEGF production [268]. We speculate that under hypoxic conditions such as ischemia or tissue injury increased HIF-1α and VEGF levels together with androgen-stimulated AR signaling activate the PI3K/Akt/mTOR pathway and promote tissue repair.

Additionally, we examined the activation of Akt and mTOR by DHT at the protein level. Treatment with DHT increased the phosphorylation of Akt and mTOR within 60 minutes as compared to the non-stimulated control. Moreover, inhibition of PI3K/Akt with LY294002 abrogated DHT-induced mTOR phosphorylation, suggesting that Akt is upstream of mTOR. These results confirm that DHT activates the Akt/mTOR pathway in EPCs.

Taken together, we observed that the PI3K/Akt/mTOR/VEGF axis as well as AR signaling is necessary for DHT-mediated vasculogenesis in EPCs. Hence, in order to investigate whether there is a cross-talk between AR and Akt/mTOR, we stimulated EPCs with DHT in the presence of the AR antagonist flutamide or AR siRNA and examined the phosphorylation of Akt. We conclude that the inhibition of AR signaling blocks DHT-induced Akt activation. Therefore, our results provide evidence for a possible interplay between AR and the Akt/mTOR/VEGF pathways.

The link between AR and Akt has been shown in various cell types. Sun et al. reported that AR directly interacts with Src and the regulatory p85 subunit of PI3K, and this interaction is necessary for androgen-induced Akt activation in AR-positive breast cancer and prostate cancer cells [275]. Moreover, in HUVECs, AR appeared to directly interact with VEGF receptor KDR, p85 PI3K and Src, and this association was further enhanced by DHT treatment. Interestingly, AR inhibition abolished KDR/PI3K/Src complex despite VEGF stimulation and ultimately blocked Akt/eNOS activation, thereby supporting the role of AR in a cross-talk with the Akt/VEGF signaling [85]. Accordingly, with regard to the observed AR-PI3K/Akt interactions, it is reasonable to assume that there is a cross-talk between AR and the PI3K/Akt/mTOR signaling, responsible for the androgen-mediated stimulatory effects on EPC function.
5.3 ALK1/Smad1/5/8 Signaling Mediates DHT-Induced Barrier Function but Not Vasculogenesis in EPCs

OBJECTIVE

To further elucidate the molecular mechanisms regulating the stimulatory effects of DHT on EPC function, we examined the role of TGFβ signaling. In the present study, we assessed the effects of DHT on the expression of the downstream effectors of TGFβ/BMP signaling and the underlying mechanisms. Finally, we studied the role of this pathway in modulating EPC functions (vasculogenesis and barrier function) in response to DHT.

INTRODUCTION

TGFβ pathway is implicated in various cellular processes. The TGFβ family includes TGFβ, BMP subfamily, activins and growth differentiation factors (GDF) [203][204]. Members of the TGFβ family bind to type receptor I and type receptor II and induce the formation of a heteromeric complex, leading to the activation of downstream transcription factors called Smads [207][208]. The same member of the TGFβ family can trigger diverse responses by recruiting a distinct set of receptors depending on the context and cell type. Hence, accumulating evidence provides inconsistent results in TGFβ signaling in postnatal angiogenesis. However, the research suggests a key role of TGFβ signaling in embryonic angiogenesis, since mutations in ALK1, Endoglin and Smads lead to severe defects in embryogenesis and to early lethality [222][223][224].

The impact of TGFβ signaling on ECs has been well documented. In the present study, we investigated whether DHT treatment can modulate TGFβ signaling in EPCs and whether this pathway contributes to DHT-induced vasculogenesis. Deficiency in TGFβ signaling is associated with the genetic disease HHT, characterized by hyperdilated, leaky and prone to rupture blood vessels with impaired endothelial function [229][230][231][232][233]. Hence, we investigated the role of TGFβ pathway in regulating the barrier function in EPCs in response to DHT.

METHODS

Described in section 4.
RESULTS

5.3.1 DHT Triggers the Activation of the ALK1/Smad1/5/8 Signaling

To study the role of the ALK1/Smad1/5/8 signaling in EPC function, we examined whether DHT activates this pathway in EPCs. First, we confirmed that both ALK1 and ALK5 are expressed in EPCs (Figure 25A). To study further this signaling, EPCs were starved for 24 hours in the basal medium containing endothelial supplements and 0.4% FCS and subsequently treated with 100nM DHT for 5, 10, 30, 60 and 120 minutes. We observed a strong induction of Smad1/5/8 phosphorylation (downstream target of ALK1) by DHT within 60 minutes, with a maximal induction at 30 minutes (Figure 25B). Importantly, there was no significant modulation of ALK5 downstream effectors Smad2/3 (Figure 25B). Thereafter, to examine whether DHT modulates the expression of direct targets of the transcription factors Smad1/5/8, EPCs were treated for 4, 8 and 24 hours with 100nM DHT. As shown in Figure 25C, DHT treatment up-regulated Id-1 expression from 100% to 157.6%, 124.1%, 314.7% after 4, 8 and 24 hours, respectively. Interestingly, the expression of Endoglin, which has been shown to play an important role in the activation of the ALK1 signaling, was increased by DHT in a similar manner from 100% to 378.4%, 233% and 575.9% after 4, 8 and 24 hours of treatment, respectively (Figure 25C).
Results and Discussion

Figure 25: The effects of DHT on the activation of TGFβ signaling. (A) Western blot showing that ALK1 and ALK5 both expressed in EPCs. (B) DHT 100nM induced an increase in phosphorylation of Smad1/5/8, however, no change was observed in phospho-Smad2 and phospho-Smad3 levels. (C) The expression of Id-1 and Endoglin was significantly up-regulated in the presence of 100nM DHT for 4, 8 and 24 hours. Graph values are from representative experiment and similar results were obtained in three separate experiments.

Next, we investigated the role of ALK1 and ALK5 in the induction of the Smad1/5/8 pathway. EPCs were starved for 24 hours and pre-treated for 1 hour with 100ng/ml ALK1 Fc or 500nM SJN2511, the ALK1 and ALK5 inhibitors, respectively, and then stimulated with 100nM DHT for 30 minutes. In cells pre-treated with the ALK1 inhibitor, DHT did not increase phospho-Smad1/5/8 levels (Figure 26A), indicating that ALK1 is required for promoting its downstream signaling. In the presence of the ALK5 inhibitor, SJN2511, phosphorylation of Smad2 was completely abrogated (Figure 26B), nonetheless, DHT induced Smad1/5/8 phosphorylation. These results suggest that ALK5 is not involved in DHT-mediated activation of the ALK1/Smad1/5/8 mechanism. Growing evidence demonstrate that ligands other than TGFβ can trigger ALK1 signaling, amongst which are bone morphogenetic proteins (BMPs). BMP2 and BMP4 are known to regulate the function of endothelial cells by promoting new blood vessel formation. Hence, we studied whether BMP2 and BMP4 may play a role in mediating DHT-induced Smad1/5/8 activation. To accomplish this, EPCs were pre-treated for 1 hour with 250ng/ml Noggin, the BMP2 and BMP4 inhibitor, and subsequently stimulated with 100nM DHT for 30 minutes. As shown in Figure 27, Noggin indeed prevented an increase in phospho-Smad1/5/8 levels. These findings suggest that DHT may require BMP2 or BMP4 to initiate the ALK1/Smad1/5/8 signaling.
Results and Discussion

Figure 26: The role of ALK1 and ALK5 in mediating DHT-increased phosphorylation of Smad1/5/8. (A) ALK1 Fc 100ng/ml abrogated DHT-mediated Smad1/5/8 activation, whereas (B) Smad2 blockade with 500nM SJN2511 did not affect DHT-induced Smad1/5/8 phosphorylation, however, the phosphorylation of Smad2 was inhibited in the presence of SJN2511. EPCs were starved for 24 hours in the basal medium containing supplements and 0.4%FCS and were pre-treated with ALK1 fc for 4 hours and with SJN2511 for 1 hour prior to 100nM DHT treatment for 30 minutes in the basal medium supplemented with 0.4%FCS. Representative of three experiments.

Figure 27: The effects of Noggin in DHT-induced activation of Smad1/5/8. Western blot representing inhibition of DHT effects on Smad1/5/8 phosphorylation in the presence of 250ng/ml Noggin. EPCs were serum-derived for 24 hours and incubated with Noggin for 1 hour in the basal medium containing 0.4%FCS. 100nM DHT treatment was added to EPCs for 30 minutes. Representative of three experiments.

5.3.2 DHT-Induced Activation of Smad1/5/8 Signaling Is Mediated by Intracellular Calcium Influx

We further addressed the question how DHT triggers the ALK1/Smad1/5/8 signaling. Since DHT induces Smad1/5/8 phosphorylation within 15 minutes of treatment, we postulate that the stimulatory effects of androgens are mediated via non-genomic mechanism(s). Because rapid non-genomic actions are mediated by second messengers and androgens are known to engage calcium to mediate their signaling, we investigated the role of intracellular calcium in the activation of the ALK1/Smad1/5/8 pathway upon DHT treatment. First, we assessed
whether DHT increases intracellular calcium in EPCs. To accomplish this goal, EPCs were loaded with the calcium-sensitive agent fluo4 and the changes in basal fluorescence levels were measured with a fluorescence microscope. As shown in Figure 28A, we detected alterations in the intracellular calcium level upon DHT stimulation. Calcium levels were elevated in response to 100nM DHT within 2 minutes, and this effect was abrogated in the presence of the calcium chelator BAPTA-AM (10μM) (Figure 28B). Hence, we show that DHT treatment increases intracellular calcium concentration in EPCs.

![Figure 28: The effects of DHT on the intracellular calcium flux.](image)

To measure fluctuations in intracellular calcium levels, EPCs were loaded with the calcium indicator fluo4 (1µM). After detecting basal fluorescence intensity with fluorescence microscope, 100nM DHT was added to the cells. (A) DHT increased intracellular calcium levels within 2 minutes. (B) The calcium chelator BAPTA-AM (10 μM) completely abrogated an increase of intracellular calcium triggered by DHT. Representative of four experiments.

Next, we investigated whether calcium mediates DHT-induced phosphorylation of Smad1/5/8. Since it has been reported that G-protein coupled receptors (GPCR) mediate the non-genomic effects of androgens and their activation is linked to the calcium signaling, we used the GCPR inhibitor pertussis toxin (PTX) to address this question. Indeed, we observed that DHT-induced phosphorylation of Smad1/5/8 was reduced from 303±34.5% to 238±10.3% in the presence of 1μM PTX (Figure 29A). To further confirm calcium participation in the activation of Smad1/5/8 pathway by DHT, EPCs were treated with 1µM
U73122 – the phospholipase C inhibitor. We observed that the inhibition of the intracellular calcium release by U73122 reduced DHT-induced phosphorylation of Smad1/5/8 from 158.8% to 49.8% (Figure 29B). Moreover, in the presence of the calcium chelator BAPTA-AM (10µM) DHT could not trigger Smad1/5/8 signaling (Figure 29C). These results suggest that calcium plays a central role in mediating Smad1/5/8 activation by DHT.

Figure 29: The effects of the calcium signaling inhibitors on DHT-induced phosphorylation of Smad1/5/8. EPCs were pre-treated with the inhibitors for 1 hour prior to 100nM DHT treatment for 30 minutes. (A) The G-protein coupled receptor inhibitor 1µM PTX, (B) the phospholipase C inhibitor 1µM U73122 and (C) the calcium chelator 10µM BAPTA-AM blocked the effects of DHT on Smad1/5/8 phosphorylation. Graph values are from representative experiment and similar results were obtained in three separate experiments.
5.3.3 The Role of Smad1/5/8 in Vasculogenesis

Growing evidence supports the pro-angiogenic role of the ALK1/Smad1/5/8 pathway, which is activated by the members of the TGFβ family. Our findings provide evidence that DHT induces the phosphorylation of Smad1/5/8, resulting in the up-regulation of its downstream target Id-1, which has been shown to contribute to the capillary formation in endothelial cells. Therefore, we examined the role of DHT-stimulated Smad1/5/8 signaling in mediating vasculogenesis in EPCs. Surprisingly, silencing Smad1 expression with siRNA did not affect the microvessel formation in EPCs. As shown in Figure 30, vasculogenesis was induced by 100nM DHT from 100±22% to 152±5.4%, and in the presence of Smad1 siRNA the capillary formation was not inhibited (157±0.6% and 166±38.1% in the vehicle and DHT-treated cells, respectively). Moreover, Noggin (100ng/ml), the inhibitor of BMP2 and BMP4, did not block DHT-induced vasculogenesis significantly (from 123±0.2% to 94±9.6%) (Figure 31). Therefore, our data suggest that Smad1/5/8 signaling does not play a crucial role in the regulation of new vessels formation in EPCs.

Figure 30: The role of Smad1 in mediating the stimulatory effects of DHT on vasculogenesis in EPCs. (A) The matrigel-based vasculogenesis assay was used to evaluate the effects of Smad1 siRNA in DHT-stimulated tubulogenesis. EPCs were transfected with Smad1 siRNA (50nM) for 72 hours, following the matrigel assay for 16 hours. (B) Smad1
silencing was confirmed by treatment with 10ng/ml BMP9 for 30 minutes and Western blotting. All the data are expressed as means ±SEM. Representative of four experiments.

![Figure](image)

**Figure 31:** Noggin did not affect vasculogenesis in EPCs. The capillary formation by EPCs was evaluated by the matrigel assay in the presence of 250ng/ml Noggin and 100nM DHT. All the data are expressed as means ±SEM. Representative of three experiments.

5.3.4 The Role of the ALK1-Smad1/5/8 Pathway in Regulating EPC Barrier Function

Our data indicate that DHT stimulates vasculogenesis in EPCs and also activates the ALK1/Smad1/5/8 pathway, a pivotal mechanism in mediating the capillary formation. However, in our experiments with EPCs, we did not observe the link between Smad signaling and the microvessel formation. Interestingly, the role of ALK1 in endothelial function was discovered in patients with hereditary hemorrhagic telangiectasia (HHT), characterized by leaky and fragile blood vessels, prone to ruptures. Therefore, the role of ALK1 pathway might be associated with other endothelial cell functions, distinct from blood vessel formation. Hence, we examined the effects of DHT on the endothelial barrier function, important for the integrity of the endothelium and the vascular homeostasis.

First, we investigated how DHT treatment affects the barrier function in EPCs by studying the expression of the tight junction proteins. The tight junction proteins include occludin and claudin5, which are present in the endothelium. Interestingly, 100nM DHT increased the expression of occludin and claudin5 already within 4 hours of treatment (Figure 32). The treatment with the inflammatory cytokines 10ng/ml IL-1β, 10ng/ml TNF-α and 2U/ml Thrombin served as a control, since these agents are known to disrupt the integrity of
endothelial monolayer. Indeed, occludin and claudin5 expression was down-regulated in the presence of IL-1β, TNF-α and Thrombin.

Figure 32: The modulatory effects on the expression of occludin and claudin5 in EPCs. Western blot showing up-regulation in occludin and claudin5 protein levels after 6 hours of 100nM DHT treatment. 2U/ml Thrombin, 10ng/ml IL-1β and 10ng/ml TNF-α decreased the expression of occludin and claudin5, respectively. Representative of three experiments.

Next, we studied the role of Smad1/5/8 in mediating the DHT effects on barrier function by using Smad1 siRNA and the ALK1 inhibitor. As shown in Figure 33A, 100nM DHT failed to up-regulate the expression of occludin in Smad1-deficient EPCs, however, claudin5 expression was not altered by Smad1 silencing. Moreover, we observed that ALK1 inhibition with 100ng/ml ALK1 fc resulted in decreased occludin expression, whereas claudin5 levels remained unaffected by ALK1 fc (Figure 33B). These observations suggest that ALK1 specifically regulates the expression of occludin, but not claudin5, via Smad1/5/8 in EPCs.

Figure 33: The differential effects of DHT-activated ALK1/Smad1 signaling in occludin and claudin5 expression. (A) Transfected EPCs with Smad1 siRNA (50nM) for 72 hours were treated with 100nM DHT for 6 hours in the basal medium containing supplements and 0.4%FCS. Smad1 silencing abrogated DHT-induced occludin expression, however, had no effect on claudin5 expression. (B) EPCs were pre-treated with 100ng/ml ALK1 fc for 4 hours prior to the stimulation with 100nM DHT for 6 hours. In the presence of ALK1 occludin expression was reduced and claudin5 was nonetheless up-regulated by DHT. Representative of two experiments.
In order to prove the functional role of our findings, we performed the permeability assay using FITC-Dextran. We measured the barrier function of EPCs, grown to confluence on collagen-coated inserts with 3µm pores. Using 10kDa FITC-Dextran with the capacity of passing between the endothelial cells, we detected the permeability of the cells in response to DHT with or without the ALK1 inhibitor. Our results show that 100nM DHT decreased the permeability from 100±0.2% to 80±0.2% starting at early time point. Importantly, the ALK1 inhibitor reversed this effect (from 163±1.9% in the presence of DHT alone to 254±1.9% after 3 hours of treatment) (Figure 34A). Moreover, Noggin (250ng/ml) blocked in part the effect of DHT by increasing the permeability from 105±0.7% to 120±1.8% at 3 hour time point (Figure 34B). Interestingly, in the experiments with thrombin (2U/ml), a known barrier disruptor, the permeability of EPCs was increased by more than 80% after 3 hours of treatment. Importantly, DHT rescued the cells by reversing the effects of thrombin from 217±2.9% to 145±0.4% at 3 hour time point (Figure 34C). Taken together, our findings suggest that DHT promotes EPC barrier function through the ALK1/Smad1/5/8 pathway and may, in part, involve the participation of BMP2/BMP4.

![FITC-Dextran Flux](image-url)
Figure 34: The role of DHT and ALK1 signaling in regulating the permeability in EPCs. The barrier function of EPCs was measured in the permeability assay. EPCs were grown on collagen-coated inserts with 3μm pores in the complete medium. Upon reaching confluence, 100nM DHT and/or 2U/ml thrombin in combination with 10ng/ml 10kDa FITC-Dextran were added to the upper chamber, and the samples were taken from the lower chamber at different time points to measure fluorescence with Tecan. (A) EPCs were pre-treated with 100ng/ml ALK1 fc in the basal medium containing 0.4%FCS and without supplements for 4 hours. 100nM DHT decreased the permeability, and this effect was reversed in the presence of the ALK1 inhibitor. (B) In the presence of Noggin (250ng/ml) the permeability was increased compared to DHT alone. (C) Thrombin significantly increased the permeability in EPCs, however this effect was reduced in the presence of DHT. All the data are expressed as means ±SEM. *P< 0.05 versus control, § P< .05 versus Thrombin. Representative of four experiments.
DISCUSSION

Numerous studies have demonstrated that TGFβ signaling is an important regulator of endothelial cell function. It has been shown to play a central role in embryonic development by governing angiogenic processes. The defects in major TGFβ signaling mediators such as ALK1, Endoglin and Smad1/5/8 result in embryonic lethality due to vascular abnormalities in murine models [222][223][224]. Moreover, a prominent role of TGFβ pathway in modulating postnatal angiogenesis has been demonstrated by many studies [217][218][225][226][227].

Regarding the role of DHT in TGFβ signaling, we found that DHT treatment induces the phosphorylation of transcription factors Smad1/5/8, downstream of ALK1, whereas no change has been observed in the phosphorylation of Smad2/3, which are downstream effectors of ALK5. ALK1 is known to be expressed mostly in endothelial cells [276][277]. Interestingly, we could detect ALK1 expression in EPCs, which are phenotypically similar to endothelial cells. Moreover, ALK5 was also present in EPCs, although some studies point out that ALK5 is typical for vascular smooth muscle cells [277]. DHT increased the expression of Id-1, a downstream target of Smad1/5/8, implying a full activation of transcription factor Smad1/5/8 upon DHT exposure. An increase in Endoglin expression in response to DHT treatment was also observed in EPCs. Importantly, both Id-1 and Endoglin play a key role in angiogenesis. These findings indicate that most elements of TGFβ signaling are present in EPCs and activated upon DHT treatment.

Some groups reported a cross-talk between ALK1 and ALK5 signaling [278]. According to their findings, ALK5 is necessary for the activation of ALK1 pathway. To address this question in EPCs, we used the ALK1 inhibitor ALK1 fc and the ALK5 inhibitor SJN2511. In our experiments, we were able to show that blocking ALK1 abolished DHT-stimulated Smad1/5/8 phosphorylation. Moreover, ALK5 inhibition decreased baseline phospho-Smad2 levels, however, it did not affect DHT-mediated Smad1/5/8 activation. These results suggest that DHT promotes the ALK1/Smad1/5/8 signaling independent of ALK5. The possible explanation for these differences may be associated with the cell type dependent interactions between these receptors and the distinct expression pattern of ALK1 and ALK5 in EPCs.

Recently, besides TGFβ, new ligands for ALK1 have been described: BMP9 and BMP10. BMPs belong to the TGFβ family, and have been intensively investigated for their role in endothelial cell function. Among other BMPs, BMP2 and BMP4 appear to have an important role in ECs. In contrast to TGFβ, BMPs recruit a distinct set of receptors upstream of Smads. BMPs were found to have a binding affinity for type II receptors: BMP receptor II (BMPRII)
and activin receptor II (ACVRII). Moreover, ALK3 and ALK6 are shown to mediate BMP2 and BMP4 effects. In our study, we observed that inhibition of BMP2 and BMP4 using Noggin reduced DHT-stimulated increase in phospho-Smad1/5/8 levels. Since we also demonstrated that ALK1 is involved in DHT-activated Smad1/5/8 signaling, these findings raise some questions. We speculate that ALK1 might interact with ALK3 and ALK6 or ALK1 might substitute ALK3/6 in EPCs, therefore mediating BMP2/4 actions. Additionally, the precise mechanism of the inhibition by Noggin and the possibility that this may block other BMPs remain unclear. However, the participation of BMP9 and BMP10 can be ruled out, as they have been shown to be insensitive to Noggin [279]. As TGFβ/BMP signaling is not well explored in EPCs, further investigations are required to decipher these findings.

Next, we explored the mechanism of DHT-induced ALK1/Smad1/5/8 activation. The rapid induction of Smad1/5/8 by DHT in AR-independent manner led us to the conclusion that the non-genomic actions might be responsible for the observed effects of DHT on ALK1 signaling. As the non-genomic mechanisms involve second messengers, and androgens are known to exert their effects by triggering intracellular calcium signaling, we assumed that calcium might mediate DHT-induced activation of the ALK1/Smad1/5/8 pathway in EPCs. To test this hypothesis, we first measured the flux of the intracellular calcium in response to DHT treatment. Within few minutes after DHT treatment, we were able to detect an increase in calcium levels in EPCs. This intracellular calcium release was blocked by the calcium chelator BAPTA-AM. The calcium signaling cascade can be initiated by GPCR, a receptor located in the membrane. Its activation leads to phospholipase C (PLC) activity, which in turn produces inositol 1,4,5-triphosphate (InsP₃). Ultimately, InsP₃ triggers calcium release via InsP₃ receptors in the endoplasmic reticulum (ER). Androgens have been shown to mediate their non-genomic effects through membrane-associated GPCR [280][281]. Therefore, we used the GPCR inhibitor pertussis toxin (PTX) to study the role of the non-genomic pathway in DHT-stimulated Smad1/5/8 signaling. PTX reduced the stimulatory effects of DHT on the Smad1/5/8 activation. To prove that DHT stimulates calcium release from the ER, we used the PLC inhibitor U73122. We observed that U73122 blocked DHT-induced Smad1/5/8 signaling. Finally, the cytoplasmic calcium chelator BAPTA-AM abolished the phosphorylation of Smad1/5/8 by DHT. Our findings support the hypothesis that calcium signaling is responsible for the non-genomic actions of DHT in stimulating the ALK1/Smad1/5/8 pathway in EPCs.

With regard to the role of calcium in Smad signaling, Eapen et al. reported that in pluripotent mouse mesenchymal cells, calcium release from the ER and subsequent calmodulin activation
led to the phosphorylation of Smad1, therefore indicating the link between calcium and Smad pathways [282]. Interestingly, Dragoni et al. showed the role of intracellular calcium in mediating VEGF-induced growth in EPCs [283]. Our observations provide evidence for DHT-induced calcium signaling in EPCs and for the role of calcium in activating the ALK1/Smad1/5/8 pathway. We also observed that DHT-stimulated Smad1/5/8 activation was inhibited by Noggin. In this regard, we speculate that BMPs might mediate the effects of DHT on EPC function. In fact, BMP4 increases tubulogenesis in bovine aortic endothelial cells (BAEC) [284], whereas BMP2 stimulates cell survival in pulmonary artery endothelial cells (PAEC) [285]. However, contrary results have also been demonstrated. For example, BMP4 inhibits choroidal neovascularization [250]. Growing evidence points towards an important role of the BMP/ALK1/Smad1/5/8 pathway in angiogenesis. Hence, we assessed its role in mediating angiogenic effects of DHT in EPCs. Surprisingly, in our study, we did not observe any effect of the BMP/ALK1/Smad1 signaling on vasculogenesis in EPCs. Neither Noggin nor Smad1 siRNA influenced DHT-induced vasculogenesis in EPCs. Therefore, our results are in contrast with previously published data and highlight the complexity of BMP signaling, which is context- and cell type-dependent.

Since the modulatory effects of BMPs in EC have been documented, and in our study we observed the activation of the BMP/ALK1/Smad1/5/8 signaling by DHT, we questioned what may be the other potential role of this signaling in EPCs.

Several studies have described the role of TGFβ signaling in the vascular vessel maturation. Lamouille et al. reported that constitutively active ALK1 in EC inhibited migration and cell proliferation [288]. These findings suggest a key role of ALK1 signaling in the maturation process of the vessel formation, characterized by inhibition of the proliferation and by vessel establishment via increased EC integrity and recruitment of pericytes and smooth muscle cells. Another study demonstrated that BMP9-induced ALK1/Smad1/5/8 signaling inhibits VEGF-induced sprouting by preventing the differentiation into tip cells in HUVECs and by reducing retinal hypervascularization in vivo [228]. In their study, a cross-talk between ALK1 and Notch is necessary for this effect. Interestingly, we observed that ALK1 inhibition increased dramatically the sprouting of EPCs, also in the presence of DHT or 10%FCS (data not shown). Moreover, BMP9 has been shown to mediate the vessel quiescence [251]. Hence, these findings support the importance of the ALK1/Smad1/5/8 signaling in maintaining the vessel stability and quiescence.
Results and Discussion

Notably, in patients with pulmonary arterial hypertension (PAH) and hereditary hemorrhagic telangiectasia (HHT), mutations in Endoglin, ALK1 and BMPRII are associated with vascular abnormalities. The leaky and fragile blood vessels observed in these patients are likely to be caused by impaired endothelial function and the loss of endothelial integrity. This led us to the hypothesis that the ALK1/Smad1/5/8 pathway might also regulate endothelial integrity and, hence, the barrier function of EC.

Endothelial integrity and selective permeability are provided by dynamic junction complexes, consisting of adherens and tight junctions, desmosomes and gap junctions. Tight junctions (TJ) are highly expressed in EC, especially in such specialized tissues as the BBB and the BRB. In order to verify whether DHT has an effect on modulating TJs in EPCs, we examined the expression of occludin and claudin5, the major TJ proteins in EC. DHT treatment increased the expression of occludin and claudin5. We used thrombin, IL-1β and TNF-α as positive controls, since they are known to inhibit endothelial integrity. In contrast to DHT, these agents decreased occludin and claudin5 expression. We further investigated the role of the ALK1/Smad1/5/8 pathway in mediating the DHT-stimulated up-regulation of TJs in EPCs. Interestingly, it appears that only occludin is regulated by the ALK1/Smad1/5/8 mechanism, since the ALK1 inhibitor and Smad1 siRNA abrogated an increase in occludin but not in claudin5 expression. Occludin and claudin5 do not share homological sequence, suggesting that they have different roles in TJs. For example, *in vivo* study showed that occludin knockout mice elicited several defects in prostate, infertility, gastric epithelium dysplasia, suggesting the role of occludin in regulating the permeability. However, occludin is not required for TJs assembly [145].

Performing a functional assay to measure the cell permeability upon DHT treatment, we found that DHT decreased the paracellular flux of 10kDa Dextran through EPC monolayer compared to control. Moreover, ALK1 inhibition reversed the effects of DHT on the barrier function. Taken together, these results show that DHT may decrease the vascular permeability in EPCs via activation of ALK1 signaling and the subsequent induction of occludin expression. Furthermore, we hypothesize that BMP2 and BMP4 are involved in the regulation of DHT-mediated barrier function, since the permeability in EPCs was increased in the presence of Noggin. Interestingly, DHT was able to block the negative effects of thrombin on permeability, therefore protecting EPC barrier integrity. Whether in this case DHT protection is mediated by the ALK1/Smad1/5/8 needs to be investigated further.

Some evidence points towards a role for the TGFβ/BMP signaling in the endothelial barrier function. For example, TGFβ induced vascular permeability via ALK5 in bovine pulmonary
artery EC [256]. A recent finding has showed the protective role of BMP4 in pulmonary endothelial barrier function [289]. The role of androgens in the barrier function has been demonstrated in regulating TJs in the blood-testis barrier (BTB) [290][291][292]. Moreover, occludin deficient mice are infertile, indicating an important role of occludin in androgen-dependent tissues [145].

Taken together, our findings provide evidence for a novel role of DHT in regulating the endothelial barrier function via the ALK1/Smad1/5/8 signaling. Future investigations are required to reveal the mechanisms of Smad-occludin interaction, which might involve cytoskeleton rearrangements regulated by MAPK and Rho mechanisms as described by others [293][294][295].
5.4 DHT Up-Regulates AR Expression in EPCs and AoSMCs but Induces Differential AR-Mediated Effects on EPC and AoSMC Growth

**OBJECTIVE**

We have demonstrated that the pro-angiogenic effects of DHT in EPCs are mediated by AR. The AR signaling is important in both physiological and pathological conditions, hence, understanding the precise mechanism(s) regulating AR expression in EPCs might be relevant for therapeutic approaches. Hence, we investigated the mechanisms how DHT regulates AR expression in EPCs. Since abnormal growth of smooth muscle cells is another important factor in vascular remodeling processes associated with CVD, we also examined the effects of DHT on AR expression and the proliferation of male human aortic smooth muscle cells (AoSMCs).

**INTRODUCTION**

AR mediates the biological actions of androgens in various cell types. AR is a 110 kDa cytoplasmic protein and belongs to the family of the nuclear receptors, acting as a transcription factor upon ligand binding and subsequently leading to the activation of androgen signaling [24]. Since sex hormones are known to regulate the expression of their receptors, the regulatory effects of DHT on AR expression in EPCs may be of therapeutic importance. In the present study, we investigated the regulation of AR expression by DHT in EPCs and AoSMCs. Quantitative real-time PCR was used to assess the changes in AR mRNA levels, whereas western blotting was applied for the protein expression analysis. The transcription and proteasome inhibitors were applied to study how DHT regulates AR activation.

Several *in vitro* studies have demonstrated that testosterone and DHT augmented migration and proliferation of vascular smooth muscle cells [76][77][78][79]. These results indicate that androgens might induce the deleterious effects in vascular health by stimulating the media-intima thickness of the vascular wall.

**METHODS**

Described in section 4.
RESULTS

5.4.1 DHT Regulates AR by Stabilizing AR Protein in EPCs

Since androgens mediate their biological actions via androgen receptor (AR), we assessed the role of AR in mediating the effects of androgens in EPCs as well as DHT-dependent regulation of AR activity. As shown in Figure 35, AR expression was up-regulated by 100nM DHT in a time-dependent manner. A significant up-regulation of AR was observed at the earliest time point of 4 hours (183%) and after 24 hours the expression reached 430%.

Figure 35: The effects of DHT on AR expression. EPCs were treated with 100nM DHT for 4, 8 and 24 hours. DHT up-regulated AR expression already after 4 hours of treatment. Representative of three experiments.

To further study the exact mechanism of DHT-induced up-regulation in AR expression, we examined the role of transcription, translation and ubiquitin proteasome degradation. To measure AR mRNA levels we first performed RT-PCR. In contrast to AR protein, there was no change in AR mRNA levels in EPCs treated with 100nM DHT for 30 hours (Figure 36A). Moreover, the transcription inhibitor actinomycinD (ActD, 10ng/ml) had no effect on AR mRNA. The fact that β-actin mRNA was decreased by approximately 50% in the cells treated with ActD under identical conditions demonstrated that the transcription was indeed inhibited (Figure 36B). Finally, at the protein level, AR expression was not inhibited by ActD in the presence DHT (176±19.9% and 241±30.5% in DHT- and DHT+ActD-treated cells, respectively) (Figure 36C), implying that DHT does not affect AR gene transcription.
Results and Discussion

Figure 36: DHT does not affect AR mRNA levels. The effects of DHT on the transcription of AR gene were assessed by RT-PCR. (A) AR mRNA levels were not affected neither by treatment with 100nM DHT for 30 hours nor by the transcription inhibitor actinomycinD (10ng/ml). (B) The efficiency of transcription inhibition was confirmed by measuring mRNA levels of β-actin, where ActD decreased β-actin mRNA up to 50%. (C) Western blot representing no effect of ActD on AR expression. All the data are expressed as means ±SEM. Representative of three experiments.

To investigate the role of the translation in regulating DHT-stimulated AR expression, we studied the effects of the protein synthesis inhibitor cycloheximide (CY) on AR expression. As shown in Figure 37, 1μM CY significantly reduced DHT-induced AR expression from 414±0.3% to 201±19.2%. These results suggest a participation of protein synthesis in DHT-induced AR expression.
Results and Discussion

Figure 37: DHT modulates the translation of AR. Western blot showing the effect of protein synthesis inhibitor cycloheximide on AR expression. EPCs were pre-treated with 1μM CY for 1 hour prior to DHT treatment for 24 hours. All the data are expressed as means ±SEM. Representative of three experiments. P< 0.05 versus control, DHT+CY versus DHT.

To ascertain whether protein stabilization plays a role in mediating AR up-regulation, we assessed the effects of the proteasome inhibitor MG132 on AR expression. Treatment with 100nM MG132 mimicked the effects of DHT on AR protein expression by up-regulating AR from 100±24.7% to 361±52.1%. Moreover, when combined with DHT, the stimulatory effects on AR expression were found to be additive and reached 519±15.9% (Figure 38A). To decipher the relative role of proteasomal degradation and protein stabilization, we evaluated the effects of MG132 and DHT on Raf-1, known to undergo proteasomal degradation and is accumulated in the cytoplasm when inhibition of ubiquitin proteasome activity is inhibited. Treatment with 100nM DHT did not affect Raf-1 expression (100±0.3% and 98±10.3% in control and DHT-treated EPCs, respectively), whereas MG132 increased the expression of Raf-1 from 100±0.3% to 127±1.4% (Figure 38B), implying that MG132 and DHT increase AR expression via distinct mechanisms. These findings suggest that DHT may up-regulate AR via the protein stabilization instead of a direct inhibition of the proteasome activity.
Results and Discussion

Figure 38: The role of ubiquitin proteasome degradation in regulating AR expression. (A) 24 hours treatment with 100nM DHT and 100nM proteasome inhibitor MG132 increased AR expression. (B) Raf-1 expression in the presence of DHT and MG132 was assessed by western blot. Only in response to MG132 Raf-1 expression was upregulated. All the data are expressed as means ±SEM. Representative of three experiments. P< 0.05 versus control.

Interestingly, from the functional aspect, we observed a further increase in the capillary formation by DHT-treated EPCs in the presence of the proteasome inhibitor MG132 (Figure 39), which up-regulated AR expression as shown above. These findings confirm the participation of AR in mediating the angiogenic effects of DHT in EPCs.

Figure 39: The proteasome inhibitor promotes vasculogenesis in EPCs. EPCs were pretreated with 100nM MG132 for 24 hours, following the matrigel-based assay for 16 hours. The stimulatory effects of DHT on the capillary formation were enhanced in the presence of MG132. P<0.05 versus control.
Results and Discussion

MG132. All the data are expressed as means ±SEM. Representative of two experiments. *P* < 0.05 versus DHT, DHT + MG132 versus MG132.

5.4.2 DHT Up-regulates AR Expression by Stabilizing AR Protein in AoSMCs

In the present study, we also investigated DHT-dependent AR regulation in male aortic smooth muscle cells (AoSMCs). Here, we observed that treatment with 100nM DHT for 24 hours significantly increased the expression of AR (Figure 40A). Similar to EPCs, the transcription inhibitor actinomycinD (100ng/ml) had no effect on DHT-induced AR expression (Figure 40B). However, DHT was not able to induce AR expression in the presence of the translation inhibitor cycloheximide (10μM) (Figure 40C). Moreover, the proteasome inhibitor MG132 (1μM) further increased AR expression and Raf-1, whereas DHT did not augment Raf-1 levels (Figure 40D). Taken together, these findings demonstrate that similar to EPCs the up-regulation of AR expression by DHT in AoSMCs involves AR protein stabilization and in part modulation of the translation.

![Figure 40: DHT-mediated regulation of AR expression in AoSMCs.](image)

(A) AoSMCs were treated with 100nM DHT for 24 hours in the basal medium containing 0.4%FCS and without supplements. DHT increased significantly AR expression. (B, C, D) ActD (100ng/ml), CY (10μM) and MG132 (1μM) were added to the cells in the presence or absence of 100nM DHT for 24 hours. ActD did not affect AR expression, whereas CY abrogated DHT-induced AR expression and MG132 further increased AR levels. Raf-1 expression was increased only in the presence of MG132. Representative of three experiments.
5.4.3 The Mitogenic Effects of DHT on Male AoSMCs

The contribution of smooth muscle cells (SMC) to the pathogenesis of CVD is well known. For example, increased SMC migration and proliferation within the vascular wall contributes to neointima formation, which subsequently leads to vascular occlusion and deleterious effects on the cardiovascular system. Hence, in this study, we investigated the effects of DHT on the proliferation of male AoSMCs and whether these effects are AR mediated.

First, we evaluated the effects of DHT on the proliferation (cell number) of AoSMCs. AoSMCs were grown in the medium supplemented with 0.4%FCS for 3 days in the presence or absence of test agents. Treatment with 100nM DHT stimulated the growth of male AoSMCs from 100±1.4% to 122±3.3% (Figure 41A). Moreover, flutamide, an AR antagonist, blocked the mitogenic effects of DHT in male AoSMCs from 120±7.4% to 102±0.7% (Figure 41B).

![Figure 41: The stimulatory effects of DHT on AoSMC growth.](image)

(A) AoSMCs were treated with 100nM DHT for 3 days, followed by cell counting on the day 4. Treatment with DHT induced the proliferation of AoSMCs. (B) The AR antagonist flutamide (1µM) significantly reduced the stimulatory effects of DHT on AoSMC growth. All the data are expressed as means ±SEM. Representative of four experiments. *P*<0.05 versus control, DHT+FLU versus DHT.

5.4.4 DHT Modulates the Cell Cycle Regulators in AoSMCs

To further confirm the stimulatory effects of DHT on AoSMC growth, we evaluated the expression of cell cycle regulatory proteins in response to DHT treatment. As shown in Figure 42A, DHT increased the expression of CyclinA and CyclinD1. Moreover, treatment with
100nM DHT down-regulated the expression of cell cycle inhibitory proteins p27 and p21 (Figure 42B).

**Figure 42:** The modulatory effects of DHT on the expression of cell cycle proteins. Western blotting was used to assess the changes in cell cycle protein expression. (A) CyclinD1 and CyclinA were up-regulated after 24 hours of treatment with 100nM DHT. (B) The expression of p27 and p21 were decreased by 100nM DHT. Representative of three experiments.

Taken together, our data suggest that androgens induce the growth of male AoSMCs and these effects are AR mediated. Therefore, androgens may contribute to vascular disorders and remodeling associated with cardiovascular disease in men.

**DISCUSSION**

ARs are known to be expressed in the vascular wall (in endothelial cells, smooth muscle cells) [19] and we have confirmed this finding in our study. Moreover, in the present study, we examined the modulatory effects of DHT on AR expression in EPCs and AoSMCs. We found that DHT up-regulates the expression of AR in both EPCs and AoSMCs. To assess the underlying mechanisms for the AR up-regulation, we investigated the role of transcription, translation and proteasomal degradation.

In both EPCs and AoSMCs, DHT-mediated up-regulation of AR was inhibited by protein synthesis inhibitor cycloheximide but not by the transcription inhibitor actinomycinD. Androgens have been shown to regulate AR expression in other cell types, however, the mechanisms mediating these effects seem to vary. For example, in contrast to our finding in EPCs and AoSMCs, increase in AR protein by DHT was associated with down-regulation of
AR mRNA expression in breast and prostate cancer (AR-positive) cells [297]. Moreover, in osteoblasts DHT increased AR mRNA [298]. These data suggest that the regulation of AR by DHT at a transcriptional level may be cell specific. Since DHT has been shown to be more effective than T in stabilizing AR, it is feasible that AR stability following ligand binding contributes to these effects. In this context, the dissociation rate of T from AR has been shown to be three times faster than DHT [299]. To assess whether AR stability following DHT binding is indeed responsible for increased AR expression, we studied the role of proteasomal degradation in AR expression. Our finding that inhibition of proteasomal activity by MG132 led to an increased AR expression indicates that proteasomal degradation regulates AR turnover. Interestingly, Raf-1 expression, which is known to be degraded solely via the proteasomal mechanism, was up-regulated by MG132. However, Raf-1 expression was not increased in the presence of DHT, suggesting that DHT increases AR expression by promoting AR stabilization and not by inhibiting proteasomal activity. Taken together, our findings demonstrate that mechanisms of AR regulation by DHT in EPCs and AoSMCs involve modulation of translation and AR stabilization.

To assess the functional importance of AR in regulating vascular biology, we examined the effects of DHT on the growth of AoSMCs (contribute to neointima formation and vascular occlusion). Surprisingly, our finding that DHT induced the proliferation of AoSMCs suggests that DHT may exert a negative effect on cardiovascular health via its action on SMC growth. This notion is further supported by the fact that DHT up-regulated the expression of cell cycle regulators CyclinA and CyclinD1, whereas down-regulated the expression of negative regulators of cell cycle p21 and p27. The fact that the growth effects of DHT were blocked by AR antagonist suggests that these effects are AR mediated. Our results are consistent with previous findings from both in vivo and in vitro studies [76][77][78][79].

In EPCs, treatment with DHT promoted vasculogenesis, a hallmark of endothelial repair. Interestingly, the vasculogenic effects of DHT were enhanced when AR expression was induced by inhibitor of proteasomal degradation, and blocked in cells treated with AR antagonist (flutamide) as well as in cells treated with AR siRNA to down-regulate AR expression. Together these findings provide evidence that the vasculogenic effects of DHT on EPCs are AR mediated. Since AR expression in EPCs is induced by DHT, it is tempting to speculate that in elderly men with low androgens receiving EPC therapy, priming of EPCs prior to infusion may facilitate better CV repair and endothelial regeneration, however, in vivo studies are required to investigate this possibility.
The fact that DHT induced disparate effects on AoSMCs and EPCs suggests that androgens might have a dual role in the cardiovascular system of men. On one side androgens may promote vascular repair by stimulating the function of EPCs, on the contrary they may elicit deleterious effects on the cardiovascular system by promoting the growth of AoSMCs and contributing to the remodeling processes. It is feasible that the mitogenic or pro-growth effects of DHT on AoSMCs only occur in the absence of endothelium. This notion is further supported by the fact that EC produce several vasoactive molecules (NO, prostaglandins) which inhibit SMC growth. Moreover, androgens are known to induce these endothelium-secreted growth inhibitors [82][83].

We speculate that androgen effects on the cardiovascular system might depend on the accumulation of other risk factors, therefore TRT may be protective in healthy men, however, it may induce deleterious effects when applied in older men with pre-existing CVD. This might explain the disparate outcomes observed in clinical studies using TRT. It is feasible that the pro-growth effects of DHT on SMCs are lost in the presence of healthy endothelium, however, this hypothesis needs to be experimentally tested.
6 Conclusions and Perspectives

Increase in life expectancy is accompanied with an enhanced demand for improvement in quality of life and prevention of age-related diseases. Since testosterone deficiency has been suggested to be associated with cardiovascular disease (CVD), increased number of aging men undergo testosterone replacement therapy (TRT). However, some studies have reported adverse effects of TRT and increased mortality due to CVD. Hence, there is a need for understanding the role of T in cardiovascular health and whether TRT is beneficial.

The role of EPCs in the cardiovascular repair has been well established and they are currently used in cardiovascular therapy. Interestingly, hypodonalad men have lower levels of EPCs and their function is impaired. Consistent with these findings, our results show the stimulatory effects of DHT on EPC function by promoting vasculogenesis. To ascertain these results, we confirmed them by three in vitro angiogenesis models. Additionally, we were able to demonstrate that a cross-talk between DHT-activated AR and the Akt/mTOR/VEGF signaling pathway mediates the pro-angiogenic effects of DHT in EPCs. Therefore, our research suggests a positive role of androgens in EPC-mediated tissue regeneration, which may be important for the improvement of vascular health.

Our key findings decipher another role of DHT in EPC function. We found that DHT regulates the barrier function of EPCs by increasing the integrity of the endothelial monolayer and decreasing the permeability. We demonstrated that TGFβ signaling through the ALK1/Smad1/5/8 mechanism is implicated in these effects. Interestingly, deficiency in ALK1 signaling is associated with vascular abnormalities related to impaired endothelial integrity in pulmonary arterial hypertension and hereditary hemorrhagic telangiectasia [229][230][231][232][233]. Moreover, we show that DHT up-regulated the expression of the tight junction proteins occludin and claudin5. However, the ALK1/Smad1/5/8 pathway was involved only in the activation of occludin expression. Therefore, we speculate that DHT activates the ALK1/Smad1/5/8 signaling and via occludin it enhances the barrier function. Additionally, since Noggin increased the permeability of EPCs, our findings suggest a novel role of BMP/ALK1/Smad1/5/8 in regulating DHT-induced barrier function in these cells. Enhanced permeability of the endothelium is linked to a hyperdilated structure and leakage of blood vessels, leading to vascular pathologies such as hypertension and stroke. Hence, our research supports the concept of the protective effects of androgens on EPC function by stimulating new blood vessels formation and by improving endothelial integrity.
Conclusions and Perspectives

With regard to AR regulation, our findings demonstrate that DHT up-regulates AR expression by increasing the stability of AR upon ligand binding and therefore preventing proteasome-dependent degradation and in part by modulating its translation. These results allow us to determine the regulatory mechanisms of AR expression, which might be helpful in developing therapeutic approaches to target AR signaling. More importantly, modulation of AR expression in EPCs may be of clinical relevance in men receiving these cells for CV repair.

In the present study, we also evaluated the effects of DHT on the growth of male AoSMCs which contribute to neointimal thickening in vascular remodeling processes. Surprisingly, we found that DHT induces the proliferation of AoSMCs. Accordingly, the cell cycle regulators CyclinA and CyclinD1 were up-regulated by DHT, whereas the cell cycle inhibitors p21 and p27 were down-regulated. Hence, we observed the differential modulation of EPCs and AoSMCs by DHT treatment, suggesting a dual role of DHT in regulating vascular remodeling processes. We hypothesize that a balance between the protective and deleterious effects of androgens may determine the outcome of TRT and the mechanisms regulating this balance have to be investigated further. It is reasonable to assume that androgens exert their protective actions by improving endothelial function and repair, however, under pathological conditions with severe vascular damage or remodeling associated with CVD, they may promote the deleterious effects by stimulating SMC growth and vaso-occlusion. It is feasible that by improving EC function androgens can counteract the proliferative actions on SMCs.

Figure 43: A schematic representation of the observed effects and mechanisms of DHT on EPC function. DHT modulates EPC function by promoting vasculogenesis via the Akt/mTOR/VEGF mechanism and by stimulating the barrier function via the ALK1/Smad1/5/8 signaling.
Taken together, our study provides evidence for the beneficial effects of DHT on EPC function. However, it may also induce the deleterious effects on the vasculature by stimulating SMC proliferation. It is feasible that the proliferative effects of DHT on SMCs occur only in the absence of endothelial cells or presence of endothelial damage. Hence, co-culture studies are required to investigate this possibility. Our findings suggest that prior to infusion into patients priming EPCs with DHT would induce AR expression and improve their endothelial repair. Moreover, treating older men with TRT may stimulate endothelial repair, however, SMC growth may also be induced in the presence of dysfunctional ECs. Based on these findings, we hypothesize that androgens may induce vasoprotective actions in men with intact/normal but not damaged endothelium.
7 References


Natali, and A. Lenzi, “Testosterone-induced relaxation of rat aorta is androgen structure specific and involves K⁺ channel activation Testosterone-induced relaxation of rat aorta is androgen structure specific and involves K⁺ channel activation,” pp. 2742–2750, 2014.


References


[73] P. Alexandersen, J. Haarbo, I. Byrjalsen, H. Lawaetz, and C. Christiansen, “The online version of this article, along with updated information and services, is located on the World Wide Web at: http://circres.ahajournals.org/cgi/content/full/84/7/813,” 1999.


K. Sumiko Yoshida, MD, PhD1,*, Ken-ichi Aihara, MD, PhD1,*, Yasumasa Ikeda, MD, PhD2, Yuka Sumitomo-Ueda, MD, PhD3, Ryoko Uemoto, BSc1, Kazue Ishikawa, BSc4, Takayuki Ise, MD3, Shusuke Yagi, MD, PhD3, Takashi Iwase, MD, PhD3, Yasuhiro Mouri, PhD5, Matomo Sak and P. Walsh, PhD8, Shigeaki Kato, PhD7, and Toshio Matsumoto, MD, “Androgen receptor promotes sex-independent angiogenesis in response to ischemia and is required for activation of vascular endothelial cell growth factor receptor signaling,” vol. 128, no. 1, pp. 3–18, 2014.
References


mesenchymal stem cells are enriched at different gestational ages in human umbilical cord blood.,” *Pediatr. Res.*, vol. 64, no. 1, pp. 68–73, Jul. 2008.


References


References


of Hypoxia-inducible Factor 1 α Expression by the Epidermal Growth Factor / Phosphatidylinositol 3-Kinase / PTEN / AKT / FRAP Pathway in Human Prostate Cancer Cells: Implications for Tumor Angiogenesis and Therapeutics Advances in Brief Prosta,” 2000.


“Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2.”


induced endothelial cell proliferation and VEGF-stimulated angiogenesis.,” *J. Cell Sci.*, vol. 120, no. Pt 6, pp. 964–72, Mar. 2007.


References


[289] Thomas Helbing; Elena Ketterer; Bianca Engert; Jennifer Heinke; Sebastian Grundmann; Christoph Bode; Martin Moser, “Bone Morphogenetic Protein Activity Modulates Endothelial Barrier Function,” in *Arterioscler Thromb Vasc Biol.*, 2012, no. 32: A73, p. 32: A73.


Curriculum Vitae

Personal Data

Name: Yuliya Plutino
Date of Birth: 14 December 1985
Nationality: Belarus
Marital Status: married

Education

05/2011-present
PhD at the Clinic for Reproductive Endocrinology, University Hospital Zurich/ETH, D-BIOL
PhD thesis: “The Role of Androgens in Vascular Remodeling Associated with Cardiovascular Disease”

04/2009-02/2011
Master of Science in Human Biology, University of Zürich
Thesis: “The role of the RAS in β-cell failure in Type 2 Diabetes”

07/2009-08/2009
Participation in Biology Undergraduate Summer School
University of Zurich, Institute of Molecular Cancer Research

09/2003-07/2008
Diploma in Cell Biology
International Sakharov Environmental University, Minsk, Belarus

09/1992-06/2003
Gymnasium, Lida, Belarus

Professional experience

08/2008-04/2009
Junior research assistant, Research Institute of Medical Expertise and Rehabilitation, Minsk

02/2008-03/2008
Practice in teaching biology, Gymnasium, Minsk
Publications and Presentations

Publications in Preparation

- *Regulation of Androgen Receptor Expression in Endothelial Progenitor Cells (EPCs) by DHT: Potential Implications for EPC Mediated Cardiovascular Repair in Men* (in preparation) (Y.Plutino et al.)
- *Intracellular Calcium Mediates Non-Genomic Phospho-Smad1/5/8 Activation by DHT in EPCs* (in preparation) (Y.Plutino et al.)
- *DHT Induces Vasculogenesis and Barrier Function in EPCs: Evidence for Differential Role of ALK1/Smad1/5/8 and Akt/mTOR/VEGF pathways* (in preparation) (Y.Plutino et al.)

Oral Presentations:

- *Dihydrotestosterone Regulates Androgen Receptor Expression in Endothelial Progenitor Cells*, 12th Day of Clinical Research, University Hospital Zürich, 2013
- *The role of androgens in cardiovascular disease*, Gynäkologisch-Endokrinologisches Kolloquium, University Hospital Zürich, 2012

Poster Presentations:

- Y. Plutino, F. Barchiesi, B. Imthurn, R. Dubey. *Activation of Intracellular Calcium Triggers DHT-induced phospho-Smad1/5/8 Signaling in EPCs*, 13th Day of Clinical Research, University Hospital Zürich, 2014.
- Y. Plutino, F. Barchiesi, N. Simigdala, B. Imthurn, R. Dubey. *Capillary Formation and Autologous Regulation of Androgen Receptor Expression in Endothelial Progenitor Cells (EPCs) by DHT: Potential Implications for EPC Mediated Cardiovascular Repair in...*


