Role of estrogen receptor signaling in the control of the hair follicle cycle

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INDEX

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figures</td>
<td>7</td>
</tr>
<tr>
<td>Tables</td>
<td>8</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>9</td>
</tr>
</tbody>
</table>

1. INTRODUCTION .................................................................................................................. 11
1.1 Physiology of hair growth ......................................................................................... 12
  1.1.1 Morphogenesis ........................................................................................................ 12
  1.1.2 The hair follicle cycle .......................................................................................... 14
  1.1.3 Control of hair follicle development and cycling .............................................. 16
  1.1.4 Hair follicle structure .......................................................................................... 17
1.2 Estrogens ......................................................................................................................... 21
  1.2.1 Ovarian steroidogenesis ............................................................................................ 22
  1.2.2 Extraovarian steroidogenesis .................................................................................... 24
1.3 Estrogen receptors .......................................................................................................... 24
  1.3.1 ER-structure .............................................................................................................. 25
  1.3.2 ER-Expression ............................................................................................................ 27
  1.3.3 Signaling pathways ..................................................................................................... 28
  1.3.4 Coactivators/Corepressors ......................................................................................... 32
  1.3.5 ERα versus ERβ- agonist or antagonist ...................................................................... 34
  1.3.6 Internal regulation of ERs ......................................................................................... 36
  1.3.7 Cross-talks ................................................................................................................ 37
  1.3.8 Endogenous modulators of ER-expression .................................................................. 39
  1.3.9 Animal models for estrogen research ...................................................................... 41
1.4 Role of estrogens in hair biology ..................................................................................... 43
  1.4.1 Role of estrogens in skin biology .............................................................................. 43
  1.4.2 Steroidogenic enzyme-activity in the skin ................................................................. 45
  1.4.3 Role of estrogens in hair growth and hair follicle cycling ...................................... 46
  1.4.4 ER-expression in the skin and the pilosebaceous unit ............................................ 47
1.5 E2-responsive genes ......................................................................................................... 47
1.6 Experimental design ........................................................................................................ 48
1.7 Specific questions addressed ........................................................................................ 49

2. MATERIALS AND METHODS .................................................................................................. 50
2.1 Animals and tissue collection ......................................................................................... 50
  2.1.1 C57BL/6 mice .............................................................................................................. 50
  2.1.2 Anagen-induction by depilation .................................................................................. 50
  2.1.3 Skin-organ-culture ..................................................................................................... 54
  2.1.4 ERβ-deficient mice .................................................................................................... 55
  2.1.5 Organ-culture of microdissected human hair follicles ............................................. 56
2.2 Immunohistochemistry ...................................................................................................... 57
  2.2.1 Hematoxylin & Eosin staining ..................................................................................... 57
  2.2.2 Ki-67-staining for human hair follicles ..................................................................... 58
  2.2.3 TUNEL-staining (in-situ apoptosis) .......................................................................... 58
  2.2.4 Estrogen receptor alpha, beta, beta ins 18aa .......................................................... 59
## Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Image Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. 1.1</td>
<td>Comparison of human scalp and murine pelage anagen VI hair follicle</td>
<td>12</td>
</tr>
<tr>
<td>F. 1.1.1.1</td>
<td>Beginning of hair follicle morphogenesis</td>
<td>14</td>
</tr>
<tr>
<td>F. 1.1.1.2</td>
<td>Development and cycling of hair follicles</td>
<td>15</td>
</tr>
<tr>
<td>F. 1.1.4.1</td>
<td>HF in three different hair cycle stages</td>
<td>19</td>
</tr>
<tr>
<td>F. 1.1.4.1</td>
<td>HF in three different hair cycle stages</td>
<td>20</td>
</tr>
<tr>
<td>F. 1.2.1</td>
<td>Conversion of inactive hormonal precursors into active sex steroids</td>
<td>21</td>
</tr>
<tr>
<td>F. 1.2.2</td>
<td>Steroidogenic pathway in the ovary</td>
<td>23</td>
</tr>
<tr>
<td>F. 1.3.1.1</td>
<td>Structure and domains of human ERα and ERβ</td>
<td>27</td>
</tr>
<tr>
<td>F. 1.3.3.1</td>
<td>ER-signaling pathways</td>
<td>29</td>
</tr>
<tr>
<td>F. 1.3.5.1</td>
<td>Structure of E2, tamoxifen and ICI 182,780</td>
<td>36</td>
</tr>
<tr>
<td>F. 1.3.7.1</td>
<td>Scheme of proposed IGF-I/ER cross-talk in the uterus</td>
<td>39</td>
</tr>
<tr>
<td>F. 2.1.2.1</td>
<td>Depilation of telogen back skin hairshafts (I)</td>
<td>52</td>
</tr>
<tr>
<td>F. 2.1.2.2</td>
<td>Depilation of telogen back skin hairshafts (II)</td>
<td>53</td>
</tr>
<tr>
<td>F. 2.3.2.1</td>
<td>Ki-positive matrix keratinocytes below Aubers’ line</td>
<td>64</td>
</tr>
<tr>
<td>F. 2.3.3.1</td>
<td>Measurement of the dermal thickness in the catagen back skin</td>
<td>65</td>
</tr>
<tr>
<td>F. 2.5.1</td>
<td>Microarray analysis</td>
<td>67</td>
</tr>
<tr>
<td>F. 3.1.1</td>
<td>ERα IR during the depilation-induced murine hair cycle</td>
<td>70</td>
</tr>
<tr>
<td>F. 3.1.1</td>
<td>ERα IR during the depilation-induced murine hair cycle (scheme)</td>
<td>71</td>
</tr>
<tr>
<td>F. 3.1.2</td>
<td>ERβ IR during the depilation-induced murine hair cycle</td>
<td>72</td>
</tr>
<tr>
<td>F. 3.1.2</td>
<td>ERβ and ERβ ins IR during the depilation-induced murine hair cycle (scheme)</td>
<td>73</td>
</tr>
<tr>
<td>F. 3.1.2</td>
<td>ERβ ins IR during the depilation-induced murine hair cycle</td>
<td>74</td>
</tr>
<tr>
<td>F. 3.1.3</td>
<td>Semi-quantitative RT-PCR of ERα and ERβ in the depilation-induced murine hair cycle</td>
<td>75</td>
</tr>
<tr>
<td>F. 3.2.1</td>
<td>IR of ERα after murine skin organ culture</td>
<td>78</td>
</tr>
<tr>
<td>F. 3.2.2</td>
<td>IR of ERβ after murine skin organ culture</td>
<td>79</td>
</tr>
<tr>
<td>F. 3.2.3</td>
<td>IR of ERβ after murine skin organ culture</td>
<td>80</td>
</tr>
<tr>
<td>F. 3.2.4</td>
<td>mRNA-levels of ERα and ERβ after short term murine skin organ culture</td>
<td>82</td>
</tr>
<tr>
<td>F. 3.3.1</td>
<td>Semi-quantitative RT-PCR of ERβ after short term murine skin organ culture</td>
<td>84 - 85</td>
</tr>
<tr>
<td>F. 3.4.1</td>
<td>IR of ERα after melatonin treatment in murine skin</td>
<td>87</td>
</tr>
<tr>
<td>F. 3.4.2</td>
<td>IR of ERβ after melatonin treatment in murine skin</td>
<td>88</td>
</tr>
<tr>
<td>F. 3.4.3</td>
<td>IR of ERβ ins after melatonin treatment in murine skin</td>
<td>89</td>
</tr>
<tr>
<td>F. 3.5.1</td>
<td>IR of ERβ in female scalp HF after melatonin treatment</td>
<td>91</td>
</tr>
<tr>
<td>F. 3.5.2</td>
<td>IR of ERβ in female scalp HF after prolactin treatment</td>
<td>92</td>
</tr>
<tr>
<td>F. 3.6.1</td>
<td>IR of ERβ in female and male frontotemporal scalp HF</td>
<td>93</td>
</tr>
<tr>
<td>F. 3.7.1</td>
<td>IR of ERβ in male scalp HF after E2 treatment</td>
<td>95</td>
</tr>
<tr>
<td>F. 3.8.1</td>
<td>Hair shaft elongation after E2-treatment in male scalp HF</td>
<td>96</td>
</tr>
<tr>
<td>F. 3.8.2</td>
<td>Hair shaft elongation after E2-treatment in male scalp HF</td>
<td>97</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>F. 3.8.3</td>
<td>Hair shaft elongation after E2-treatment in female scalp HF</td>
<td>98</td>
</tr>
<tr>
<td>F. 3.8.4</td>
<td>Proliferation of matrix keratinocytes after E2-stimulation in male scalp HF</td>
<td>99</td>
</tr>
<tr>
<td>F. 3.8.5</td>
<td>Proliferation of matrix keratinocytes after E2-stimulation in female scalp HF</td>
<td>99</td>
</tr>
<tr>
<td>F. 3.8.6</td>
<td>E2 administration slightly prolongs the anagen phase</td>
<td>100</td>
</tr>
<tr>
<td>F. 3.9.1</td>
<td>E2 treatment changes the distribution pattern of IGF-I</td>
<td>102</td>
</tr>
<tr>
<td>F. 3.10.1</td>
<td>Catagen development in ERβ-deficient mice</td>
<td>103</td>
</tr>
<tr>
<td>F. 3.10.2</td>
<td>Dermal thickness in ERβ-deficient mice</td>
<td>103</td>
</tr>
<tr>
<td>F. 3.10.3</td>
<td>TUNEL positive cells in ERβ-deficient mice</td>
<td>104</td>
</tr>
<tr>
<td>F. 3.11.1</td>
<td>IR of ERα in the skin of ERβ-deficient mice</td>
<td>105</td>
</tr>
<tr>
<td>F. 3.12.1</td>
<td>Scatter plot of cDNA microarray from human scalp hair follicles</td>
<td>107</td>
</tr>
<tr>
<td>F. 3.12.2</td>
<td>IR of K6HF in murine skin</td>
<td>110</td>
</tr>
<tr>
<td>F. 3.12.3</td>
<td>IR of K6HF in human scalp HF</td>
<td>111</td>
</tr>
</tbody>
</table>

### Tables

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. 1.1.3.1</td>
<td>Molecular mediators of hair follicle growth (I)</td>
<td>16</td>
</tr>
<tr>
<td>T. 1.1.3.1</td>
<td>Molecular mediators of hair follicle growth (II)</td>
<td>17</td>
</tr>
<tr>
<td>T. 1.1.4.1</td>
<td>Glossary of anatomical terms frequently used in hair research</td>
<td>18</td>
</tr>
<tr>
<td>T. 1.4.1</td>
<td>Growth and transcription factors, cytokines and hormones (selection) with connections to the hair follicle cycle and E2/ER</td>
<td>43</td>
</tr>
<tr>
<td>T. 3.12.1</td>
<td>E2 responsive genes in human scalp HF (up/down-regulation)</td>
<td>108</td>
</tr>
<tr>
<td>T. 3.12.2</td>
<td>E2 responsive genes in human scalp HF (sex-dependent regulation)</td>
<td>109</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Androstendione</td>
</tr>
<tr>
<td>AF</td>
<td>activation function</td>
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<td>AIB1</td>
<td>amplified in breast cancer</td>
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<td>AP-1</td>
<td>activating protein-1</td>
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<td>APM</td>
<td>M. arrector pili</td>
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<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>ArKO</td>
<td>Aromatase-knock out mouse</td>
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<tr>
<td>BERKO</td>
<td>estrogen receptor beta-knock out mouse</td>
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<tr>
<td>BG</td>
<td>bulge region</td>
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<tr>
<td>CBP</td>
<td>cAMP response element-binding protein</td>
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<tr>
<td>CL</td>
<td>club hair</td>
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<td>CR</td>
<td>cortex</td>
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<tr>
<td>CTS</td>
<td>connective tissue sheath</td>
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<tr>
<td>CU</td>
<td>cuticle</td>
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<td>DAB</td>
<td>diaminobenzamine</td>
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<td>DAPI</td>
<td>4`, 6-diamidine -2´-phenylindole-dihydrochloride</td>
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<td>DBD</td>
<td>DNA-binding domain</td>
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<td>DHEA</td>
<td>Dihydroepiandrostendione</td>
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<td>DHT</td>
<td>Dihydrotestosterone</td>
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<td>DP</td>
<td>dermal papilla</td>
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<td>DRM</td>
<td>dermis</td>
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<tr>
<td>E1</td>
<td>Estrone</td>
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<td>E2</td>
<td>17ß-estradiol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EPI</td>
<td>epidermis</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<td>ERE</td>
<td>estrogen response element</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
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<td>estrogen receptor ß</td>
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<td>ERß ins</td>
<td>estrogen receptor ß ins</td>
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<tr>
<td>ERα</td>
<td>estrogen receptor ß α</td>
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<tr>
<td>ERγ</td>
<td>estrogen receptor ß γ</td>
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<td>ES</td>
<td>epithelial strand</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GC</td>
<td>germ capsule</td>
</tr>
<tr>
<td>GM</td>
<td>glassy membrane</td>
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<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>HF</td>
<td>hair follicle</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HM</td>
<td>hair matrix</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HS</td>
<td>hair shaft</td>
</tr>
<tr>
<td>HSD</td>
<td>17ß-hydroxysteroid-dehydrogenase</td>
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<tr>
<td>hsp</td>
<td>heat shock protein</td>
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<tr>
<td>ICI 182,780</td>
<td>Imperial Chemical Industries 182,780</td>
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<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactivity</td>
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<td>IRS</td>
<td>inner root sheath</td>
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<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
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<tr>
<td>LBD</td>
<td>ligand binding domain</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>MK</td>
<td>matrix keratinocytes</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MR-KO</td>
<td>mineralocorticoid receptor-knock out mouse</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
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<td>ORS</td>
<td>outer root sheath</td>
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<tr>
<td>p/p.p.</td>
<td>post partum</td>
</tr>
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<td>P450arom</td>
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</tr>
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<td>17ß-hydrolase</td>
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<td>P450scc</td>
<td>CYP11A1</td>
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<td>PBS</td>
<td>phosphate-buffered saline solution</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
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<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SG</td>
<td>sebaceous gland</td>
</tr>
<tr>
<td>SP-1</td>
<td>GC-box binding protein</td>
</tr>
<tr>
<td>SRC-1</td>
<td>steroid receptor coactivator 1</td>
</tr>
<tr>
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<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>STS</td>
<td>steroid sulfatase</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
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<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIF-2</td>
<td>transcriptionally intermediary factor-2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

The hair follicle (HF) is a characteristic feature of mammals and the only organ that underlies a life-long cyclic transformation which is characterized by three distinct stages: growth (anagen), regression (catagen) and resting (telogen). While only the minority of the human patients with hair growth disorders have a disturbed hair shaft production, more often the hair loss is due to alterations in hair follicle cycling. The most common type of alopecia, male and female pattern baldness is due to an increased shedding of the club hair (telogen effluvium) which results from a progressive shortening of the anagen phase, entering in a miniaturization of the follicle (ROOK and DAWBER 1991). Patients with alopecia or excessive hair growth (hirsutism, hypertrichiosis) suffer tremendously and although previous investigations on this topic made great effort in the past, there is still too little known about the pathophysiology and biology of the HF and an effective treatment of hair growth disorders is still missing. Estrogens (17-ß-estradiol, E2) can profoundly modulate hair growth, acting largely as hair growth inhibitors and are frequently used in trichological practise (STERRY and PAUS 2000). Therefore, the aim of this study is to obtain more detailed information about mechanisms and influence of estrogens and estrogen receptor signaling on the hair follicle cycle.
1.1 Physiology of hair growth

The skin and the HF have multiple tasks, the produced hair shaft itself fulfills several functions, i.e. protection against environmental trauma, thermoregulation, social communication and mimicry (STENN and PAUS 2001).

Each HF is rhythmically transformed and regenerated, termed the hair follicle cycle. The cycle length varies species-specific, e.g. it takes years in human, weeks in mice. But the developmental and cycle stages as well as the basic follicle transformations underly the same pattern in human and murine skin (KLIGMAN 1959) (Fig. 1.1) and will be shown by means of mice.

![Comparison of human scalp (left) and murine pelage (right) anagenVI hair follicles. from: (MILLAR 2002)](image)

1.1.1 Morphogenesis

It has been known that HF morphogenesis (Fig.1.1.1.1, Fig. 1.1.1.2) is governed by a series of inductive events or “messages” that the epidermal keratinocytes committ to hair follicle specific differentiation and the mesenchymal cells that form follicular papilla send each other to achieve progression to the next developmental stage (HARDY 1992). This results in the construction of the hair bulb, in which keratinocytes rapidly proliferate and differentiate into six distinct cell compartments,
Introduction

forming the medulla, cortex and cuticle of the hair shaft, as well as the cuticle, Huxley and Henle layers of the inner root sheath (IRS). The latter separates the hair shaft from the outer root sheath (ORS), which forms the external concentric layer of epithelial cells in the HF (SENGEL 1976). The hair follicle morphogenesis can be divided into eight consecutive stages, each characterized by a unique expression pattern, which is governed by a plethora of growth factors, growth factor antagonists, adhesion molecules and intracellular signal transduction components (BOTCHKAREV and PAUS 2003). The different stages start with an accumulation of nuclei, the so-called pregerm (PINKUS 1958), which develops into circumscribed epidermal thickening of enlarged keratinocytes in the basal layer of the epidermis, termed the hair peg (DRY 1926), forming a broad column, in which the epithelial keratinocytes become concentrically arranged around the follicular axis (Fig.1.1.1.1).

At this time, the dermal papilla (DP) is recognizable as a condensation of fibroblasts at the proximal end of the column. The hair peg elongates and the IRS starts to develop as a cone-shaped structure, in pigmented skin visible melanin formation can be found microscopically (PAUS et al. 1999). By reaching the hypodermal muscle layer, the panniculus carnosus, the HF has its maximal length and its prominent hair shaft emerges through the epidermis. This constitutes the end of morphogenesis and thereafter the onset of the first hair cycle (day 8 p.p. in C57BL/6 mice). First recognizable cyclic changes of HF activity start on day 15-17 p.p., when the HF enters a stage of physiological apoptosis-driven involution (catagen) (STRAILE et al. 1961; DEPLEWSKI and ROSENFIELD 2000; STENN and PAUS 2001).

In mice, the hair growth cycle occurs in a wave-like synchronous pattern starting from neck to tail. So, it is possible to find regions where all follicles have entered the next stage in contrast to a more distant region on the back skin within a single mouse. In contrast to rodents, each follicle in the scalp of human skin behaves independently of its neighbours, thus it is a “mosaic” pattern of hair replacement (CHASE 1954).
**Fig.1.1.1.1 Beginning of hair follicle morphogenesis (stage 0-stage 3)**

Stage 0: accumulation of nuclei (pre-germ), stage 1: epidermal thickening (hair peg), stage 2: forming of a broad column with concentrically arranged keratinocytes, stage 3: dermal papilla (DP) at the proximal end of the hair peg.

**1.1.2 The hair follicle cycle**

The cycling of the hair follicle (**Fig.1.1.1.2**) is divided into three distinct phases and characterized by a life-long regression and proliferation activity. The **anagen** or growth phase (anagen I-VI) starts with the proliferation of secondary germ cells in the bulge region and is characterized by a massive proliferation and differentiation of keratinocytes of the hair matrix, as well as the remodelling of perifollicular innervation, the HF immune system and the pigmentation of the hair shaft by follicular melanogenesis. The **catagen** or regression phase (catagen I-VIII) is characterized by a programmed, apoptosis-driven involution of the lower part of the HF, the termination of follicular melanogenesis, formation of the club hair, condensation and upward movement of the DP. The **telogen** or resting phase implicits the relative quiescence of the HF, since the epithelial remnants of the telogen HF (distal ORS, secondary hair germ, bulge) are engaged, e.g. in substantial biochemical activity and some degree of proliferation (PAUS and COTSARELIS 1999; MULLER-ROVER et al. 2001).
Fig. 1.1.1.2 Development and cycling of hair follicles (PAUS and COTSARELIS 1999)
Key stages of morphogenesis (0-8). Anagen (growth), catagen (regression), telogen (resting).
1.1.3 Control of hair follicle development and cycling

The HF transition between distinct stages of development and postnatal cyclic regeneration is governed by a bidirectional signal exchange between follicular keratinocytes and fibroblasts of the follicular dermal papilla. Obviously, many growth stimulators and inhibitors which are involved in the regulation of HF development also control the cyclic activity in postnatal HFs (Table 1.1.3.1). The DP is supposed to be the control centre of follicle growth, initiating and terminating anagen. The hair follicle development is due to DP fibroblasts and its contact to hair matrix keratinocytes (JAHODA and REYNOLDS 1993), which signals act on the epithelial stem cells of the follicle to initiate anagen (bulge activation hypothesis). The stem cells are supposed to generate rapidly dividing transit amplifying cells, which migrate toward the DP for constructing a new hair bulb (LAVKER et al. 1993). The exact signaling of this mechanism remains to be elucidated.

Table 1.1.3.1 Molecular mediators of hair follicle growth (part I), modified after (STENN and PAUS 2001):

<table>
<thead>
<tr>
<th>Molecular Mediator</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast growth factor (FGF), -receptor</td>
<td>terminates anagen</td>
<td>(DU CROS 1995; ROSENQUIST and MARTIN 1996)</td>
</tr>
<tr>
<td>Transforming growth factor (TGF)-ß, -receptor</td>
<td>catagen development</td>
<td>(LITTLE et al. 1994)</td>
</tr>
<tr>
<td>Bone morphogenic protein (BMP)</td>
<td>suppresses hair growth</td>
<td>(BOTCHKAREV 2003)</td>
</tr>
<tr>
<td>Keratinocyte growth factor (KGF)</td>
<td>induces anagen</td>
<td>(ROSENQUIST and MARTIN 1996)</td>
</tr>
<tr>
<td>Insulin-like growth factor (IGF)-I, receptor, binding proteins</td>
<td>stimulates hair growth</td>
<td>(PHILPOTT et al. 1994; HEMBREE et al. 1996)</td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>stimulates cell growth in the ORS</td>
<td>(MOORE et al. 1981)</td>
</tr>
<tr>
<td>Hepatocyte growth factor (HGF), -receptor</td>
<td>epithelial-mesenchymal interactions</td>
<td>(LINDNER et al. 1997)</td>
</tr>
<tr>
<td>Vitamin D receptor</td>
<td>mutation results in alopecia</td>
<td>(REICHRATH et al. 1994)</td>
</tr>
<tr>
<td>Noggin</td>
<td>antagonist to BMP</td>
<td>(BOTCHKAREV et al. 2001)</td>
</tr>
<tr>
<td><strong>Table 1.1.3.1 Molecular mediators of hair follicle growth (part II), modified after (STENN and PAUS 2001)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>17-β-estradiol</strong></td>
<td>Inhibits hair growth</td>
<td>(OH and SMART 1996)</td>
</tr>
<tr>
<td><strong>Prolactin</strong></td>
<td>Stimulates catagen development</td>
<td>(PEARSON et al. 1999)</td>
</tr>
<tr>
<td><strong>Androgen receptor</strong></td>
<td>“paradoxical” site-dependent</td>
<td>(ITAMI et al. 1995)</td>
</tr>
<tr>
<td><strong>Aromatase cytochrome</strong></td>
<td>Steroidogenic enzyme located in the hair follicle and sebaceous gland</td>
<td>(SAWAYA and PENNEYS 1992)</td>
</tr>
<tr>
<td><strong>5alpha-reductase type I/II</strong></td>
<td>Steroidogenic enzyme located in the hair follicle and sebaceous gland</td>
<td>(SAWAYA and PRICE 1997)</td>
</tr>
</tbody>
</table>

### 1.1.4 Hair follicle structure

Various types of hair shafts exist, in mice at least 8 major hair types have been described: pelage or truncal hair, vibrissae, cilia/eyelashes, tail hair, ear hair, hair of the foot area, genital/perianal hair and nipple hair. Regardless of the type, all of them have similar structural features. The shaft is composed of a cuticle (thin, overlapping scales), a cortex (hollow cylinder of hardened, cornified material) and a medulla (cells separated by air-filled spaces in different arrangements) (SUNDBERG and HOGAN 1994).

The HF can be divided into two cell types, the epithelial and the mesenchymal cells. During anagen, the lower portion of the thick, onion-shaped bulb consists of mitotically active, pluripotential matrix cells, becoming cells of the medulla, cortex, cuticle and IRS. The cuticle forms the outermost layer of the emerging hair. The cuticular cells are situated between the cortical and IRS cells in the bulb region and are the last to start their differentiation. The IRS contains three different layers, the cuticle, the Huxley and the Henle layer, which cells are shed into the pilary canal at the level of the sebaceous gland. The ORS, surrounding the IRS, is multilayered and continuing with the epidermis at the emerging end of the HF, attenuated at the lower level of the bulb. Matrix cells, medulla, cortex and IRS/ORS represent ectodermal derivatives, whereas the dermal papilla, the connective tissue sheath and the hyaline membrane are ectomesodermal derivatives (neural crest). During anagen, the DP is enclosed by the bulb and the connective tissue sheath which surrounds the HF on
the dermal side. Noteworthy the anagen-coupled activity of melanocytes, embedded on top of the DP into matrix keratinocytes (Fig. 1.4.1, Table 1.4.1) (MONTAGNA and PARAKKAL 1974).

**Table 1.4.1: Glossary of anatomical terms frequently used in hair research** (MULLER-ROVER et al. 2001)

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulb</td>
<td>Prominent onion-shaped thickening on the proximal end of the HF, relatively undifferentiated matrix cells, melanocytes and cells from the proximal ORS</td>
</tr>
<tr>
<td>Bulge</td>
<td>Convex extension of the distal part of the ORS, near the epidermis, location of epithelial follicle stem cells and insertion of M. arrector pili</td>
</tr>
<tr>
<td>Club hair</td>
<td>Resting hair shaft with a hollow brush of keratinized keratinocytes on the proximal end, tightly attached to the cortical cells of the hair cortex</td>
</tr>
<tr>
<td>Connective tissue sheath (CTS)</td>
<td>Part of the dermal connective tissue, tightly attached to the outer side of HF, composed of fibroblasts, macrophages and connective tissue</td>
</tr>
<tr>
<td>Dermal papilla (DP)</td>
<td>Mesodermal part of the HF, closely packed mesenchymal cells, framed by the bulb matrix during anagen</td>
</tr>
<tr>
<td>Epithelial strand (ES)</td>
<td>Column of epithelial cells between the germ capsule and the compact DP, laterally demarcated by the thickened glassy membrane</td>
</tr>
<tr>
<td>Secondary germ capsule (GC)</td>
<td>Syn. Secondary hair germ, Bag-like structure of glycogen-free cells of distal ORS, surrounding the club hair</td>
</tr>
<tr>
<td>Hair shaft</td>
<td>Terminally differentiated HF keratinocytes (trichocytes), divided into hair cuticle, cortex and medulla</td>
</tr>
<tr>
<td>Hyaline membrane</td>
<td>Outermost noncellular part of the HF, basal lamina and two layers of orthogonally arranged collagen fibers, separates ORS from CTS</td>
</tr>
<tr>
<td>Isthmus</td>
<td>Middle portion of the HF extending from the sebaceous duct to the insertion of m. arrector pili (bulge region)</td>
</tr>
<tr>
<td>Inner root sheath (IRS)</td>
<td>Multilayered structure composed of terminally differentiated HF keratinocytes surrounded by the ORS, surrounds the hair shaft up to to hair canal</td>
</tr>
<tr>
<td>Outer root sheath (ORS)</td>
<td>Outermost sheath of HF keratinocytes, merges distally into the basal layer of the epidermis and proximally into the hair bulb</td>
</tr>
<tr>
<td>Sebaceous gland (SG)</td>
<td>Glandular structure close to the insertion of the m. arrector pili with holocrine function, lipid-filled sebocytes</td>
</tr>
</tbody>
</table>
Fig. 1.1.4.1 HF in three different hair cycle stages (part I):
schematic drawing of anagen VI, catagen and telogen HF (left to right)
abbreviations: apm-arrector pili muscle, club-club hair, cts-connective tissue sheath,
gm-glassy membrane, irs-inner-root sheath, HM-matrix cells, mel-melanocytes, ors-
outer-root sheath, sg-sebaceous gland
Fig. 1.1.4.1 HF in three different hair cycle stages (part II)
Hematoxylin and Eosin staining, structure of scalp HF during anagen (A,B), catagen (C) and telogen (D) from: (PAUS and COTSARELIS 1999). Abbreviations: apm-arrector pili muscle, bg-bulge, cl-club hair, Cr-cortex, cts-connective tissue sheath, cu-cuticle, drm-dermis, epi-epidermis, gm-glassy membrane, hm-hair matrix, hs-hair shaft, iec-involuting epithelial column, irs- inner-root sheath, m-matrix cells, ors-outer-root sheath, sg-sebaceous gland, sc-subcutaneous fat
1.2 Estrogens

The gonads are the major source of circulating sex steroid hormone levels in the body. Besides these, several peripheral tissues contain enzyme activities to synthesize active androgens and estrogens. Precursors are locally converted into active sex steroids so that they can exert biological actions directly. The most important enzymes involved are the aromatase (p450arom, mediating the conversion of the androgens androstenedione (A) and testosterone (T) into the estrogens estrone (E1) and 17ß-estradiol (E2)), the type I 17ß-hydroxysteroid dehydrogenase (HSD, A into T and E1 into E2) and the type II 17ß-HSD, which catalyzes the conversion in the opposite direction (Fig.1.2.1). Steroid sulfatase (STS) catalyzes the formation of DHEA and E1 from their respective sulfated precursors, and 5α-reductase irreversibly converts T into dihydrotestosterone (DHT) (VAN DER EERDEN et al. 2002).

Fig. 1.2.1 Conversion of inactive hormonal precursors into active sex steroids
1.2.1 Ovarian steroidogenesis

The preovulatory follicle secretes estradiol during the first half of the menstrual cycle, whereas the corpus luteum secretes both estradiol and progesterone during the second half of the cycle. The production of these two biologically active steroids is orchestrated in the follicle and corpus luteum in a cell-specific manner under the control of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (BULUN and ADASHI 2003). The major C19-steroid product of the ovary, androstendione, is biologically inactive but functions as a dual precursor and contributes to circulating levels of estrone and testosterone through its conversion in extraglandular tissues such as adipose tissue and skin (HEMSELL et al. 1974). The estrogenically weak estrone is locally converted to the potent estrogen estradiol in target tissues, e.g. brain, genital skin. The naturally occurring estrogens are C18-steroids characterized by the presence of an aromatic A ring, a phenolic hydroxyl group at C-3, and either a hydroxyl group (estradiol) or ketone group (estrone) at C-17. P450_{arom} is the key enzyme for estrogen production in the ovary. The specific activity of the aromatase enzyme complex and its production in the granulosa cells is regulated primarily by FSH. All C18-steroids including estrone, estradiol and estriol are commonly referred to as estrogens. Estrone and estriol are only weak estrogens and must be converted to estradiol to exert full estrogenic activity. The principal and most potent estrogen secreted by the ovary is 17ß-estradiol (E2). Although estrone is secreted by the ovary too, another important source of estrone is the extraglandular conversion of androstenedione in peripheral tissues (BULUN and ADASHI 2003). Estriol (16-hydroxyestradiol) is the most abundant estrogen in urine and is produced by the metabolism of estrone and estradiol in extraovarian tissues. There are at least seven enzymes in the 17ß-hydroxysteroid-dehydrogenase (HSD) family with overlapping activities, which are capable of converting estrone to estradiol in the ovary and extraovarian tissues (PELTOKETO et al. 1999).

The ovary secretes a variety of C19-steroids, including dehydroepiandrosterone (DHEA), androstendione and testosterone. They are produced by the thecal cells and to a minor degree by the ovarian stroma. Androstenedione can be converted to estrogen or testosterone in the ovary as well as extraglandular tissues. Steroids in
general derive from cholesterol. There are several sources of cholesterol that can provide the ovary with substrate for steroidogenesis: plasma lipoprotein cholesterol, cholesterol synthesized de novo within the ovary and cholesterol from intracellular stores. The first and rate-limiting step in the synthesis of all steroid hormones is the movement of cholesterol into the mitochondrion, which is regulated by StAR (steroidogenic acute regulatory protein). This step is followed by conversion of cholesterol to pregnenolone, catalyzed by the mitochondrial enzyme complex consisting of p450\textsubscript{scC}, adrenodoxin and flavoprotein (BULUN and ADASHI 2003) (Fig.1.2.2).

![Steroidogenic pathway in the ovary](image)

Fig. 1.2.2 Steroidogenic pathway in the ovary

Estradiol production requires the activity of six steroidogenic proteins and six enzymatic steps. Abbr.: 3β-HSD II (3β-hydroxysteroid dehydrogenase isomerase type II), 17β-HSD I (17β-hydroxysteroid dehydrogenase type I), P450 arom (aromatase), P450 c17 (17α-hydroxylase/17,20 lyase), P450 scC (CYP11A1), StAR (steroidogenic acute regulating protein) (BULUN and ADASHI 2003)
1.2.2 Extraovarian steroidogenesis

Estradiol formation takes place in a number of tissues. Although small quantities of estrogens are produced by an individual adipocyte or skin fibroblast in a continuous fashion, these cell types contribute to circulating estradiol levels because of their relative abundance. P450<sub>arom</sub> in adipose and skin fibroblasts is responsible for peripheral aromatization of A (BULUN and ADASHI 2003). Estrogens are able to modify the androgen metabolism within distinct subunits of the hair follicle, i.e. DP, thus diminishing the amount of DHT formed after incubation with T. It is not known whether this conversion is mediated directly by an inhibition of 5-α-reductase within the hair follicle (HF) or indirectly through estrogen-induced increased conversion of T to weaker androgens, thereby diminishing the amount of T available for the conversion to DHT (NIYYAMA et al. 2001).

The biology of estrogens in their diverse target cells is determined by the structure of the ligand, the estrogen receptor (ER) subtype, the nature of the gene promoter responsive unit as well as the character and balance of co-activators and co-repressors present in different target cells (KATZENELLENBOGEN et al. 2000), termed as tripartite ER pharmacology (KATZENELLENBOGEN and KATZENELLENBOGEN 2000).

1.3 Estrogen receptors

Around 1960 it was reported that the effects of estrogen had to be mediated by a receptor protein (JENSEN 1962). The first ER was cloned in human tissue (WALTER et al. 1985; GREEN et al. 1986; GREENE et al. 1986), chicken (KRUST et al. 1986), mice (WHITE et al. 1987) and rats (KOIKE et al. 1987) and aptly renamed ERα. A decade later the discovery of rat ERβ (KUIPER et al. 1996) was subsequently followed by the cloning of ERβ in humans and mice (MOSSELMAN et al. 1996; TREMBLAY et al. 1997) and the identification of several ERβ isoforms with extended
N-termini (LEYGUE et al. 1998), a variant with an 18 amino acid residue insertion into the ligand-binding domain with altered ligand-binding characteristics (PETERSEN et al. 1998) and C-terminal splice variants unable to bind ligand or activate reporter gene transcription (MOORE et al. 1998; OGAWA et al. 1998; SAJI et al. 2002). Also for ERα various alternatively spliced forms have been described (ZHANG et al. 1996), to date at least eight isoforms of the human ERalpha mRNA are reported and still there are new splicing events found for ERα (OKUDA et al. 2003) and ERβ (SHODA et al. 2002). The two receptors are not isoforms of each other, but rather distinct proteins encoded by genes on different chromosomes (hERα on 6, hERβ on 14) (ENMARK et al. 1997).

1.3.1 ER-structure

The nuclear receptor superfamily describes a related but diverse array of transcription factors, which include nuclear hormone receptors and orphan nuclear receptors, whose ligands are unknown, at least at this time the receptor is identified (OLEFSKY 2001). ERs are Class I members of the steroid receptor gene superfamily that includes androgen receptor, progesterone receptor, glucocorticoid receptor, thyroid hormone receptor and retinoic acid receptors. They are defined as ligand-inducible transcription factors (COUSE et al. 2001).

All receptors of the nuclear receptor superfamily have a similar architecture with independent but interacting functional domains: The aminoterminal A/B domain, containing the autonomous activation function(AF)-1, which enables the receptor to interact with members of the transcriptional apparatus; the C-domain contains a two-zinc finger structure, playing an important role in receptor specific DNA-ligand-binding and receptor dimerization; the D domain, also referred to as a "hinge-region", necessary to give the receptor some degree of flexibility between the DNA and the ligand binding domains E, binding heat shock protein hsp 90 and probably harbouring the sequence representing the nuclear localization signal; the multifunctional carboxyl-terminal E/F domain is crucial for binding receptor specific ligands, nuclear translocation, receptor dimerization and modulation of target gene
expression in association with co-repressors and co-activators (MANGELSDORF et al. 1995). These six functional regions show various degrees of sequence conservation. The N-terminal A/B domain is of extreme variable size, poorly conserved between the two ERs and has an identity of only 17%. The highly conserved C region, harbouring the DNA-binding domain (DBD), shows 96% homology between the two receptors, as a consequence, ERα and ERβ bind to very similar if not identical response elements. The ligand-binding domain E (LBD) has approximately 60% amino acid homology between the two subtypes and contains the activation function(AF)-2, which is dependent on the binding of agonists (DECHERING et al. 2000), suggesting that ERβ recognizes and binds to similar EREs as ERα, but each receptor has a different spectrum of ligands. Investigations of the crystal structure of LBD of ER in complex with E2 and antagonist raloxifene show that both bind at the same site but demonstrate different binding modes; each class of ligand induces a distinct conformation in the transactivation domain of the LBD (BRZOZOWSKI et al. 1997; PIKE et al. 2000). The hinge region (domain D), as well as the carboxy-terminal F-domain are both not well conserved and somewhat shorter in length in ERβ compared to ERα (RUFF et al. 2000) (Fig.1.3.1.1).
Fig. 1.3.1.1 Structure and domains of human ERα/ERβ, above the receptors is given the total protein number, the percentages show homology of the domains between the receptors, AF (activation function), DNA (DNA-binding domain, DBD) LIGAND (ligand-binding domain, LBD)

### 1.3.2 ER-Expression

The two subtypes have overlapping but distinct tissue distribution patterns in vivo and distinct activation profiles at promoter elements of known target genes (LOVEN et al. 2001). It is still not clear how ERα and ERβ contribute (individually or cooperatively) to the physiological effects of estrogens (BRANDENBERGER et al. 1997; BRANDENBERGER et al. 1999; GREENE 2003).

E2 target tissues can be devided into two groups, the classical and non-classical E2 target tissues, respectively. The classical targets are the uterus, mammary gland, placenta, liver, central nervous system, cardiovascular system and bone. ERα is highly expressed in these tissues and the transcriptional rate of E2-responsive genes increases after E2-stimulation.
The non-classical target tissues include prostate, testis, ovary, pineal gland, thyroid gland, parathyroids, adrenals, pancreas, gallbladder, skin, urinary tract, lymphoid and erythroid tissues. There the ERα-expression is very low, whereas ERβ is highly expressed in non-classical E2 target tissues, i.e. prostate epithelium, skin, urogenital tract, ovarian follicles, lung, intestinal epithelium, certain brain regions and muscle (GUSTAFSSON 1999).

In tissues which contain both receptor subtypes, such as ovary, testis and various regions of the brain, a cell-specific localization for each ER subtype has been generally observed. In adipose tissue five ERβ variants and ERα were compared to their distribution and cell differentiation. ERβ showed a constant expression pattern, whereas ERα was dominant in undifferentiated preadipozytes (PEDERSEN et al. 2001). In breast cancer the co-expression of both receptors was shown by RT-PCR (SPEIRS et al. 1999). In the rat prostate ERβ is expressed in prostatic epithelium, while ERα is confined to stromal cells (CHANG and PRINS 1999). More than 50% of the rat mammary cells do not express one type of ER, as the presence of these receptors is not a prerequisite for estrogen-mediated proliferation (SAJI et al. 2000). The rat ovary expresses more Erβ than ERα. There was no apparent modulation of ERα mRNA levels observed during the estrous cycle, in contrast the LH-surge down-regulates ERβ preovulatory on mRNA level (BYERS et al. 1997).

It is noteworthy that the androgen receptor (AR) is expressed at the majority of sites at which ERs are also expressed. Since aromatase, the enzyme which converts testosterone to estradiol, is also found at many of the same sites (SHARPE 1998), it may be suggested that local balance between estrogen and androgen action could lead to a fine regulation of their target cells (PELLETIER 2000).

1.3.3 Signaling pathways

After ligand binding, the gene modulatory effect of a receptor depends on the conformational change of the receptor induced by the ligand and several subsequent events. These include the release of inhibitory proteins (heat shock proteins), receptor dimerization, receptor/DNA-interaction, recruitment of and interaction with co-activators and other transcription factors and the formation of a preinitiation
complex (NILSSON and GUSTAFSSON 2002). The existence of a second ER conjured up many appealing applications in estrogen-responsive tissues, depending on whether the two receptors perform specific or redundant functions and redefines concepts of the estrogen signaling pathways.

![ER-signaling pathways](image)

**Fig. 1.3.3.1 ER-signaling pathways**, from (HALL et al. 2001)

1.3.3.1 **Classical ligand-dependent signaling**

Upon ligand binding the receptor conformation changes from an inhibitory heat shock protein-bound complex into a transcriptionally active form. This enables the receptor protein to bind to specific response elements, estrogen response elements (ERE), 15-bp palindromic sequences in the promoter region of target genes, either as a dimer or as a monomer and modulate the rate of transcription of these target genes. The DNA-bound receptors contact the general transcription apparatus either directly or indirectly via cofactor protein (MCKENNA et al. 1999), of which several have been identified (HORWITZ et al. 1996; ROSENFELD and GLASS 2001). Maximum
transcriptional activity requires the concerted actions of the ligand-independent AF-1 domain (an area of site-specific phosphorylation) in the amino terminus and the ligand-dependent AF-2 in the carboxy terminus (Fig.1.3.3.1).

1.3.3.2 Ligand-independent signaling
ER function can be modulated by extracellular signals in the absence of E2. These findings focus primarily on the ability of polypeptide growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-I) as well as the intracellular effector analog 8-bromo-cyclic adenosine monophosphate through second messenger pathways that alter intracellular kinase and phosphatase activity, resulting in altered phosphorylation of the ER and finally increase the expression of ER target genes (SMITH 1998; LEE and BAI 2002).

The mechanisms by which ER and growth factor pathway converge are not entirely clear, therefore several studies were performed to show the dependence of each pathway on the other, e.g. in the mouse uterus co-treatment with anti-EGF antibodies was able to attenuate the uterine response to E2; as well it was shown that administration of the ER antagonist ICI164,384 reduced the uterine response to EGF (IGNAR-TROWBRIDGE et al. 1992).

Phosphorylation of serine 118 in the AF-1 domain of ERα enhances the transcriptional activity of AF-1. This phosphorylation is mediated by mitogen-activated protein kinase, which may in turn be triggered by EGF or IGF-I. Although AF-1 is divergent in the two receptors, the serine residue at position 118 is structurally and functionally conserved in ERβ. In addition, both ERs contain a tyrosine phosphorylation site in the C-terminus, which induces a conformational change in the receptor that affects hormone binding and dimerization (DECHERING et al. 2000) (Fig.1.3.3.1).

1.3.3.3 ERE-independent signaling
The E2-ER complex is also able to alter transcription of target genes containing alternative response elements, such as the GC-box binding protein (SP-1), nuclear factor κB (NFκB) (MARIN-CASTANO et al. 2003) or activating protein-1(AP-1). At the
AP-1 site in association with other DNA-bound transcription factors (Fos/Jun) the
activated ER is tethered to DNA and finally increase the expression of target genes.
It is known that ERα and ERβ regulate some gene-promoters on AP-1 sites in an
opposite manner (WEBB et al. 1995). Furthermore, ERβ exerts a negative
transcriptional regulation at AP-1 sites when complexed to its natural hormone E2,
whereas antiestrogens positively activate gene transcription (PAECH et al. 1997)
(Fig.1.3.3.1).

1.3.3.4 Non-genomic effects

Non-genomic signaling elicits rapid cellular effects that peak minutes after stimulation
in multiple cell types. The rapidity of activation makes modulation gene transcription
less likely and cannot be blocked by inhibitors of protein or RNA synthesis,
suggesting non-nuclear effects of E2. The signalling cascades recruit second
messengers, including calcium and NO, receptor tyrosine kinases including EGF-
receptor and IGF-I-receptor, G-protein-coupled receptors (GPCRs), and protein
kinases including phosphoinositide-3 kinase (PI3K), serine-threonine kinase Akt,
mitogen-activated protein kinase (MAPK), nonreceptor kinase SRC, and protein
kinases A and C (HO and LIAO 2002).

Rapid effects support several possible theories: first, when the effects occur
at the plasma membrane and not receptor-mediated, as reported from experiments
with MR-KO-mice; second, when the signal is mediated through membrane-bound
receptors of other than classical steroid receptor (insensitive to classical steroid
hormone antagonists, e.g. G protein-coupled receptors, as oxytocin receptor
interacts with progesterone or the activation of MAPK extracellular signal-regulated
kinases 1 and 2 (ERK1 and2) by E2 in human breast cancer cells that lack ERα and
β); or third, when the signal is mediated through membrane-localized classical
steroid receptor (confirmed as classical receptors by the use of antibodies) (CATO et
al. 2002) (Fig.1.3.3.1).

Recently, the idea of estrogen binding entities in subcellular fractions as the
plasma membrane or the endoplasmatic reticulum is has come into focus in the
recent years, though it is far from being confirmed (COLLINS and WEBB 1999; GOVIND and THAMPAN 2003). The existence of the third ER, ERγ (HAWKINS et al. 2000) or pER, an estrogen-binding-protein, cloned from the mouse liver which may contribute estrogenic action in non-reproductive organs (RAO 1998) has therefore been seriously taken into account. An ER ortholog was previously found in the mollusk *Aplysia* (THORNTON et al. 2003), which is constitutively active and does not bind estradiol or related molecules (SCHWABE and TEICHMANN 2004).

### 1.3.4 Coactivators/Corepressors

As mentioned above transcription activation requires the coordinated interaction of multiple transacting factors with DNA recognition sites and other regulatory proteins. In response to cellular signals, transcription factors bind to specific DNA sequences residing in target genes and interact with numerous regulatory proteins to form an active transcription complex and initiate changes in gene expression. This multistep process provides a mechanism by which cells expressing different populations of proteins can differentially regulate expression of target genes (LOVEN et al. 2001). This takes place by the recruitment of adapter proteins characterized as co-regulators (HORWITZ et al. 1996), with ligand-dependent exchange of co-repressors for co-activators serving at the basic mechanism for switching gene repression to activation (ROSENFELD and GLASS 2001).

The most investigated are the co-activator actions on the ligand-binding AF-2 domain. Crystallographic analysis of the ERα-ligand binding domain occupied with an agonist has indicated that the AF-2 domain is structurally complex (BRZOZOWSKI et al. 1997). Upon binding an agonist four of twelve alpha helices that constitute the ligand binding domain of ERα are rearranged to form a hydrophobic cleft with docking sites for the co-activators important for AF-2 function.

The most clearly validated AF-2-interacting co-activators are SRC-1 (steroid receptor coactivator 1) (TREMBLAY et al. 1999), TIF-2 (transcriptionally intermediary factor-2, also called GRIP1, glucocorticoid receptor-interacting protein1) and AIB1 (amplified in breast cancer, also called RAC3, receptor-associated coactivator-3, and
ACTR, activator of thyroid and retinoic acid receptors). All are members of the p160 family of co-activators (named on their weight, 160kD). One of the primary functions of p160 is to recruit other transcriptional co-activators and histone acetyltransferase, i.e. p300, CBP (CREB, cAMP response element-binding protein) and pCAF (p300/CBP-associated factor), to ERα-dependent enhancers in target genes (MCDONNELL and NORRIS 2002).

In addition, steroid receptor co-regulators co-ordinate the transcription and exon-splicing. As splicing is a tissue-specific regulated process and the receptors have different tissue-specific functions, ERα and ERβ do not mediate the same splicing events (AUBOEUF et al. 2002). The co-regulatory complexes are suggested for differential use in both a cell- and promoter-specific fashion to activate or repress gene transcription. These co-regulatory components, themselves being targets of diverse intracellular signaling pathways, provide a combinatorial code for tissue- and gene-specific responses, utilizing both enzymatic and platform assembly functions to mediate the actions of the receptors for development and homeostasis (ROSENFELD and GLASS 2001).

With few exceptions the majority of cofactors are widely expressed in similar amounts in most cells, while concentration levels are not responsible for different cell-selective responses to agonists and antagonists. It appears likely that different ways of regulation of co-activator activity rather than control of protein abundance may be more important (MCDONNELL and NORRIS 2002).

Various cellular pathways are involved in these interactions, e.g. Stat3 stimulates the transcriptional activity of Ers and acts synergistic with SRC1 and CBP on transcriptional activity. Co-activators act as bridging factors and most of them are expressed in a wide variety of cell types and can interact with more than one type of nuclear receptor. The JAK/STAT (Janus kinase/Signal transduces and activates transcription) pathway is involved in many cytokines, hormones and growth factors mediated signaling pathways to regulate a variety of biological responses, i.e. development, cell differentiation, proliferation and survival. Once STAT proteins are activated by tyrosine-phosphorylation they form homo- or heterodimers that are
translocated to the nucleus, where they can bind to specific sequences of the DNA and stimulate gene transcription (DE MIGUEL et al. 2003).

Till today, there are still new cofactors being detected, e.g. recently an estrogen receptor alpha associated protein, template activating factor Iß, which binds to unoccupied ERα in MCF cells and regulates transcription of estrogen responsive genes by modulating acetylation of ERα as well as probably having effects on other nuclear receptors (LOVEN et al. 2003).

1.3.5 ERα versus ERß- agonist or antagonist

The major differences in ligand-binding between ERα and ERß lie in the affinity to various compounds and the transcriptional response a given compound is able to elicit. The ligand-binding activity of ERß is more different than has been suggested from the primary structure of the subtypes (BARKHEM et al. 1998; NETTLES et al. 2004). It has been suggested that each subtype of ER might exert a different function, ERα being involved in activation and ERß in suppression of cellular functions including cellular division. Thus, E2 binds with high affinity to both subtypes.

There are several synthetic so-called SERMs (Selective estrogen receptor modulators) with mixed agonistic/antagonistic potential. For example, tamoxifen is a cell- and tissue-specific mixed agonist-antagonist for ERα, but a pure antagonist on ERß (KUIPER et al. 1997; TREMBLAY et al. 1998; CHUNG et al. 2002; DARDES et al. 2002), whereas ICI182.780, “Fulvestrant” is a pure ER-antagonist with 89% affinity acting via competitive binding to both ERs to prevent endogenous estrogen from exerting the effects in the target cells (JONES 2002; FRASOR et al. 2004) (Fig.1.3.5.1).

Defining the AF-1 site is of great interest as it is required for the partial agonist activity of tamoxifen and it is positively affected by MAPK-directed phosphorylation. P160 and CBP interact weakly with NH2-terminus of ERs. Studies were performed using deletion and ligation for investigating the transcriptional activity. The
agonist/antagonist balance and activity of antiestrogens are determined by specific sequences within the A/B domain, and may be influenced by differences in levels of specific factors interacting with ER (MCINERNEY and KATZENELLENBOGEN 1996).

A transgenic mouse model for in vivo identification of SERMS was created, expressing the reporter luciferase which is induced by E2. Finally, the luciferase content reflects ER transcriptional activity and can be used for ER dynamics during physiological fluctuations of estrogen (CIANA et al. 2001). This model reports that ER transcription activity is tissue-specific and it reveals that ER-action happens in immature animals and neutered males, concluding a hormone-independent activation of ERs (CIANA et al. 2003). Recently, another new animal model for selective ERß-agonist evaluation was created for use in inflammatory treatment (HARRIS et al. 2003).

Phytoestrogens are nonsteroidal polyphenolic compounds present in several plants, exerting estrogenic effects, and can alternatively used for hormone replacement therapy to avoid side effects. On the basis of their chemical structure phytoestrogens may be divided into four subclasses: isoflavonoids (genistein, daidzein), flavonoids (chrys, naringenin), coumestans (coumestrol) and mammalian lignans (enterolactone, enterodiol). Many of these agents are designated as non-estrogenic because they do not cause uterine growth. Today it is known that the non-activation is only a non-activation of ERα, e.g. genistein is a ligand for ERß, therefore formally known as non-estrogenic or anti-estrogenic (GUSTAFSSON 2003).
1.3.6 Internal regulation of ERs

In addition to the various signaling pathways and the distribution pattern in different tissues, as well the species-specific differences (COUSE et al. 1997), cross-talk to other regulatory pathways, endogenous modulators and the effect of one ER to the other has important influence on the finally E2/ER-mediated response.

The splice variants ERßcx and ERß ins are dominant negative regulators of ERα, a mechanism which is independent of the E2 concentration (MOORE et al. 1998; OGAWA et al. 1998; PETERSEN et al. 1998). One may conclude that the antagonism may be the source for insensitivity to estrogens of the lactating mammary gland in vitro, i.e. no progesterone receptors are being induced by E2-treatment (SAJI et al. 2001). The complex interactions and species-specific differences will be explained by the uterus, as all gonadal steroid hormone receptors, ERα/ß, PR and AR are expressed in the uterus (COOKE et al. 1997; WEIHUA et al. 2000).
ERα is involved in uterine proliferation and oxytocin receptor expression, whereas the progesterone receptor is involved in cell differentiation and metabolic activity. E2 down-regulates the expression of ERα in specific cell types in the rat uterus on mRNA- and protein-level after hormone treatment (NEPHEW et al. 2000), uterinic responses are mainly ERα-mediated (LINDBERG et al. 2002). In the mouse uterus, ERβ modulates ERα, E2 down regulates ERβ and up-regulates ERα (WEIHUA et al. 2000), in the rat uterus differences of regulation of ERα and β mRNA expression were found (MURATA et al. 2003).

The AR is stimulated by E2 (previous an sequential activation of ERα); with an increase of IGF-I secretion it results finally to E2 induced epithelial cell proliferation. Besides, ARs are co-localized with ERα but not with ERβ (WEIHUA et al. 2002). In vivo, after the implant of E2 in rats with following removal it has been described that E2-suppression of uterine ER-binding capacity is dose dependent and reversible. The corresponding loss of immunoreactivity distinguishes down-regulation from an alteration of an ER form that fails to bind (MEDLOCK et al. 1991).

E2 binds non-cooperatively to ER, is uncoupled from ER occupancy and down-regulates the transcriptional level in rat cells, proposing that a single estrogen binding site being involved in estrogen-mediated actions (LEE and GORSKI 1998)

1.3.7 Cross-talks

There is increasing evidence for a complex mechanism of cross-talk between peptide growth factors and steroid pathways (SMITH 1998). Acting through ER, estrogens induce expression of IGF-I which in turn exerts its actions through binding to the IGF-I-receptor, a transmembrane protein with tyrosine kinase activity. The binding of IGF-I to its receptor activates the tyrosine kinase and initiates a cascade of phosphorylations that activate intracellular kinases and nuclear transcription factors, including the ERs. IGF-I effects on ER activity are mediated in part by the protein kinase A and phosphatidylinositol-3-kinase/Akt pathways (MARTIN and STOICA 2002) (Fig.1.3.7.1). ERα-expression declines after IGF-I treatment in human cancer cells in enzyme immunoessay and on the gene level. Therefore IGF-I has additive
effects on estrogen-regulated genes as progesterone receptor and pS2 (STOICA et al. 2000).

Cross-talks between ER and peptide growth factors suggest interactions in modulation of hormonal activity. EGF, TGFalpha as well as IGF-I enhance dose-dependently the transcriptional activity, these actions can be inhibited by the pure ER-antagonist. In human cancer cells IGF-I and E2 show synergistic actions (IGNAR-TROWBRIDGE et al. 1996). E2 transcriptional and non-transcriptional effects are also mediated via EGFR: EGF activates in an E2 independent manner the nuclear ER (with the co-activators SRC-1, ERK) and the membrane ER-signaling happens through EGFR for rapid effects through various kinase cascades (LEVIN 2003). In human cell lines the ER is involved in intrauterine maturation of nerve cells cross-talking with IGF-I/IGF-II, presenting the activation of an unliganded intracellular receptor by a membrane receptor ligand (MA et al. 1994). Further it has been reported that ERß mediates increased CREB-phosphorylation after E2-treatment in the mouse brain (ABRAHAM et al. 2003) and that the insulin-receptor-I is able to modulate the protein expression, binding capacity and phosphorylation of ERs (ANDO et al. 1998).
**Fig.1.3.7.1 Scheme of proposed IGF-I/ER cross-talk in the uterus** (KLOTZ et al. 2002) 1: classical E2/ER binding mechanism, 2: ER-activation by IGF-I through PI3-Kinase(solid arrow) or MAPK (broken line arrow)

### 1.3.8 Endogenous modulators of ER-expression

The interplay of ER and other hormones (FREYSCHUSS et al. 1994; VAN DER EERDEN et al. 2002) as well as the regulatory effect between the steroid hormone family (KATZENELLENBOGEN 1996) are to be further investigated.

Prolactin (PRL) regulates ER-expression in mammals. The corpus luteum is a transient ovarian endocrine gland with a finite life span. One of its major functions is to produce progesterone, a hormone crucial for the establishment and maintenance of pregnancy. Defects in luteal function resulting in diminished progesterone production have been associated with human infertility, abortion and cyclus
abnormalities. In the ovarian cycle the LH-surge is responsible for maturation of the oocyte and the process of ovulation and as well for the transformation of the follicle into the corpus luteum. These changes underly the complex regulation of luteotropic hormones.

PRL stimulates both estradiol production and action in the corpus luteum. It is neccessary for maintaining luteal function by stimulating expression of the LH-R in addition to ERα and ERβ, thereby enabling the corpus luteum to respond to these hormones with an increase of progesterone production. In addition, PRL down-regulates 20α-HSD, and therefore prevents progesterone catabolism. 17β-HSD is associated with the short form of the PRL-R and responsible for the conversion of estrone to estradiol.

Additional to the corpus luteum, prolactin and E2 share numerous target tissues, e.g. uterine decidua (FRASOR and GIBORI 2003), where the RNA-levels of both ERs are detected and modulated by PRL in a different manner (a down-regulation of ERβ mRNA was monitored after high-dose treatment) (TESSIER et al. 2000). Progesterone, which usually inhibits ER expression in the uterus and is used to prevent estradiol-induced endometrial cell proliferation, has opposite effects in the decidua. It decreases ERα and increases ERβ-RNA-levels (TESSIER et al. 2000). An increase of ER-levels was reported after PRL supplement in vitro (SHAFIE and BROOKS 1977).

Melatonin (N-acetyl-5-methoxytryptamine) is also associated to the ERs (MAESTRONI and CONTI 1996). It suppresses ERα transcriptional activity, mRNA, protein and basal phosphorylation state by interaction with cofactors (KIEFER et al. 2002). Melatonin inhibits proliferation of human endometrial cancer with estrogen-positive character via MT2 receptor (KOBAYASHI et al. 2003).

Treatment with melatonin leads to a rapid reduction in steady state ERmRNA levels in human cancer cells, as physiological concentrations of 10nM significantly suppressed mRNA levels after 48 hours (MOLIS et al. 1994). Two possible pathways were discussed: first, Melatonin may inhibit ER gene transcription through the membrane-associated melatonin receptor or, secondly, that melatonin being highly
lipophilic, diffuses into the nucleus to directly bind to promoter sequences and inhibit transcription of ER gene (MOLIS et al. 1994). In the liver of the rainbow trout melatonin showed *in vitro/in vivo* no effects on basal or E2-stimulated ER-expression (10nM-100µM) (MAZURAIS et al. 2000). Melatonin inhibits the activation of ERs after E2-stimulation without affecting the ER localization, nor the E2-ER binding. E2-binding is necessary for melatonin in order to interfere with the ER-DNA-binding at ERE. Thus, the antiestrogenic effect of melatonin and the exact interaction between E2-ER to DNA is still unclear (RATO et al. 1999).

1.3.9 Animal models for estrogen research

Several mouse models were created for estrogen research (COOKE et al. 1997; FLODBY et al. 2001; HISHIKAWA et al. 2003). A new mutant was recently cloned by introduction of ERα into transgene mice overexpressing mammary tumors, creating a triple –transgene (TILLI et al. 2003).

The reproductive phenotype of single (ERα-/- (ERKO) (LUBAHN et al. 1993), ERβ-/- (BERKO) (KREGE et al. 1998) and double (ERα-/-, ERβ-/- (DERKO) (COUSE et al. 1999)) KO-mice are reported as following: the ERKO females are sterile, BERKO females fertile or subfertile, both with normal folliculogenesis, and DERKO adults are deficient in granulosa cells. Male BERKO mice are fertile, ERKO and DERKO males are infertile (DUPONT et al. 2000; COUSE et al. 2001). BERKO female become very rarely pregnant, and they usually deliver not more than two offsprings. Implantation occurs in only one horn and is usually accompanied by several dead or resorbed fetuses.

Loss of ERβ causes an increase of cell proliferation and enhances responsiveness to E2, suggesting an anti-uterotrophic function. BERKO mice lose the capacity of E2 to down-regulate PR, indicating that induction of PR is ERα-mediated and repression of epithelial PR is ERβ-mediated or due to hyperresponsiveness to E2 (WEIHUA et al. 2000). Furthermore, ERβ partially replace ERα in gene transcriptional activity, this being the so-called “ying-yang”-effect (LINDBERG et al. 2003).
Both ERs are involved in inhibiting LH levels at times of estrogen-negative feedback in vivo and LH is able to decrease ERβmRNA levels (GUO et al. 2001). Only ERα appears to be critical for the estrogen-negative feedback suppression of Gonadotropin-releasing hormone (GnRH) mRNA expression in female mice. Paradoxically, GnRH neurons only express ERβ, in BERKO mice GnRH mRNA expression appears entirely normal, suggesting no major role in the estrogen-dependent suppression of GnRH gene expression, not excluding any regulatory function of ERβ but insufficiency to suppress gene expression on its own (DORLING et al. 2003).

In DERKO-mice there are reported estrogenic responses, indicating that E2 could have receptor-independent effects. On the other hand, ERKO-mice are incomplete ERα-inactivated, suggesting a functional AF-2 in ERKO and DERKO mice (LINDBERG et al. 2002). The lack of both receptors appears to lead to a different ovarian phenotype than the lack of estrogen, as shown in aromatase (Ar)KO-mice. The ovaries of ArKO have follicles in different developmental stages, but females are infertile, have no corpora lutea and no detectable estrogen serum levels, but they are still responsive to estrogens. The differences to ERKO lie probably in prenatal exposure of maternal estrogens on the developing uterus or non-estradiol ER signaling pathway/ligand-independent estrogen receptor activation. This would explain the fertility in male ArKo in contrast to ERKO/BERKO (KORACH et al. 2003).

An alternative for ER-investigation is a serotonergic neuronal cell line of the rat, which express ERβ, AR, NF-kB and very low serotonin but not ERα or PR (BETHEA et al. 2003).
1.4 Role of estrogens in hair biology

Hair growth is affected by several growth and transcription factors, cytokines and hormones (DEPLEWSKI and ROSENFIELD 2000; STENN and PAUS 2001), which are in parts known to be influenced by estrogens (Table 1.4.1).

Table 1.4.1 Growth and transcription factors, cytokines and hormones (selection) with connections to the hair follicle cycle and E2/ER

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>Stimulates hair growth (PHILPOTT et al. 1994)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Catagen induction (FOITZIK et al. 2003)</td>
</tr>
<tr>
<td>Substance P</td>
<td>Prolongs human and murine hair growth (PAUS et al. 1994; LEE 2003)</td>
</tr>
<tr>
<td>TNFα, IL6</td>
<td>Overexpression leads to distorted hair follicles (STENN and PAUS 2001)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Development of cutaneous vascular system (MECKLENBURG 2002)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Catagen development (LITTLE et al. 1994)</td>
</tr>
</tbody>
</table>

Estrogens are furthermore involved in sebaceous glands function, proliferation and differentiation of epithelial cells in the epidermis and adnexa, functional activity of dermal fibroblasts, wound healing and skin immunoactivity (CALVIN 2000; HACZYNSKI et al. 2002; KANDA and WATANABE 2003).

1.4.1 Role of estrogens in skin biology

About 70 years ago, it was first recognized that hair growth and sexual hormones in animals are closely connected, as in clipped guinea pigs the regrowth of the hair was higher in spayed than in breeding females (DAWSON 1933). A few years later it was reported that injections of estrogenic hormones inhibit hair growth in rats (EMMENS...
1942; HOOKER and PFEIFFER 1943) as well as its topical application inhibits hair growth in rats and dogs (EMMENS 1942; WILLIAMS et al. 1946). After plucking of the hair differences between male and female rats were reported, as well as inhibition of spontaneous hair growth during lactation and pregnancy, this leads to the assumption that female hormones (estrogens, prolactin, progesterone) might influence the hair growth cycle, as hair waves of females lag behind males and the same effects were seen in gonadectomized animals (JOHNSON 1958; MOHN 1958). Changes in the duration of anagen were not seen in any rodent, in contrast to lengthening of the telogen phase (JACKSON and EBLING 1972). Investigations of estrogens on the rat skin demonstrate E2 effects on mitosis and differentiation of epidermis and sebaceous glands (EBLING 1954).

In humans E2 causes an increase of the cutaneous blood flow, proliferating effects on skin and mucous membranes, fortification of the cutaneous membranes of connective tissue by increase of elasticity, decrease of lipid-production on the skin surface as well as inhibition of sebum secretion were reported (WINKLER 1969).

Localization of estrogens in the skin and differences in estrogen binding affinities in different regions within the murine skin were first demonstrated by using tritiated estradiol in autoradiographical studies (radioactivity in the epidermis, dermal fibroblasts and the hair follicle) (STUMPF et al. 1974). The ER protein was detected in the skin and it was reported, that tritiated estradiol binds specifically to the cytosol of the mouse skin tissue and the estrogen–binding protein translocates from the cytoplasm into the nucleus of skin cells seen in mice (UZUKA et al. 1978) as well as in humans, where in addition the possibility of a second receptor suggested for the first time, as at that time it had been known for progesterone receptor (HASSELQUIST et al. 1980; PUNNONEN et al. 1980).

Either with tritiated estradiol the reservoir function for steroids of the skin was reported. Especially the sebaceous glands and the stratum corneum showed radioactivity for more than 24 hours, suggesting two penetration pathways to the dermis, one through the stratum corneum and vital epidermis, the other through the hair canals and hair sheaths (BIDMON et al. 1990).
1.4.2 Steroidogenic enzyme-activity in the skin

In cell cultures ERs are expressed in keratinocytes (HUGHES et al. 1997) and melanocytes (JEE et al. 1994), whereas cultured dermal fibroblasts contain the key enzyme for the conversion of T to E2, aromatase (SCHWEIKERT et al. 1976). It is very important to consider that a large amount of estrogens in men and women are locally synthesized in the skin from inactive adrenal precursors. Recently, using immunohistochemistry and/or tritiated testosterone, more detailed informations were obtained concerning the localization of enzymes which are involved in the androgen/estrogen-synthesis in vitro: steroid sulfatase was detected in the dermal papilla (HOFFMANN et al. 2001), 3ß-HSD in the SG (DUMONT et al. 1992), 17ß-HSD in the CTS (NIYAMA et al. 2001) and type-I and -II 5α-reductase in the DP and fibroblasts (differences with regard to specific scalp regions of the amount of the enzyme towards the beard HF vs. occipital skin) and ORS (ITAMI et al. 1990; SAWAYA and PRICE 1997; LACHGAR et al. 1999).

Female microdissected HF express higher aromatase-level than male donors, and aromatase is mainly expressed in the epithelial parts of the HF, not in the DP. The levels differ between the sexes comparing samples from occipital (4fold) to frontotemporal (6fold) (SAWAYA and PRICE 1997; HOFFMANN et al. 2002). Heterozygous inversions in the aromatase gene result in a severe estrogen excess in many tissues (gynecomastia in men) (SHOZU et al. 2003).

The AR-expression on human scalp is site-dependent with paradoxical effects of androgens on human hair growth (ITAMI et al. 1995; JAHODA and REYNOLDS 1996; INUI et al. 2002), which remains to be proven for estrogen action. The multiple interactions of several hormones and enzymes in the skin and hair follicles especially during and after lactation and pregnancy or in postmenopausal women (BRAUNSTEIN 2003) are getting more and more important in hair research, though it is difficult to dissociate E2-based hair growth effects from those that other hormones might exert (LABRIE et al. 2000; ZOUBOULIS 2000).
1.4.3 Role of estrogens in hair growth and hair follicle cycling

E2 can profoundly modulate hair growth and E2-containing topical preparations are frequently used in trichological practice (SINCLAIR 1999; STERRY and PAUS 2000). In rats, dogs and mice E2 exhibits hair growth inhibitory properties (EMMENS 1942; WILLIAMS et al. 1946; EBLING et al. 1991), and decreases the telogen rate and prolongs the anagen phase in human, therefore topical E2 is useful in the management of hair growth disorders, i.e. androgenetic alopecia (AGA) and telogen effluvium (LYNFIELD 1960; BARMAN et al. 1969; WÜSTNER and ORFANOS 1974; SCHUHMACHER-STOCK 1981). In pregnant women it was previously reported, that scalp hair diameters increases compared to nonpregnant women, but it remains to be proven, to which hormonal influence that belongs (NISSIMOV and ELCHALAL 2003). On human occipital scalp hair E2 inhibits hair shaft elongation in male and female in vitro (KONDO et al. 1990; NELSON et al. 2003).

In animals topical E2 administration results in CD1 mice to arrest the HF in telogen stage (telogen arrest), which is reversible by the pure ER-antagonist ICI 182,780, which, when applied alone, leads to telogen-anagen transition (OH and SMART 1996; CHANDA et al. 2000). These results were later confirmed in C57BL/6 and C3H male and female mice and showed in addition that topical 17α-estradiol inhibits hair growth in male mice, as there were no effects seen in the previous studies (SMART et al. 1999). E2 had no systemic effects on hair growth, suggesting a direct cutaneous effect (SMART et al. 1999). Orchidectomy induces telogen-anagen transition and a supposedly ERα mediated increase of epidermal thickness by an increase in the proliferation rate of the keratinocytes in the basement layer of the epidermis after E2 treatment (MOVERARE et al. 2002). Furthermore, E2 accelerates the development and damage of cyclophosphamide induced catagen (OHNEMUS et al. 2004).
1.4.4 ER-expression in the skin and the pilosebaceous unit

As shown in the rat cardiac fibroblasts (LEE and EGBALI-WEBB 1998), human fibroblasts co-express ERβ and α (HACZYNSKI et al. 2002) matching the recent report that ERβ is the predominant receptor in human skin and pilosebaceous unit, distinct to ERα and AR expression (THORNTON 2002; THORNTON et al. 2003). ERβ is expressed in both male and female human non-balding scalp hair follicles in the epidermis and in the sebaceous gland (ERα nuclear and cytoplasmatic). Most predominant expression of ERβ in the HF was detected in the bulb, DP, matrix keratinocytes, CTS, bulge, ORS, whereas ERα showed weak cytoplasmic staining (THORNTON et al. 2003).

The ER-expression in mice is hair cycle dependent, highest levels are described in telogen in the dermal papilla, the mesenchymal control center of the hair follicle (THORNTON 2002). ER-activation is assumed to have some influence on the telogen-anagen follicle transition. Therefore E2 seems to be an endogenous paracrine regulator of the hair cycle (OH and SMART 1996) and in animals ERα is called to be predominant in the hair follicle (CHANDA et al. 2000; MOVERARE et al. 2002).

1.5 E2-responsive genes

The molecular mechanisms of estrogen action are relatively well investigated, but only a few target genes with consensus ERE (primary-responsive genes) are known so far, such as progesterone receptor, prolactin, lactoferrin, ovalbumin, vitellogenin, cathepsin D1, pS2, glucose-6-phosphate dehydrogenase, c-fos, c-jun, c-myc and choline acetyltransferase. There are more genes activated eventually by estrogen but without apparent ERE: EGF, EGFR, cyclin D1 and others, which can be termed the secondary E2-responsive genes (MURAMATSU and INOUE 2000; ROLLEROVA and URBANCIKOVA 2000).
The method of DNA microarray is becoming one of the most efficient tools for comprehensive analysis of gene expression. It is potentially useful for the study of the signaling cascade, especially for analyzing the biological events the cells triggered by estrogen (OMOTO and HAYASHI 2002; FRASOR et al. 2003; MORENO-BUENO et al. 2003; OLTRA et al. 2003).

1.6 Experimental design

Because the effect of topical applied estrogens has been performed successfully (OH and SMART 1996; CHANDA et al. 2000; OHNEMUS et al. 2004) and the effects were suggested to be strictly cutaneous (SMART et al. 1999) this study was aimed to investigate the systemic effects of estrogens on the hair follicle. The mouse skin organ culture mimicks \textit{in vivo} conditions, treatment with substances have similar systemic-like effects on the HF and it has been well-established (PAUS et al. 1990; BOTCHKAREV et al. 1999; FOITZIK et al. 2003). In addition, the concentrations, especially pharmacological, can be given in a wider range without any side-effect, in contrast to topical application or intraperitoneal injection. The number of animals needed for statistical reason (spot check) can also be held much smaller because the single hair follicle is the statistical feature.

Reported effects in the murine pelage hair follicle are not automatically transferable to differentially located hair follicles, therefore we extended the organ culture towards the human scalp hair follicle. The microdissected HF is a well-established tool in hair research, as well mimicking \textit{in vitro in vivo} conditions (PHILPOTT et al. 1990). Furthermore, an advantage is the isolation of the single follicle without any surrounding structures, i.e. sebaceous gland (ZOBOULIS 2000). This “naked” investigation assay allows to detect autocrine/paracrine mechanisms within the HF. The \textit{in vitro} cultured free-floating follicles show no loss of hair follicle architecture and the elongation rate is not restricted.

Both models simulate physiological conditions and as the hair growth must be addressed species-specific and location-dependent, murine pelage hair follicles as
well as human frontotemporal scalp hair follicles were investigated according to their specific questions addressed.

1.7 Specific questions addressed

1. How are the ERs expressed throughout the murine hair follicle cycle?

2. Are ER gene expression and ER-immunoreactivity correlated?

3. Is it possible to modulate ER-expression in the human and murine hair follicle in vitro? (How is ER-expression endogenously controlled?)

4. Are there differences in the ER-expression in male vs female scalp hair follicles?

5. Does the effect of E2 on hair follicle growth differ between occipital vs frontotemporal and male vs female scalp skin hair follicles in vitro?

6. Cross-talk between peptide growth factors and ERs: Does E2 modulate IGF-I /IGF-IR expression in organ-cultured human hair follicles?

7. Are there differences in the catagen development between ER ß-deficient and wild type mice?

8. Can new the target genes for E2-stimulation be identified in the hair follicle?
2. MATERIALS AND METHODS

2.1 Animals and tissue collection

2.1.1 C57BL/6 mice

Female six to eight weeks old C57BL/6 mice were purchased from the animal facility of the University Hospital Eppendorf and were housed in community cages under standardized conditions (12 hours light/dark-cycle, water and mouse chow *ad libitum*).

The hair growth in mice is synchronized and occurs as a wave from posteriorly and dorsally from the throat region so that all follicles in a particular region are in the same hair cycle stage. This allows investigations of a large amount of hair follicles within the same phase of the hair growth cycle. In C57BL/6 mice, all melanin pigmentation is coupled to the hair follicle which makes it easy to correlate macroscopic skin colours to the underlying hair cycle stage: Anagen (black, active hair growth), Catagen (grey, hair follicle regression), Telogen (pink, resting of the hair production).

2.1.2 Anagen-induction by depilation

For synchronizing all hair follicles of the back skin of telogen mice (recognizable at the pink skin colour), a wax and rosin mixture is applied on the back under ketamin-hydrochloride general anesthesia and peeled off after hardening, thus stripping all telogen hair shafts (PAUS et al. 1990; FOITZIK et al. 2000; MECKLENBURG 2002).

First the depilation mixture (50% Rosin Gum (Sigma, no R-3755)) and 50% Beeswax bleached white (Aldrich Chem. Comp. Inc., no 24,322-1)) is carefully heated in the microwave (A). Than the animals are generally anaesthesized using 0,1 ml of the stock solution per 10 g bodyweight for intraperitoneal injection (stock solution: 2,4 ml Ketanest (50 mg Ketaminhydrochlorid per ml), 0,8 ml Xylazin (20 mg Xyl. per ml), 6,8
ml NaCl 0.9%) (B). The heated wax-rosin-mixture should be controlled for non-irritating temperature on the own skin before using on the mice (C). When the wax-mixture reached a convenient temperature and is still liquid, it is pasted on the back of the mouse from neck to tail in a thin layer (D). The wax must be dried before it could be removed, visible by change of the colour (E-F). The hardened wax can be stripped off with the fur carefully in tail-to-neck direction (G/H). This step might be repeated three to four times, after finishing all wax has to be removed from the animals (I/J). The depilated animals are kept at a warm place (30-37°C) for at least one hour (K) (Fig. 2.1.2.1, Fig. 2.1.2.2). By this technique, all depilated telogen hair follicles immediately begin to transform into anagen follicles with the associated melanogenesis. This predictably results in progressive skin pigmentation and thickening within five to six days, in the development of mature anagen VI follicles and a grey to black skin color within eight to nine days, and the subsequent appearance of new, uniformly black hair shafts. Histologically, functionally and macroscopically, depilation-induced anagen VI follicles are indistinguishable from spontaneously developing anagen follicles. Under reconversion of the skin color from black to pink, follicle regression and its associated cessation of melanogenesis occur spontaneously 17 to 20 days after anagen induction without loss of hair shafts.

**Fig. 2.1.2.1 Depilation of back skin hairshafts in telogen mice(I) (page 52)**
Depilation mixture is carefully heated in the microwave (A), animals are generally anaesthetized (B), control of the temperature of the wax on the own skin (C), wax is pasted on the back of the mouse from neck to tail in a thin layer (D), the wax must be dried before it could be removed, visible by change of the colour (E-F).
Materials and methods

Fig. 2.1.2.1 Depilation of back skin hairshafts in telogen mice (I)
Fig. 2.1.2.2 Depilation of back skin hairshafts in telogen mice (II)
Strip-off the hardened wax (G/H) until all wax is removed from the animals (I/J). The depilated animals are kept at a warm place (30-37°C) for at least one hour (K).
Materials and methods

2.1.3 Skin-organ-culture

Before starting the murine skin-organ-culture, the medium has to be prepared in a sterile glass bottle containing DMEM (Dulbecco’s Modified Eagle Medium, Gibco, #31885-023) with 10% of heat-deactivated and sterile filtered fetal calf serum (Berlin: Seromed, #S 0115), 1% of L-Glutamin (50µg/mL, Seromed, #K0282) and 1% of antibiotic/antimycotic mixture (100fold, Sigma, #A 4668, with 10 000 IU of penicillin G, 10 000 µg Streptomycin and 25 µg Amphotericin). The sterile gel-foam sponges (Upjohn Co, France, # 317681) were cut in approximately five mm to ten mm big squares with a sterile scalpel blade under sterile conditions and preincubated in complete medium in CO2-incubator in a sterile petri dish at least 3 hours, that the sponges were soaked with medium.

The mice were anaesthesized and gently shaved with an electrical clipper. Than the mice were killed by cervical dislocation. The skin must be washed two times with 70% pure alcohol which was spilled over the back of the mouse until it is fully covered and left to dry. Afterwards, the skin was rinsed two times with sterile phosphate-buffered saline-solution. The skin was harvested carefully from the tail region to the neck without any fat and transferred to the sterile hood and placed on sterile filter. The skin was spreaded on a few drops of medium upon a sterile board. Four-mm-punch biopsies (Stiefel, # Y221801G18) were taken from the skin in as many places as possible but not too close to the edge of the sample and always with a little rim of untouched skin in between biopsies to avoid squeeze artefacts. The biopsies must be transferred immediately into medium in a sterile petri dish to avoid the drying of the skin. After taken all biopsies they were randomized and placed into a six-well-plate upon a sterile gel-foam-sponge, six biopsies per well, containing 5 ml of mixed medium. The plates were pre-incubated in CO2-incubator (5% CO2, 37°C) for one hour. Afterwards the chosen substances were added in an calculated concentration to each well and the biopsies went back into the incubator. Medium and substances had to be renewed every day.
Materials and methods

After the end of incubation (72h) the biopsies were taken off the sponges and washed in PBS. One half of the biopsies were placed in 4% formalin or 4% paraformaldehyde for paraffin embedding and the other half was frozen in a drop of medium in liquid nitrogen for cryosections.

The culture was modified in order of the biopsie punches. Because of the difficulties during the freezing process (the epithelial layer splitted off the dermis, therefore no acceptable cryosections could be cut from those samples), we decided to cut the skin into slides of three-mm to ten-mm, to get a bigger skin sample which is not affected in the liquid nitrogen. In addition we shortened the culture period down to 48, 24, 12 and at last to 6 hours, because of the rapid decrease of the estrogen receptor expressions during the culture period.

The investigated substances were added to the medium as follows:

- **17β-estradiol** (Sigma, St. Louis, MO, USA, # E8875), 1-100 nM
- **ICI 182,780** (Tocris, Ellisville, MO, USA, #1047), 1-100 nM
- **Melatonin analogue** (Immune system Ltd., GB, #126-6293), 1 nM-10 pM
- **Luteotropic Hormone / prolactin** (Sigma, St. Louis, MO, USA, #013K1258), 200/400 ng/mL

### 2.1.4 ERβ-deficient mice

To study hair cycling abnormalities and phenotypic differences in ERβ-deficient mice, wildtype and BERKO female mice in catagen (p.p. 19) stage were investigated. These mice were sent from Jan-Ake Gustafsson, Department of Medical Nutrition and Biosciences, Karolinska Institutet, NOVUM, Sweden.

Genomic P1 clones of the mouse ERβ gene from a129/SvJ library were isolated by Genome Systems (St. Louis) using primers and probes supplied by the Department of Pathology, Chapel Hill, NC, USA. Sequence analysis was performed to confirm the validity of the clones. A targeting construct was generated that included 1.3 kb and 7.4 kb of genomic sequence as the 5´and 3´homology regions. A copy of the neomycin-resistance gene (Neo) driven by a phosphoglucomkinase promoter was inserted in the reverse orientation into the PstI site in exon 3. The targeting construct
was linearized at the indicated NotI site and introduced into the BK4 subline of the E14TG2a strain 129 embryonic stem (ES) cell line (SMITHIES and KIM 1994). Screening for correctly targeted ES cells were accomplished by using PCR (KIM and SMITHIES 1988). Targeted ES cells were confirmed by using Southern blot analysis with EcoRI-digested ES cell genomic DNA and a probe specific to exon 2; the unmodified ERß gene gives a 12-kb band, whereas the disrupted ERß gene gives a 5.4-kb band.

Genotyping of the tail was performed in the Karolinska Institutet, Sweden as follows: Tail DNA was accomplished by using PCR with the intron 2 primer, the Neo primer and a third primer from exon 3 (5´CATCCTTACAGGACCAGACAC3´); a 1,435-bp band (intron 2 and exon 3 primers) is amplified for homozygous wildtype (+/+ ) mice, a 1,479-bp band (intron 2 and Neo primers) for homozygous mutant (-/- ) mice, and both bands for heterozygous (+/- ) mice.

### 2.1.5 Organ-culture of microdissected human hair follicles

Human anagen VI hair follicles were isolated by microdissection from human scalp skin, taken from healthy females and males obtained with informed consent during facelift plastic surgery. All experiments were performed in order to Helsinki guidelines. Isolation of hair follicles was achieved by using a scalpel blade to cut through the skin at the dermo-subcutaneous fat interface. The intact hair follicle bulb was removed from the subcutaneous fat under a stereo dissecting microscope by gently gripping the outer root sheath of the follicle in the forceps and pulling the hair follicle from the subcutaneous fat. This results in the isolation of intact hair follicle bulbs without sustaining any visible damage.

Isolated hair follicles were maintained in serum free Williams E medium (Biochrom, # F1115) supplemented with 1% L-Glutamin (50µg/mL, Seromed, # K0282), 0.02% hydrocortisone (Sigma, # M-0888), 0.1% insulin (Sigma, # I1882) and 1% of antibiotic/antimycotic mixture (100fold, Gibco, # 15240-062, with 10 000 IU of penicillin G, 10 000 µg Streptomycin).
The hair follicles were placed in a 36-well plate with 500µL complete medium and incubated in the CO₂-incubator (5% CO₂, 37°C) for one hour. Afterwards the chosen substances were added in an calculated concentration to each well. Medium and substances had to be renewed every second day. Hair shaft elongation was measured using an inverted microscope on day 0, 1, 3, 5, 7 and 9. After the end of the culture period (nine days) the follicles were washed in PBS and frozen in a drop of medium for cryosections.

Substances were added to the medium as follows:

- **17ß-estradiol** (Sigma, St. Louis, MO, USA, # E8875), 1-100 nM
- **Melatonin analogue** (Immune system Ltd., GB, #126-6293), 1 pM, 10 nM
- **Luteotropic Hormone / prolactin** (Sigma, St. Louis, MO, USA, #013K1258), 400 ng/mL

### 2.2 Immunohistochemistry

#### 2.2.1 Hematoxylin & Eosin staining

The skin samples from the murine skin organ culture and the human hair follicles were harvested after finishing their culture period, nine days or six hours, respectively.

All samples were cryopreserved and cryosections of 4 µm were fixed in acetone for ten minutes at -20°C. The slides dried 30 minutes at room temperature and were washed two times in Tris buffered saline (TBS, pH 7.6) and 10 minutes in destilled water. The sections are stained in Mayers hemalaun (Merck, #1.04302) for 10 minutes and rinsed unter tap water approximately 15 minutes. The counterstaining was performed with 0.1% eosin (Sigma, #E4382) for maximum one minute. The finally differentiation is as follows: one time in 70%, two times in 96%, two times in 100% ethanol and two times in XyloL, all steps with dipping carefully 10 to 15 times. The slides are mounted with Eukitt (O.Kindler GmbH&Co, Germany).
2.2.2 Ki-67-staining for human hair follicles
Frozen sections were fixed in acetone for 10 minutes, dried and washed in phosphate-buffered saline (PBS, pH 7.4). After preincubation with 10% normal goat serum in PBS for twenty minutes, the slides were incubated with the primary antibody mouse-anti human Ki-67 antigen (DAKO, clon MIB-1) diluted 1:20 in PBS with 2% normal goat serum overnight at 4°C. The slides were washed three times for five minutes in PBS and the secondary antibody goat anti-mouse IgG-rhodamine red (Jackson Immuno Research) diluted 1:200 in PBS with 2% normal goat serum was incubated for 45 minutes at room temperature. The slides were washed three times in PBS for five minutes and counterstained with 4´,6-diamino-2-phenylindole (DAPI) (Roche, #236 276, Germany) solution (0.1µg/mL) in phosphate buffered saline solution for 30 seconds at room temperature to delineate nuclei, washed three times for five minutes in TNT and mounted in Fluoromount (Southern Biotechnology, UK). The sections were examined under an Axiophot fluorescence microscope (Zeiss, Germany), the images were recorded as computer files via Hamamatsu digital camera (Hamamatsu Photonics, Japan) and analyzed by using an Openlab program (Improvision, UK).

2.2.3 TUNEL-staining (in-situ apoptosis)
The non-fixed frozen sections were dried for 2 minutes at room temperature, fixed in 1% paraformaldehyde in phosphate-buffered saline solution (PBS, pH 7.4) and postfixed in 2:1 ethanol to azetic acid for five minutes at –20°C. The slides were washed three times for five minutes in PBS and 13µL of equilibration buffer was put on the slides for five minutes. The TdT-enzyme-diluent (70% reaction buffer, 30% TdT enzyme, Serological Corporation, #03) was incubated one hour at 37°C, followed by the stop-buffer-diluent (68ml distilled water, 2ml stop buffer, Serological Corporation, #04) for ten minutes, the slides were washed three times in PBS for five minutes. From the TUNEL-kit the fluorescein-labeled anti-digoxigenin-antibody (56µl blocking reagent, 59µl antibody, Serological Corporation, #3B014) was incubated 30 minutes at room temperature, the slides were washed three times with PBS for five minutes and counterstained with 4´,6-diamino-2-phenylindole (DAPI) (Roche, #236
276, Germany) solution (0.1µg/mL) in phosphate buffered saline solution for 30 seconds at room temperature to delineate nuclei, washed three times for five minutes in TNT and mounted in Fluoromount (Southern Biotechnology, UK). The sections were examined under an Axiophot fluorescence microscope (Zeiss, Germany), the images were recorded as computer files via Hamamatsu digital camera (Hamamatsu Photonics, Japan) and analyzed by using an Openlab program (Improvision, UK).

2.2.4 Estrogen receptor alpha, beta, beta ins 18aa
The samples were harvested as follows, for the evaluation throughout the murine hair follicle cycle the dorsal skin from untreated mice was excised and cryopreserved at day 0, day 12, day 17, day 19 and day 25 after depilation. The cultured murine skin samples and the human hair follicles were harvested after finishing their culture period, 48 hours or six hours, respectively.

All samples were cryopreserved and cryosections of 4 µm were fixed in acetone for ten minutes at -20°C. Mouse uterus was used as positive control for the murine ERα-staining, mouse ovary for ERß-stainings. In the human ERß503 staining cryosections of human lung tissue was used for positive control.

The sections were used after storage in –20°C and had to dry for 10 minutes at 20°C. Than they were washed three times for five minutes in tris buffered saline (TBS, 0.05M, pH 7.6). After inhibition of endogenous peroxidase with 3% H2O2 in phosphate buffered saline for 15 minutes the sections were washed three times for five minutes in TBS. The sections were blocked with Avidin and Biotin (Vector, SP-2001) for 15 minutes each and washed three times for five minutes. Afterwards the slides were incubated with 10% of the normal serum of the host for the secondary antibody (chicken for ERß and ERß ins, goat for ERα) supplemented with Triton-X100® 0.3% at room temperature for 20 minutes. Subsequently the sections were incubated overnight at 4°C with the primary antibody:

- ERα 1:2000 (Santa Cruz, MC20),
- ERß 503 1: 1000 (J-A Gustafsson, Karolinka Institutet Sveden),
- ERß ins 18 aa 1:1000 (J-A Gustafsson, Karolinka Institutet Sveden)
Materials and methods

After the first incubation the sections were washed in TBS three times for five minutes and incubated 45 minutes with a biotinylated goat anti-rabbit IgG (1:200 in TBS, Jackson Immuno Research) together with 2% normal goat serum and 4% normal mouse serum in case of ERα, a biotinylated rabbit anti chicken IgG (1:200 in TBS, Promega) together with 2% normal rabbit serum and 4% normal mouse serum in case of ERβ and ERβ ins. The slides were washed three times in TBS for five minutes and a routine detection was prosecuted with the avidin biotin complex, labeled with peroxidase (Perker Elmer, Vectastain, PK-6100) for 30 minutes at room temperature. The slides were washed three times in TBS for five minutes. The signal was visualized with diaminobenzadine (DAB; Vector, Burlingame, CA, USA) over eight minutes and the slides were washed three times in TBS. Finally the sections were counterstained with methylenegreene (DAKO) for five minutes, and dehydrated in 95% ethanol two times for 2 minutes, in 100% ethanol three times for 2 minutes, in Xylol two times for five minutes and mounted with Eukitt (O.Kindler GmbH&Co, Germany).

For ERα, ERβ 503 and ERβ ins 18 aa a Tyramid Signal amplification (Fluorescein system staining, NEN Life Science Products, Boston, MA) was additionally performed. Acetone fixed frozen sections were dried for 10 minutes at room temperature, washed three times in TNT (Tris HCl, Sigma, NaCl, J.T.Baker, Tween 20, Sigma, diluted in distilled water, pH 7.5) for five minutes and incubated 15 minutes with 3% H2O2 in phosphate buffered saline for inhibition of endogenous peroxidase. The slides were washed three times in TNT and preincubated with TNB Blocking Buffer (Tris HCl, Sigma, NaCl, J.T.Baker, Blocking reagent, Perkin Elmer) for 30 minutes. The slides were incubated overnight with the primary antibody:

- ERα 1:8000 (Santa Cruz, MC20),
- ERβ 503 1: 10 000 (J-A Gustafsson, Karolinka Institutet Sveden),
- ERβ ins 18 aa 1:10 000 (J-A Gustafsson, Karolinka Institutet Sveden)

After the first incubation the sections were washed in TNT three times for five minutes and incubated 45 minutes with a biotinylated goat anti-rabbit IgG (1:200 in TBS, Jackson Immuno Research) together with 2% normal goat serum and 4% normal mouse serum in case of ERα, a biotinylated rabbit anti-chicken IgG (1:200 in
TBS, Promega) together with 2% normal rabbit serum and 4% normal mouse serum in case of ERβ and ERβ ins. The slides were washed three times for five minutes in TNT and streptavidin-conjugated horseradish peroxidase (1:100 in TNB) was added for another 30 minutes. The slides were washed three times for five minutes in TNT and incubated in fluorophore stock solution (1:50 in amplification diluent, Perkin Elmer) for 30 minutes. The sections were washed three times for five minutes in TNT and dipped in 4´,6-diamino-2-phenylindole (DAPI) (Roche, #236 276, Germany) solution (0,1µg/mL) in phosphate buffered saline solution for 30 seconds at room temperature to delineate nuclei, washed three times for five minutes in TNT and mounted in Fluoromount (Southern Biotechnology, UK). The sections were examined under an Axiophot fluorescence microscope (Zeiss, Germany), the images were recorded as computer files via Hamamatsu digital camera (Hamamatsu Photonics, Japan) and analyzed by using an Openlab program (Improvision, UK).

2.2.5 IGF-I and IGF-IR immunofluorescence staining
For IGF-I and IGF-IR a Tyramid Signal amplification (Fluorescein system staining, NEN Life Science Products, Boston, MA) was performed on E2-treated human hair follicles. The E2-treated and the non-treated control human hair follicles were harvested after finishing their culture period of nine days. They were cryopreserved and cryosections of 4 µm were fixed in acetone for ten minutes at -20°C. Acetone fixed frozen sections were dried for 10 minutes at room temperature, washed three times in TNT (Tris HCl, Sigma, NaCl, J.T.Baker, Tween 20, Sigma, diluted in distilled water, pH 7,5) for five minutes and incubated 15 minutes with 3% H₂O₂ in phosphate buffered saline for inhibition of endogenous peroxidase. The slides were washed three times in TNT and preincubated in TNB Blocking Buffer (Tris HCl, Sigma, NaCl, J.T.Baker, Blocking reagent, Perkin Elmer) for 30 minutes. The slides were incubated for one hour at room temperature with the primary antibody IGF-I (Santa Cruz, SC 9013) and IGF-IR (Santa Cruz, SC-712) for 1:2000 diluted in TNB, supplemented with 2% goat normal serum. The slides were washed three times in TNT for five minutes and incubated with a biotinylated secondary antibody goat anti-rabbit IgG (Jackson Immunoresearch, 111-065-045), diluted 1:200 in TNB for 45 minutes at room temperature, afterwards, the slides were washed three times for five minutes in
TNT. The streptavidin-conjugated horseradish peroxidase (1:100 in TNB) was added for another 30 minutes. The slides were washed three times for five minutes in TNT and incubated in fluorophore stock solution (1:50 in amplification diluent, Perkin Elmer) for 30 minutes. The sections were washed three times for five minutes in TNT and dipped in 4´,6-diamino-2-phenylindole (DAPI) (Roche, #236 276, Germany) solution (0,1µg/mL) in phosphate buffered saline solution for 30 seconds at room temperature to delineate nuclei, washed three times for five minutes in TNT and mounted in Fluoromount (Southern Biotechnology, UK). The sections were examined under an Axiophot fluorescence microscope (Zeiss, Germany), the images were recorded as computer files via Hamamatsu digital camera (Hamamatsu Photonics, Japan) and analyzed by using an Openlab program (Improvision, UK).

2.2.6 K6HF-immunofluorescence staining
An immunofluorescence staining for K6HF was performed on human scalp hair follicles, back skin of C57BL/6 mice, ERß-deficient mice and the wildtype control, all hair follicles were in late anagen stage. All samples were cryopreserved and cryosections of 4 µm were fixed in acetone for ten minutes at -20°C. The sections were used after storage in –20°C and had to dry for 10 minutes at 20°C. Than they were washed three times for five minutes in phosphate-buffered saline (PBS, pH 7,4). The sections were blocked with Avidin and Biotin (Vector, SP-2001) for 15 minutes each and washed three times for five minutes in PBS. The slides were dipped two minutes in 0.2% Triton-X100® diluted in PBS. The slides were washed three times for five minutes in PBS and incubated with 5% of goat normal serum in PBS at room temperature for 20 minutes. Subsequently the sections were incubated 45 minutes at room temperature with the primary antibody K6HF (Lutz Langbein, Heidelberg) 1:2000 in PBS and washed three times for five minutes in PBS. The slides were incubated with a Cy-3-labeled secondary antibody goat anti-guinea pig IgG (Jackson Immunoresearch, 106-166003), diluted 1:500 in PBS for 30 minutes at room temperature, afterwards the slides were washed three times for five minutes in PBS. The sections were dipped in 4´,6-diamino-2-phenylindole (DAPI) (Roche, #236 276, Germany) solution (0,1µg/mL) in phosphate buffered saline solution for 30
seconds at room temperature to delineate nuclei, washed three times for five minutes in PBS and mounted in Fluoromount (Southern Biotechnology, UK). The sections were examined under an Axiophot fluorescence microscope (Zeiss, Germany), the images were recorded as computer files via Hamamatsu digital camera (Hamamatsu Photonics, Japan) and analyzed by using an Openlab program (Improvision, UK).

2.3 Quantitative histomorphometry

2.3.1 Assessment of hair cycle stages

Hair cycle stages were assessed in order to the murine system of Müller-Röver (MULLER-ROVER et al. 2001) and the human hair follicles were classified according to Kligman (KLIGMAN 1959). Morphometry was performed on 4 µm frozen, hematoxylin-eosin stained sections. No statistical analysis was performed because of the missing changes in the mean (as the spot check is the single HF) and the resulting missing standard deviations.

2.3.2 Assessment of proliferating matrix keratinocytes

Sections stained for Ki-67 were investigated at 400x magnification. All Ki-positive cells below the Aubers’line (Fig.2.3.2.1) were counted as well as the total number of keratinocytes, stained with 4´,6-diamino-2-phenylindole (DAPI). The amount of Ki-positive cells was given in per cent, the changes were evaluated as well as the standard error mean using the Mann-Whitney-U Test.
2.3.3 Assessment of the dermal thickness

4µm sections stained with hematoxylin and eosin were investigated at 100x magnification under a Axiophot fluorescence microscope (Zeiss, Germany), the thickness was measured between the basement membrane of the epidermis to the panniculus carnosus (Fig. 2.3.3.1). Images were recorded as computer files via a Hamamatsu digital camera (Hamamatus Photonics, Japan) and analyzed by using an Openlab program (Improvision, UK). For statistical significance the standard error mean as well as the changes were evaluated using the Mann-Whitney-U Test.

2.3.4 Assessment of the apoptotic cells in the telogen hair follicle

4µm sections were stained with ApopTag Fluorescein In Situ Kit (Serological Corporation) and investigated at 400x magnification under a Axiophot fluorescence microscope (Zeiss, Germany), all positive cells in the hair follicle were counted, except of the sebaceous gland. The numbers of the positive cells were given in total, for statistical significance the standard error mean as well as the changes were evaluated using the Mann-Whitney-U Test.
Fig. 2.3.3.1 Measurement of the dermal thickness in the murine back skin (catagen stage)
To investigate the catagen development the thickness of the dermis can be compared, thickness is measured between the basement membrane of the epidermis (BM) to the panniculus carnosus (PC).

2.4 Semi-quantitative RT-PCR
RNA from full-thickness back skin of C57BL/6 mice, homogenized under liquid nitrogen, was isolated by using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. Subsequently, cDNA was synthesized from 1µg of total RNA using 1st strand cDNA synthesis kit for RT-PCR (AMV) (Boehringer Mannheim, Germany). The resulting cDNA was stored at –80°C until further use. Primers used are listed as follows:
ERα: forward 5’-AAT TCT GAC AAT CGA CGC CAG-3’; reverse 5’-GTG CTT CAA CAT TCT CCC TCC TC-3’
ERβ: forward 5’-CTT GGT CAC GTA CCC CTT AC-3’; reverse 5’-GTA TCG CGT CAC TTT CCT TT-3´ (WEIHUA et al. 2000).
β-actin: forward 5’-TGT TAC CAA CTG GGA CGA CA-3’, reverse 5’-TCT CAG CTG TGG TGG TGA AG-3’.
The PCR was run on an Astec Program Temp Control System (Astec, Fukushima, Japan). The reaction consisted of 1µl cDNA, 10µl 10fold PCR buffer, 8µl dNTP mixture (2.5 mM each), 1µl forward primer (50 pmol), 1µl reverse primer (50 pmol), 0.5µl TaKaRa Ex Taq Polymerase (Takara, Otsu, Japan) and 79.5 µl water.
Amplification was performed over 32 cycles for β-actin, 35 cycles for ERα and 35 cycles for ERβ. Each cycle consisted of the following steps: denaturation at 94°C,
annealing at 55°C and extension at 72°C. The PCR products were analyzed by agarose gel electrophoresis. Densitometry was performed by using Scion Image (Scion Corporation, Maryland, USA). For statistical significance the standard error mean as well as the changes were evaluated using the Mann-Whitney-U Test.

### 2.5 Microarray analysis

For microarray analysis, the DNA complementary to the target genes is generated and spread out in microscopic quantities on a solid surface in defined positions. The DNA binds complementary and the genes are detected by fluorescence following laser excitation.

Human hair follicles from male and females were microdissected and cultured for 48 hours supplemented with E2 (10 nM). After the culture period they were frozen in RNA-later at -80°C until further use. RNA was isolated by using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. The RNA was shipped on dry ice to memorec, Cologne for further studies. After the quality control of the RNA by electrophoresis defined 200-400bp fragments of cDNA by RT-PCR were cloned and verified. The fragments get purified, checked on agarose, diluted and spotted on treated glass-slides with an ink spotter (2nL of each probe = 20 drops containing 100 pL) (Fig. 2.5.1). The samples were labeled with different fluochromes (Cy3/5), which results in different fluorescent signals. The relative intensity of Cy3/5 probes is a reliable measure of the relative abundance of specific mRNA in each sample. Every single spot is measured in a fixed circle, same procedure is repeated in a larger area with the background – ratio, resulting in an average pixel of signal and background. For statistical reason, the modulated genes are given with the standard deviation of the four replicate ratios.
Fig. 2.5.1 Microarray analysis The cultured cell/RNA is reverse transcribed, labeled, fractioned, hybridized in a hybridization chamber, cleaned and analysed by fluorescent laser excitation

2.6 Statistical analysis

To evaluate for statistical significance, the data from identical experiments were pooled and the mean and the standard error were calculated. With the statistical analysis software SPSS (SPSS Inc., Chicago, USA), significance was assessed with the Mann-Whitney U test for unpaired samples.
3. RESULTS

3.1 Estrogen Receptors alpha and beta expression throughout the murine hair cycle

The immunoreactivity of ERα, ERβ 503 and the splice variant ERβ ins 18aa throughout the depilation induced murine hair follicle cycle was studied using two different immunohistochemistry techniques, first, the ABC staining with the substrate DAB, and secondly the more sensitive Tyramid signal amplification (TSA)-technique. Changes were observed according to intensity and distribution of all ERs immunoreactivity. Immunoreactivity (IR) was detected for ERα, β 503 and β ins throughout all investigated hair cycle stages in the depilation-induced murine hair follicle cycle (telogen, late anagen, early and late catagen) with distinct expression patterns (Fig. 3.1.1, Fig. 3.1.2).

In anagen VI, ERα-like immunoreactivity was observed in the dermal papilla, matrix keratinocytes, the inner root sheath and the outer root sheath while the epidermis and the sebaceous gland showed no ERα-like immunoactivity (Fig. 3.1.1 B/F). ER β 503-like immunoreactivity was intense and ubiquitously seen in the dermal papilla, inner root sheath, outer root sheath, the sebaceous gland and the epidermis of anagen VI follicles (Fig. 3.1.2 B/F), with less intensity ER β ins was co-expressed in the same distribution pattern in the anagen VI hair follicle (Fig.3.1.2 J/N).

In early catagen, ERα-like immunoreactivity was weakly expressed in the dermal papilla, matrix keratinocytes, inner root sheath, outer root sheath and the connective tissue sheath (Fig 3.1.1 C/G). ER-β-like immunoreactivity was detected in the outer root sheath, inner root sheath, bulb matrix, dermal papilla, epidermis and the sebaceous gland (Fig. 3.1.2 C/G), again ERβ ins was similarly but weaker co-expressed (Fig.3.1.2 K/O).

In late catagen, ERα-like immunoreactivity was strongly expressed in the dermal papilla, secondary hair germ, outer root sheath and connective tissue sheath, no expression was found in the epidermis and sebaceous gland (Fig. 3.1.1 D/H). ERβ-like immunoreactivity was similarly expressed as in the early catagen stage in the outer root sheath, inner root sheath, dermal papilla, epidermis and the sebaceous
gland (Fig. 3.1.2 D/H), same distribution pattern was visible for ERβ ins expression (Fig. 3.1.2 L/P).

In telogen stage, ERα-like immunoreactivity peaked and was strongly detected in the dermal papilla and cells of the sebaceous gland. Less intense it was shown in the keratinocytes of the outer root sheath and the germ capsule (Fig. 3.1.1 A/E). Immunoreactivity of ERβ was seen in the dermal papilla, keratinocytes of the germ capsule, the sebaceous gland and the remaining outer root sheath (Fig. 3.1.2 A/E), similar distribution was again found for ERβ ins (Fig. 3.1.2 I/M).

The described immunohistochemical expression correlates with the ER mRNA levels in full-thickness back skin homogenates, analyzed by semi-quantitative RT-PCR. Skin samples were investigated using the following time points of depilation-induced hair follicle cycling, day 0 (telogen), day 12 (late anagen) and day 19 (late catagen) after depilation.

After normalisation against beta actin ERα mRNA-levels appeared to be hair cycle dependent with peak in the telogen stage (day 0 and 25). ERα mRNA-levels declined during anagen development at day 8, where the lowest levels were detected. Densitometrically, an increase was demonstrated at day 12, continued during catagen at day 19 (Fig. 3.1.3).

ERβ transcripts within murine skin were detected throughout the whole hair cycle in a constant manner (Fig. 3.1.3).

To summarize, IR and mRNA were detected for ERα, ERβ 503 and ERβ ins throughout all investigated hair cycle stages in the depilation-induced murine hair follicle cycle (telogen, late anagen, early and late catagen) with distinct expression patterns.

Fig. 3.1.1 (page 70/71) ERα-like immunoreactivity during the depilation-induced murine hair cycle
Page 70: A-D ABC staining using DAB as substrate, E-H TSA-staining
Peak in telogen follicles (A/E) within the DP and SG while ORS showed weaker IR. In anagen VI ERα-IR was observed in the DP, MK,IRS and ORS (F/B), in early catagen (C/G) ERα IR in the DP, MK, IRS, ORS and CTS, in late catagen (D/H) in the DP, secondary hair germ and ORS
Page 71: Schematic representation of immunoreactivity, red dots: ERα-IR
Fig. 3.1.1 IR of ERα in the depilation-induced murine hair follicle cycle
Fig. 3.1.1 IR of ERα in the depilation-induced murine hair follicle cycle (scheme)
Fig. 3.1.2 IR of ERβ 503 during the depilation-induced murine hair cycle
Fig. 3.1.2 IR of ERβ 503 and ERβ ins during the depilation-induced murine hair cycle (scheme)
Results

Fig. 3.1.2 IR of ERß ins during the depilation-induced murine hair cycle
Fig. 3.1.3
Fig. 3.1.2 (page 72, 73, 74) ERβ 503 and ERβins-like immunoreactivity during the depilation-induced murine hair cycle
Page 72: Immunoreactivity pattern of ERβ A-D ABC staining using DAB as substate, E-H TSA-staining
ERβ 503 IR was intense and ubiquitously in the DP, IRS, ORS, SG and epidermis in all cycle stages.
Page 73: Schematic representation of immunoreactivity, blue dots: ERβ 503 expression, yellow dots: ERβ ins expression
Page 74: IR pattern of ERβ ins I-L ABC staining using DAB as substate M-P TSA-staining ERβ ins IR was weakly expressed in the DP, IRS, ORS, SG and epidermis in all cycle stages.

Fig. 3.1.3 (page 75) Semi-quantitative RT-PCR analysis of ERα and ERβ
Upper panel: PCR analysis of expression of ERα, ERβ and beta actin in murine skin during the depilation-induced hair cycle on day 0 (non depilated telogen skin), day 8 (mid-anagen), day 12 (late anagen), day 19 (late catagen and day 25 (telogen)
Middle/lower panel: The graphs show the intensity of each band evaluated by digital image analysis based densitometry. Experiments were generated from three individual animals per time point. The samples were normalized according to the expression of β-actin mRNA (SEM).

3.2 Expression of estrogen receptors alpha and beta in murine skin organ culture

To investigate whether the ER expression in murine skin could be modulated by prolactin, melatonin and E2, skin samples from depilation-induced murine hair cycle were taken on day 0 (telogen), day 12 (late anagen) and on day 19 (late catagen) to perform a skin organ culture (PAUS et al. 1990). Immunoreactivity of ERα, ERβ and ERβins decreased within six hours of culture compared to non-cultured skin in similar hair cycle stages (Fig. 3.2.1, Fig. 3.2.2, Fig. 3.2.3). Background signal increased in immunofluorescence staining, thus no distinguished immunoreactivity could be detected using the more sensitive TSA staining method (data not shown).

In telogen stage, ERα-like immunoreactivity was detected in the dermal papilla and cells of the sebaceous gland (Fig. 3.2.1 A/B). Immunoreactivity of ERβ was seen in the dermal papilla, keratinocytes of the germ capsule, the sebaceous gland, the epidermis and the remaining outer root sheath (Fig. 3.2.2 A/B), Erβins was weakly co-expressed (Fig. 3.2.3 A/B)

In anagen VI ERα- like immunoreactivity was observed in the dermal papilla, matrix keratinocytes, the inner root sheath and the outer root sheath while the epidermis and the sebaceous gland showed no ERα-like immunoreactivity (Fig. 3.2.1 C/D). ER β
503-like immunoreactivity was very weakly expressed in the dermal papilla, the sebaceous gland and the epidermis of anagen VI follicles (Fig. 3.2.2 C,c/D,d). The immunoreactivity of ERβ ins was weakly co-expressed (Fig. 3.2.3 C/D).

In late catagen, ERα-like immunoreactivity was expressed in the dermal papilla, secondary hair germ and outer root sheath (Fig. 3.2.1 E/F). ERβ-like immunoreactivity was expressed in the outer root sheath, the dermal papilla, the epidermis and the sebaceous gland (Fig. 3.2.2 E-H), ERβ ins immunoreactivity declined (Fig. 3.2.3 E/F).

The described decrease of immunoreactivity in the murine pelage hair follicle correlates with the decreased ER-mRNA levels in full-thickness back skin homogenates, analyzed by semi-quantitative RT-PCR. Organ-cultured skin samples were investigated after short term culture (6 hours). Skin samples pooled from five individuals were taken at defined time points of depilation-induced hair follicle cycling, day 0 (telogen), day 12 (late anagen) and day 19 (late catagen). Surprisingly ERα mRNA levels decreased more rapidly than ERβ mRNA levels and were hardly detectable after 6 hours in culture, except the samples from the catagen mice (d 19) showed detectable ERα mRNA levels (Fig. 3.2.4).

To summarize, IR of ERα, ERβ and ERβins decreased within six hours of culture in a similar pattern whereas the ER mRNA levels showed differences as ERα mRNA levels was less detectable after six hours in culture compared to ERβ mRNA levels.

Fig. 3.2.1 (page 78) Immunoreactivity of ERα during the depilation-induced murine hair cycle in non-cultured skin (A, C, E) compared to 6 hours organ-cultured murine skin samples (B, D, F). The intense of the signal decreased in all shown hair cycle stages, telogen (day 0 p.d., A/B), late anagen (day 12 p.d., C,c/D,d) and late catagen (day 19 p.d., E/F). In telogen ERα IR was detected within the DP and SG (A/B). In anagen VI ERα-IR was observed in the DP, MK, IRS and ORS (C,c/D,d). In late catagen (E/F) IR was found in the DP, secondary hair germ and ORS.

Fig. 3.2.2 (page 79) Immunoreactivity of ERβ503 in non-cultured skin (A, C, E, F) compared to 6 hours organ-cultured murine skin samples (B, D, G, H). The intense of the signal decreased in all shown hair cycle stages, telogen (day 0 p.d., A/B), late anagen (day 12 p.d., C,c/D,d) and late catagen (day 19 p.d., E/F/G/H) ERβ 503 IR was detected in the DP, IRS, ORS, SG and epidermis.
Fig. 3.2.2
Results

Fig. 3.2.3
Fig. 3.2.3 (page 80) Immunoreactivity of ERβ ins during the depilation-induced murine hair cycle in non-cultured skin (A, C, E, G) compared to 6 hours organ-cultured murine skin samples (B, D, F, H), the intensity of the signal decreased in all shown hair cycle stages, telogen (day 0 p.d., A/B), late anagen (day 12 p.d., C/D) and late catagen (day 19 p.d., E/F/G/H) ERβ ins IR was detected in the DP, ORS, SG and epidermis.

Fig. 3.2.4 (page 82) ERα and ERβ mRNA levels decrease after 6 hours skin organ culture compared to the non-cultured skin sample mRNA levels in the depilation-induced murine hair follicle cycle D0 (A/B), D12 (C/D), D19 (E/F). Experiments were generated from three to five individual animals per time point. The samples were normalized according to the expression of β-actin mRNA (SEM).
Fig. 3.2.4
3.3 Effect of 17β-estradiol, ER-antagonist ICI 182,780, melatonin and prolactin on estrogen receptor beta RNA-level in short term murine skin organ culture

To investigate whether E2 (alone or combined with the pure ER-antagonist ICI 182.780), melatonin or prolactin exert modulatory effects on ERβ-mRNA expression, these substances were added over 6 hours to the organ-cultured skin in two different concentrations each. Skin samples from every group were used to perform a semi-quantitative RT-PCR. Although all mRNA-levels decreased (see 3.2), densitometrically calculated ERβmRNA levels showed different and hair cycle-dependent modulatory levels in the treated groups, compared to the mRNA of the vehicle controls (EtOH for ICI 182.780 and melatonin, DMEM for E2 and prolactin) (Fig. 3.3.1).

In organ-cultured skin of telogen stage (D0), melatonin down-regulates ERβ mRNA levels in both applied concentrations (10pM, 1nM), the pure ER-antagonist ICI 182.780 decreased the mRNA levels in combination with E2 (10nM), whether E2 alone or prolactin had no influence on ERβ gene expression (Fig. 3.3.1 A/a).

In organ-cultured skin of late anagen stage (D12), compared to the vehicle controls only E2 (100nM/1nM) and high-dose prolactin (400ng/mL) showed slightly inhibitory effects on ERβ-mRNA levels (Fig. 3.3.1 B/b).

In organ-cultured skin of late catagen stage (D19) again melatonin decreased the mRNA by both concentrations (10pM, 1nM), low-dose prolactin (200ng/mL), E2 (100nM) alone and in combination with ICI 182.780 showed a slight decrease of ERβ mRNA gene expression. Application of E2 in a lower concentration (1nM) increased the level of ERβ mRNA, in contrast the combination of E2 (1nM) with ICI 182.780 had an inhibitory effect (Fig. 3.3.1 C/c).

Thus, melatonin (10pM, 1nM) and prolactin (400ng/mL) showed inhibitory effects on ERβ mRNA levels in a hair cycle-dependent manner.
Results

Fig. 3.3.1
Fig. 3.3.1 (page 84, 85) Semi-quantitative RT-PCR of ERβ from organ-cultured murine skin in three hair cycle stages of the depilation-induced hair cycle (A,a=D0, B,b=D12, C,c=D19), culture period six hours. Upper panel (a,b,c) PCR expression of beta-actin and ERβ503, lower panel graphs show the intensity of each band evaluated by digital image analysis based densitometry, 1*vehicle control, 2*E2 100nM, 3*E21nM, 4*E2+ICI 182.780 100nM, 5* E2+ICI 182.780 1nM, 6*melatonin 1nM, 7*melatonin 10pM, 8*prolactin 200ng/mL, 9*prolactin 400ng/mL, 10*EtOH second vehicle control.
3.4. Effect of melatonin on estrogen receptor alpha and beta immunoreactivity-like expression in short term murine skin organ culture

Immunoreactivity of ERα, ERβ503 and ERβ ins was detected in all organ-cultured samples after six hours, the signal decreased after melatonin treatment, compared to its vehicle control. The immunoreactivity was detectable in a similar distribution as in the control sample, but showed much weaker expression (Fig. 3.4.1, Fig.3.4.2, Fig.3.4.3).

In telogen stage, ERα-like immunoreactivity was detected in the dermal papilla and cells of the sebaceous gland (Fig.3.4.1 A/B). Immunoreactivity of ERβ was hardly detectable in the epidermis (Fig. 3.4.2 A/B). ERβ ins immunoreactivity was weakly expressed in the dermal papilla (Fig. 3.4.3 A/B).

In anagen VI, ERα-like immunoreactivity was hardly detectable in the matrix keratinocytes (Fig. 3.4.1 C/D). ERβ 503-like was weakly expressed in the dermal papilla and the matrix keratinocytes (Fig. 3.4.2 C/D), as well as the immunoreactivity of ERβ ins (Fig. 3.4.3 C/D).

In late catagen, ERα-like immunoreactivity decreased in the epithelial strand (Fig. 3.4.1 E/F). The intense of ERβ-like immunoreactivity (Fig. 3.4.2 E/F) and ERβ ins immunoreactivity (Fig. 3.4.3 E/F) decreased in the whole hair follicle.

Fig. 3.4.1 (page 87) ERα immunoreactivity-like expression decreased after melatonin treatment in short term murine skin organ culture compared to the vehicle controls (A,C,E), in all stages of melatonin-treated follicles (B,D,F) an ERα-IR decrease was seen, hardly detectable in the anagen VI follicle in the MK and IRS (D) and in the catagen follicle in the ES (F).

Fig. 3.4.2 (page 88) ERβ 503 immunoreactivity-like expression decreased after melatonin treatment in short term murine skin organ culture compared to the vehicle controls (A,C,E), in all stages of melatonin-treated follicles (B,D,F) an ERβ-IR decrease was seen, hardly detectable in the epidermis of the telogen HF (arrowhead A/B) and the matrix keratinocytes of the late anagen follicle (D)

Fig. 3.4.3 (page 89) ERβ ins immunoreactivity-like expression decreased after melatonin treatment in short term murine skin organ culture compared to the vehicle controls (A,C,E), in all stages of melatonin-treated follicles (B,D,F) an ERβ ins-IR decrease was seen, hardly detectable in the anagen VI follicle in the DP (D) and in the catagen follicle in the DP (F).
Results

Fig. 3.4.1
Fig. 3.4.2
Fig. 3.4.3
3.5 Effect of prolactin and melatonin on estrogen receptor beta-expression in female human scalp hair follicles in vitro

To further elucidate the modulatory properties of melatonin and prolactin in vitro, the immunoreactivity of ERß503 was investigated on microdissected organ-cultured female frontotemporal scalp hair follicles. In the melatonin-treated (1 pM and 10nM) (Fig. 3.5.1 A-C) as well as in the prolactin-treated (400ng/mL) female frontotemporal scalp hair follicles (Fig. 3.5.2 A,B) the immunoreactivity decreased in the dermal papilla fibroblasts compared to the non-treated vehicle control.

These results confirm the inhibiting properties of melatonin and prolactin in murine pelage hair follicles which we just detected in the murine model both on the gene and the protein level for melatonin (Fig. 3.3.1 A/a, C/c, Fig. 3.4.1) and on the gene level for prolactin (Fig. 3.3.1 B/b).

3.6 Estrogen receptor beta-expression in human male and female scalp hair follicles

To investigate site-dependent differences in the ERß 503 expression in human skin, we used microdissected anagen VI frontotemporal scalp hair follicles from males and females. In the female hair follicle the immunoreactivity was found in the dermal papilla fibroblasts, the matrix keratiocytes and the outer root sheath (Fig. 3.6.1A/B) as previously described in occipital female hair follicles (THORNTON et al. 2003). In the male hair follicle the immunoreactivity was predominantly expressed in the hair matrix keratinocytes (Fig. 3.6.1C) contrasting to the expression pattern in occipital male scalp hair follicles (THORNTON et al. 2003).

These results support the hypothesis of a location-dependent ERß expression in human scalp hair follicles.
Fig. 3.5.1 ERß 503-like immunoreactivity in female frontotemporal scalp hair follicles after melatonin-treatment. A: non-treated control with ERß expression in the dermal papilla (DP), matrix keratinocytes (MK) and outer root sheath (ORS), B: melatonin treatment 1pM decrease of IR in the DP, C: melatonin treatment 10nM, IR decreased in the DP and MK.
Fig. 3.5.2 ERβ 503-like immunoreactivity after prolactin-treatment in female frontotemporal scalp hair follicle, A: non-treated control, ERβ IR was detected in the DP, ORS and MK; B: treated with 400ng/mL prolactin, IR decreased in the DP and MK.
Results

Fig. 3.6.1 ERß immunoreactivity in female and male frontotemporal hair follicles
The distribution of ERß in female scalp skin (A) and female microdissected frontotemporal scalp hair follicles (B) were found predominantly in the dermal papilla fibroblasts (DP), whereas in male frontotemporal scalp hair follicles the main immunoreactivity was seen in the matrix keratinocytes (MK) (C).
3.7 Influence of 17β-estradiol on estrogen receptor beta-expression in male frontotemporal scalp hair follicles in vitro

To further elucidate the effect of systemic E2 on ERβ 503 expression, we added E2 to the organ culture at 1-100 nM. Stimulation with E2 on organ-cultured male frontotemporal scalp hair follicles increased the immunoreactivity of ERβ 503 in the dermal papilla fibroblasts at a concentration of 1-100nM (Fig. 3.7.1).

3.8 17β-estradiol and hair growth in human male and female scalp hair follicles in the frontotemporal region in vitro

In order to the sex-dependent differences in ERβ immunoreactivity in frontotemporal human scalp hair follicles (Fig. 3.6.1) we further investigated the hair shaft elongation and proliferation rate of hair matrix keratinocytes after E2 treatment in male and female frontotemporal scalp hair follicles in vitro.

The hair shaft elongation was significantly stimulated in the male frontotemporal hair follicles after an E2 supplemented organ cultured of nine days (1-100nM) (Fig. 3.8.1, Fig. 3.8.2). In contrast the hair shaft elongation was significantly inhibited in the female frontotemporal hair follicles after nine days in E2-treated organ culture (1-100nM) (Fig. 3.8.3).

The proliferation of hair matrix keratinocytes increased significantly in the male frontotemporal hair follicles (E2 10nM) (Fig. 3.8.4) whereas the keratinocytes proliferation was inhibited in the female hair follicles (Fig. 3.8.5).

The duration of anagen was slightly prolonged in both experiments (Fig. 3.8.6 A/B).

Therefore, the E2 effect on human hair growth had to be addressed sex-dependent.
Fig. 3.7.1 ERß-expression in male frontotemporal scalp hair follicles after 17ß-estradiol stimulation in vitro
Immunoreactivity of ERß in the non-treated follicle was detected predominantly in the matrix keratinocytes (arrow, A), after stimulation with E2 the intensity of immunoreactivity in the dermal papilla fibroblasts increases, E2 1nM (arrow, B) and 100nM (C).
Fig. 3.8.1 Hair shaft elongation after E2-treatment in human male frontotemporal scalp hair follicles
Starting on day 3 the elongation was significantly stimulated after E2-stimulation 1-100nM, SEM, p-values: 0.01< *<0.05, ***<0.001
Fig. 3.8.2: E2 stimulates hair shaft elongation of male hair follicles in vitro

Light microscopy on day 7 of the vehicle control male hair follicle (A/C) and the E2-treated (10nM) male hair follicle (B/D). H&E stainings of the control follicle (E, catagen) and the E2-treated follicle (F, anagenVI). Magnification at 100x (A/B), 200x (C/D), 250x(E/F)

CTS: connective tissue sheath, DP: dermal papilla, HS: hair shaft, IRS: inner root sheath, mel: melanocytes, ORS: outer root sheath
Fig. 3.8.3 Hair shaft elongation after E2-treatment in human female frontotemporal scalp hair follicles
Starting on day 5 the elongation was significantly inhibited after E2-stimulation 1-100nM, SEM, p-values: 0.01< *<0.05, ***<0.001
Fig. 3.8.4 Proliferation of hair matrix keratinocytes increased significantly in the male frontotemporal hair follicles after E2 treatment (E2 10nM), SEM, p-value 0.01< *<0.05

Fig. 3.8.5 Matrix keratinocytes proliferation decreased in the female hair follicles after E2 treatment (E2 1-100nM), SEM
Fig. 3.8.6  E2 administration slightly prolongs the anagen stage
Hair cycle stages after organ culture (nine days) supplemented with E2 (1-100nM), A: male scalp hair follicles, B: female scalp hair follicles
3.9 Effect of 17ß-estradiol on IGF-I and IGF-IR expression female frontotemporal scalp hair follicles in vitro

To confirm the evidence of a crosstalk between peptide growth factors and ERs, we used microdissected human scalp hair follicles to perform an organ-culture and added E2 to the medium. Thereafter we performed an immunofluorescence staining of IGF-I and IGF-IR. Changes were seen as in order to IGF-I expression. IGF-I-like predominant immunoreactivity changed the localizational region within the hair follicle with increased E2-levels (Fig. 3.11.1 A-C). The immunoreactivity of IGF-IR was not modulated by E2 (Fig. 3.11.1 D).

3.10 Catagen development of estrogen receptor beta-deficient mice compared to wildtype animals

The catagen development of ERß-deficient mice (p19) was compared to the age-matched wildtype animals and showed no alterations in order of the hair follicle cycling (Fig. 3.10.1). The dermal thickness, as an additional tool in the investigation of the catagen stage, was significantly increased in the tail region of ERß-deficient mice back skin compared to the wildtype control animals (Fig. 3.10.2). The number of apoptotic cells was not altered in the hair follicles of ERß-deficient mice compared to the wildtype controls (Fig. 3.10.3).
Fig. 3.9.1 E2 treatment changes the distribution pattern of IGF-I
IGF-I immunofluorescence staining, female scalp hair follicles, A
control, B E2 10nM, C E2 100nM; inner root sheath (IRS), outer root
sheath (ORS)
D IGFIR expression in female scalp hair follicle in the dermal papilla
(DP) and connective tissue sheath (CTS) (arrow), similar after E2
treatment.
Fig. 3.10.1 Catagen development of ERβ-deficient mice (p19) was compared to the age-matched wildtype animals and showed no alterations in order of the hair follicle cycling, n= 6/3, SEM.

Fig. 3.10.2 Dermal thickness was significantly increased in the tail region of ERβ-deficient mice back skin compared to the wildtype control animals, SEM, p-values:*** <0.001
3.11 Expression pattern of estrogen receptor alpha in the hair follicle of estrogen receptor beta-deficient mice in the catagen stage compared to wildtype mice

The expression pattern of ERα-like immunoreactivity did not change in the skin and hair follicles in the ERβ-deficient mice compared to the wildtype control animals and was detected in the dermal papilla, outer root sheath and epithelial strand (Fig. 3.11.1).
**Fig. 3.11.1 Immunoreactivity of ERα in ERβ-deficient mice compared to wildtype controls**

The distribution pattern of ERα in the ERβ-deficient mice (C/D) was similar to that in the wildtype animals (A/B). IR was observed in the dermal papilla (DP), the outer root sheath (ORS) and epithelial strand (ES).
3.12 Estrogen-responsive target genes in male and female scalp hair follicles

Male and female E2-stimulated hair follicles were analyzed by cDNA microarray. Of 1300 genes tested, more than 600 E2-responsive genes were detected in both experiments (Fig. 3.12.1). For significances the level was chosen for <0.5 fold suppressed or > 1.5 fold stimulated in at least one experiment, which decreases the number of relevant genes down to 70 genes.

Additional to novel E2-responsive target genes in the human hair follicle (Table 3.12.1), a sex-dependent difference in regulation was detected: as some genes were only regulated in a single sex or in a contrasting manner (Table 3.12.2), for better differentiation the genes formerly known to be involved in the hair follicle cycle were marked red, whereas the genes with a reported connection to ER or E2 were marked green.

The hair cycle is influenced by numerous cytokines, growth factors and neuropeptides and some of the genes detected in this experiment are linked with signaling pathways in the hair cycle control. For example BMP7 (Bone morphogenetic protein 7) is a member of the TGF-β superfamily known to play an important role in developmental systems including the hair follicle (STENN and PAUS 2001). FGFR2 (Fibroblast growth factor receptor 2) is located in the matrix of the hair follicle (ROSENQUIST and MARTIN 1996), EPS8 (EGF-R pathway substrate 8) is a part of the epidermal growth factor receptor pathway and EGF-R is known to be located in the anagen ORS and matrix and in catagen on all undifferentiated cells of epithelial strand and secondary hair germ (STENN and PAUS 1999). K6HF is a novel keratin type II which is only reported in the hair follicle (WINTER et al. 1998). The fact, that E2 regulates the expression of these genes in the hair follicle supports the hypothesis of an E2/ER-mediated influence on hair follicle cycling.

For confirming the results from the microarray, real-time PCR was not available therefore we performed immunohistochemistry studies. To investigate, which ER-pathway is involved in the signaling of K6HF, which is expressed in the companion
layer of the hair follicle, immunohistochemistry was performed in an immunofluorescence staining on microdissected human hair follicles, murine skin of C57BL/6 mice, ERβ-deficient mice and the age-matching wildtype animals in late anagen stage. The intensity declined in the ERβ-deficient and wildtype samples, but immunoreactivity was still detectable. The distribution pattern of the immunoreactivity of K6HF in the human hair follicles and the murine anagen skin was similar to the previous described results detected in the companion layer of the hair follicle (Fig. 3.12.2, 3.12.3) (WINTER et al. 1998).

![Fig. 3.12.1 Scatter plot of cDNA microarray from E2-treated and non-treated control hair follicles](image)

Fig. 3.12.1 Scatter plot of cDNA microarray from E2-treated and non-treated control hair follicles (Cy5/red, stimulated sample; Cy5/green, control group). Each data point represents signal intensities for one gene in a double logarithmic scale.
### Table 3.12.1

<table>
<thead>
<tr>
<th>Genes marked are known regarding to E2/ER or hair growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E2-responsive genes down-regulated in human HF</strong></td>
</tr>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>MMP1: Matrix metalloproteinase 1</td>
</tr>
<tr>
<td>Interstitial collagenase</td>
</tr>
<tr>
<td>SPP1: Secreted phosphoprotein 1/OPN: osteopontin</td>
</tr>
<tr>
<td>Secreted by secretory phase ductal epithelium</td>
</tr>
<tr>
<td>FN1: Fibronectin 1</td>
</tr>
<tr>
<td>Adhesive glycoprotein of the extracellular matrix and plasma</td>
</tr>
<tr>
<td>SOD2: Superoxide dismutase 2</td>
</tr>
<tr>
<td>Major detoxifying enzyme in cellular oxygen metabolism</td>
</tr>
<tr>
<td>PGS2_HUMAN Bone proteoglycan II precursor</td>
</tr>
<tr>
<td>Interacting with collagen (decorin)</td>
</tr>
<tr>
<td>CDKN1A: Cyclin-dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>Regulator of cell cycle progression at G1</td>
</tr>
<tr>
<td>SPARCL1, HEVIN: high endothelial venule protein</td>
</tr>
<tr>
<td>In chronic inflammatory processes acquired from endothelial of nonlymphoid tissue</td>
</tr>
<tr>
<td>GJA1: Gap junction protein alpha 1 (connexin 43)</td>
</tr>
<tr>
<td>Proteins in cell-to-cell channels</td>
</tr>
<tr>
<td>SDCBP: Syndecan binding protein</td>
</tr>
<tr>
<td>Transmembrane proteogycans</td>
</tr>
<tr>
<td>PRKCH: Protein kinase C</td>
</tr>
<tr>
<td>Major signal transduction systems</td>
</tr>
<tr>
<td>SDC1: Syndecan 1</td>
</tr>
<tr>
<td>Integral membrane protein acting as a receptor for the extracellular matrix</td>
</tr>
<tr>
<td>BGN: Biglycan</td>
</tr>
<tr>
<td>In connective tissue metabolism by binding to collagen and TGF-β</td>
</tr>
<tr>
<td>CALR: Calreticulin</td>
</tr>
<tr>
<td>Major Calcium-binding protein in the endoplasmatic reticulum</td>
</tr>
<tr>
<td>IQGAP1: IQ motif containing GTPase activating protein</td>
</tr>
<tr>
<td>Interacts with components of the cytoskeleton</td>
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</table>

<table>
<thead>
<tr>
<th><strong>E2-responsive genes up-regulated in human HF</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>K6HF: Cytokeratin type II</td>
</tr>
<tr>
<td>Form heteropolymers with type I chains in 1:1 ratio, ORS</td>
</tr>
<tr>
<td>Transgelin</td>
</tr>
<tr>
<td>Fibroblasts and smooth muscles</td>
</tr>
<tr>
<td>Thiosulfate sulfurtransferase</td>
</tr>
<tr>
<td>Mitochondrial matrix enzyme</td>
</tr>
<tr>
<td>CDK5: Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>Neuronal signal transduction</td>
</tr>
<tr>
<td>BMP7: Bone morphogenetic protein 7</td>
</tr>
<tr>
<td>Member of TGF-β superfamily</td>
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</tbody>
</table>
Table 3.12. 2

Genes marked are known regarding to E2/ER or hair growth

<table>
<thead>
<tr>
<th>E2-responsive genes regulated only in male human HF</th>
<th>female HF</th>
<th>male HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLN: Filagrin</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Important keratin filament</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK1: Protein-tyrosine kinase</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>Involved in cytokine signaling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITGAV: Integrin</td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>Major family of cell surface receptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGFR2: Fibroblast growth factor receptor 2</td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>Signals immature cells in the developing embryo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIM1: absent in melanoma</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>CDH1: Cadherin1</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>Cell-cell adhesion glycoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPS8: Epidermal growth factor receptor pathway substrate 8</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>SOCS5: Suppressor of cytokine signaling</td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>Negative regulator of JAK1 and STAT proteins</td>
<td></td>
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<tr>
<th>E2-responsive genes regulated only in female human HF</th>
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<tr>
<td>MAL proteolipid</td>
<td>13.35</td>
<td></td>
</tr>
<tr>
<td>Component of the membrane subdomains of T-lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR4A1 (nuclear receptor subfamily 4, group A, member 1)</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>Shares 20% amino acid homology with the ER</td>
<td></td>
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<tr>
<th>E2-responsive genes regulated sex-dependent different in human HF</th>
<th>0.58</th>
<th>1.48</th>
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<tbody>
<tr>
<td>FOSL2: FOS-like antigen2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forming the transcription factor complex AP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYR61: Cysteine-rich, angiogenic inducer, 61</td>
<td>0.56</td>
<td>1.05</td>
</tr>
<tr>
<td>Significant sequence homology to the insulin-like growth factor binding proteins</td>
<td></td>
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<tr>
<td>COL4A6: Collagen, type IV, alpha6</td>
<td>0.51</td>
<td>1.12</td>
</tr>
<tr>
<td>Collagen of basement membrane</td>
<td></td>
<td></td>
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</table>
Fig. 3.12.2 Immunoreactivity of K6HF in murine skin (arrows), in C57BL/6 (A/B, day 8 after depilation, late anagen), wildtype control (C/D) and ERβ-deficient mice (E/F)
Fig. 3.12.3 Immunoreactivity of K6HF in human scalp hair follicles in the companion layer (arrows), human nonbalding skin (A), male frontotemporal hair follicle (B), female frontotemporal hair follicle (C). DP: dermal papilla
4. DISCUSSION

Regarding the specific questions we addressed in this study we firstly re-evaluated the ER expression profile throughout the entire murine hair follicle cycle and showed a hair cycle dependent expression pattern of all investigated ERs (ER, ERβ 503, ERβins). They were detectable during the complete hair follicle cycle both on the gene as on the protein level (Fig. 3.1.1, Fig. 3.1.2, Fig. 3.1.3).

To gain insights into the modulatory action in the hair follicle we secondly performed murine skin organ culture. This in vitro-study was extended with regard to the human system and a organ culture with microdissected human scalp hair follicles was performed. In both experiments we added E2, the pure ER antagonist ICI 182,780, prolactin and melatonin in different concentrations to the medium to investigate their modulatory properties. The intensity of all investigated ERs-expression declined after the culture period with differences towards the non-cultured expression pattern of ERs (Fig. 3.2.1, Fig. 3.2.2, Fig. 3.2.3, Fig. 3.2.4, Fig. 3.3.1).

With respect to human hair follicles we focused on ERβ and detected sex-dependent differences as well in its expression pattern as in hair shaft elongation after E2 treatment (Fig. 3.6.1). Male frontotemporal scalp hair follicle growth was significantly stimulated whereas the hair shaft elongation in female frontotemporal hair follicles was inhibited by E2 (Fig. 3.8.1, Fig. 3.8.2, Fig. 3.8.3).

After various reports about the cross talk between ER and peptide growth factors in several tissues (IGNAR-TROWBRIDGE et al. 1995; KLOTZ et al. 2002; MARTIN and STOICA 2002) and the stimulatory effect of IGF-I on hair growth in vitro (PHILPOTT et al. 1994), we thirdly compared the immunoreactivity of IGF-I and IGF-IR in E2-treated female scalp hair follicles to the vehicle controls and found differences in the expression pattern of IGF-I after E2-treatment, suggesting an ER-IGF-I cross talk in human hair follicles (Fig. 3.9.1).

Next, we investigated the catagen development ERβ-deficient mice with wildtype control animals regarding to differences in the histomorphometry and hair cycle alterations. As expected, ERβ-deficient mice do not have profound alterations
in hair follicle cycling, confirming the concept of an ERα mediated pathway of estrogen effects in the murine skin (Fig. 3.10.1).

To finally explore the target genes of E2 in the human hair follicle we performed a cDNA-microarray using a commercial skin focus chip. The regulated genes were in parts differently modulated in a sex-dependent manner and additionally we detected novel E2-responsive genes in the human hair follicle, which are known to be involved in hair growth (Table 3.12.1, Table 3.12.2).

It is undisputed that estrogens can profoundly modulate hair growth in practically all mammalian species investigated, usually by exhibiting hair growth inhibitory properties (EMMENS 1942; WILLIAMS et al. 1946), the exact signaling pathways and the involved mechanisms still need to be elucidated. Most of the estrogen effects in mammals are mediated on the transcriptional level by the classical receptor pathway (ERα and ERβ), appearing predominantly intranuclear. They act complementarily but not redundant and are genetically and functionally distinct. Several splice variants, such as estrogen-binding proteins, putative ERs or even new ER-family members are involved in estrogenic action with different functions, locations and interactions towards, together or probably against the classical ERs (PETERSEN et al. 1998; HAWKINS et al. 2000; OKUDA et al. 2003).

To learn more about the exact mechanism of signaling pathways one must take into account that there are at least four possible pathways for estrogenic responses (HALL et al. 2001; CATO et al. 2002) and a plethora of cofactors influencing either in a stimulative or inhibiting manner (HORWITZ et al. 1996; SMITH 1998; AUBOEUF et al. 2002). Therefore, the investigative tools and parameters should be chosen carefully in order to get pioneering results.

The aim of the current study was to obtain more detailed information of estrogen and its receptors in respect to their hair growth modulatory properties. We wanted to explore the signaling pathways and modulatory options in murine and human hair follicles. This must be addressed species-specific, as the reported effects of estrogen shows differences between the species in various cases. In animals estrogens inhibit hair growth (WILLIAMS et al. 1946; SMART et al. 1999), whereas
they prolong the anagen phase in human hair follicles (SCHUHMACHER-STOCK 1981; SINCLAIR 1999). Furthermore, until today ERβ is suggested to play the predominant role of estrogenic response within human beings (THORNTON et al. 2003), whereas ERα may fulfill the main functions in mice (CHANDA et al. 2000; MOVERARE et al. 2002).

The beginning of this study was the re-evaluation of the existence of the major ER subtypes in mice, ERα and ERβ, including its splice variant ERβ ins. Performing immunohistochemistry as well as using full-thickness back skin samples for RT-PCR it was shown that all the investigated ERs were expressed during the entire depilation-induced murine hair cycle, with differences in expression and distribution in a hair cycle dependent manner (Fig. 3.1.1-3.1.3). Variations of expression intensity during the murine hair cycle on the gene level were previously described for beta actin in real time PCR (MECKLENBURG 2002) complicating the internal standards in this respect.

It has been suggested that the estrogen action within murine skin is mediated via ERα in male and female mice (CHANDA et al. 2000; MOVERARE et al. 2002). We observed a peak of ERα-expression in telogen skin (Fig. 3.1.1), which is well in line with the concept that ERα acts as a hair cycle brake and therefore prolongs telogen. The dermal papilla is known to function as a control organ on epithelial-mesenchymal interactions (STENN and PAUS 2001), here we found most intense ERα staining within the telogen follicle.

It is speculative to ask why ERβ (and ER β ins) have not been detectable in the entire murine skin earlier, maybe due to a lack of specific antibodies. ERs are known to be difficult to investigate by any kind of method: western blotting, immunohistochemistry or on mRNA level. There are numerous explanations: the low-level expression in non-reproductive tissues, the secondary and tertiary structure of the receptors which makes exploration difficult, the wide spread distribution in the nucleus, the cytosol and plasma membrane or the opposite action of the ERs on some gene promotors (WARNER et al. 2003). Anyway, the co-existence of ERα, β and especially the weaker expressed ERβ ins, namely a negative regulator on ERα (SAJI et al. 2001), in the entire murine hair follicle cycle (Fig. 3.1.2) confirm the
strong evidence for an estrogen receptor-mediated signaling pathway in the murine hair follicle cycle as reported before (OH and SMART 1996).

For further investigation on murine hair growth in vitro, the well-established murine skin organ culture was performed. Skin from defined cycle stages, after synchronization of all back skin hair follicles by depilation was studied, concentrating on three time points of the hair cycle: the relative quiescence in telogen stage, the maximum of hair growth in anagen VI and the end of regression, late catagen. To see any effects on hair growth the time of the organ culture should be at least 48 hours. Unfortunately, after two days of culture we did not detect any ERs within the murine skin neither on the gene nor on the protein level at any of the three time points of the hair cycle. As known from androgens (WARNER, 2003, personal communication) which are hardly detectable after few hours in culture, we shortened the culture period down to 24, 12 and at last down to six hours. It was still possible to expect changes in the ER-expression pattern because the chosen substances exert rapid effects, but with respect to the hair cycle a six hours culture period would be too short to have any remarkable effects in the hair follicle cycle.

Therefore, we concentrated on the ER-expression pattern in the skin and especially in the HF. We used endogenous modulators with profound influence on estrogens and the estrogen receptors towards their function in the murine hair follicle. Firstly E2 as classical ER-ligand with a high affinity to both receptors was added alone to prove its systemic effects on hair growth, as it is still believed that only topical applied E2 affects hair growth in mice (SMART et al. 1999). Secondly E2 was added in combination with the pure ER-antagonist ICI 182,780 to exclude or identify ER-mediated signaling of estrogenic response, which has been successfull performed in vivo (SMART et al. 1999) and in rat decidua cells in vitro (ARONICA and KATZENELLENBOGEN 1993). Furthermore melatonin, known to have inhibitory properties in several cancer tissues especially in estrogen-positive breast cancer cells (MOLIS et al. 1994; KIEFER et al. 2002) was proved on its effect on murine hair growth in vitro. Prolactin, which is involved in the hair growth cycle by inducing catagen (FOITZIK et al. 2003) and is expressed as its receptor hair cycle dependent, was chosen for its specific effects on ERs, e.g. prolactin decreases ERα and
increasing ERß-mRNA levels in rat decidua and after high-dose prolactin treatment (SHAFIE and BROOKS 1977; TESSIER et al. 2000). All substances were given in two concentrations, one at least supraphysiological, one physiological or supraphysiological as well, regarding to female blood levels.

As suggested, even after short culture-time a rapid decrease of ERα and ERß was detected on both the gene and the protein level (Fig. 3.2.1-3.2.4). ERα was compared to ERß less stable, which might be due to the semi-quantitative method which even densitrometrically calculated remains semi-quantitative and due to the fact that we used full thickness skin homogenates. A quantification by real-time PCR would be needed to confirm these results. Previously a report of real-time RT-PCR on breast cancer showed that the level of transcript of ERß does not correlate with ERα and therefore suggesting that ERß mRNA expression is independent of the classical parameters (DE CREMOUX et al. 2002).

However, it was interesting that melatonin showed inhibitory properties on ER-expression on the protein as well as on the gene level in murine skin (Fig. 3.3.1, Fig. 3.4.1-3.4.3). Probably these effects are due to membrane receptor associated signaling because of the low-dose concentration (1pM). Other melatonin signaling pathways as direct interaction of ER-DNA binding (RATO et al. 1999) do not remain realistic in this experiment. None of the other treatments had paralleling results on both levels.

All parameters which are routinely used in hair research i.e. hair cycle score, apoptotic cells or the dermal thickness in catagen development failed in this study because of the shortness of the culture period.

To summarize the results in the murine model, the expression of the major subtypes ERα and ERß throughout the entire hair follicle cycle is an important new feature in estrogenic signaling and murine hair growth. It remains to be proven in which relation the ERß functional acts in hair growth towards ERα. Only the murine skin organ culture did not show up as a perfect model for ER-research. It might be possible to further improve this model, i.e. changing the ingredients of the medium. Previously detection of ER-expression in cultured human tissue in a long-term study
to after a culture period over more than one week has been reported (ZHUANG et al. 2003).

Turning to the human system we found matching results using the microdissected human scalp hair follicle for \textit{in vitro} investigations.

In human beings the 1996 cloned ER\(\beta\) (MOSSELMAN et al. 1996) is known to be predominant in several non-reproductive tissues, as just recently reported in oral epithelium and salivary glands (VALIMAA et al. 2004). The current opinion for human nonbalding skin is that ER\(\beta\) is predominant in human hair follicles (THORNTON et al. 2003). Regarding to the paradoxical, site-dependent effects of androgens on hair growth (JAHODA and REYNOLDS 1996) and the fact that the yet known reports are based on experiments in occipital scalp skin (THORNTON et al. 2003), we were interested if this would be confirmed for both sexes in frontotemporal scalp hair follicles and detected ER\(\beta\) expression in both male and female frontotemporal scalp hair follicles in a different expression pattern (Fig. 3.6.1). In the female hair follicle the ER\(\beta\) expression was located in the dermal papilla fibroblasts and hair matrix keratinocytes, whereas in male scalp hair follicles ER\(\beta\)-like immunoreactivity was mainly found in the hair matrix keratinocytes. The reported expression pattern of ER\(\beta\) in male and female occipital hair follicles in the dermal papilla cells, hair matrix keratinocytes, sebaceous gland, epidermis and outer root sheath matches with the expression we detected in female microdissected frontotemporal hair follicles and scalp skin sections (THORNTON et al. 2003).

After stimulation with E2 the distribution pattern of ER\(\beta\) in the male hair follicle changed towards an increase of immunoreactivity in the dermal papilla fibroblasts, being the control organ of epithelial-mesenchymal interactions (STENN and PAUS 2001) (Fig. 3.7.1). This is even more interesting as the hair shaft elongation of male frontotemporal hair follicles significantly increased after E2 treatment (Fig. 3.8.1, 3.8.2). This result was not in line with previous reports of inhibiting properties of E2 on human hair growth in women and men (KONDO et al. 1990; NELSON et al. 2003), and again the controversial result was due to the location. The previous studies concentrated on occipital scalp hair follicles whereas we used samples from
the frontotemporal site. The inhibiting activity of E2 on the hair shaft elongation of occipital female scalp hair follicles (KONDO et al. 1990; NELSON et al. 2003) was confirmed in this study for female frontotemporal hair follicles (Fig. 3.8.3). In both experiments E2-treatment slightly prolongs the anagen stage (SCHUHMACHER-STOCK 1981) (Fig. 3.8.6) and the proliferation rate was increased in the stimulated male hair follicles (Fig. 3.8.4) whereas the proliferation of the hair matrix keratinoctyes decreased in the female hair follicles (Fig. 3.8.5). These results raise the question whether the signaling and gene expression response of defined human hair follicle population to E2-stimulation differ in a stringently location-dependent manner, as it has been postulated for the response of beard hair versus scalp follicles to androgen stimulation with respect to TGFβ1 expression in the dermal papilla (INUI et al. 2002).

Regarding the results of the animal model we wanted to explore the interaction of ERβ, melatonin and prolactin in human scalp hair follicles. As in mice, in female frontotemporal scalp hair follicles the intensity of ERβ immunoreactivity declined after supplementing melatonin (10nM) to the culture, an effect probably due to membrane-associated receptor signaling (Fig. 3.5.1). In a similar pattern a decrease of immunoreactivity especially in the dermal papilla fibroblasts was detected after prolactin treatment (400ng/mL) (Fig. 3.5.2). Prolactin and E2 share numerous non-reproductive target tissues (FRASOR and GIBORI 2003) and prolactin is known to modulate ER mRNA levels in a different manner, i.e. after high-dose treatment a down-regulation of ERβ was seen in rat decidua (TESSIER et al. 2000), whereas in human breast cancer cells an increase of ER-levels was reported after prolactin supplement in vitro (SHAFIE and BROOKS 1977). Prolactin is furthermore involved in the hair follicle cycle and induces catagen (FOITZIK et al. 2003) in contrast to E2 which is believed to prolong the anagen stage. Therefore a decrease of ER-expression by prolactin in the hair follicle may be possible. Another connection between prolactin and E2 is given as the prolactin receptor is associated with the short form of 17β-HSD, one of the key enzymes of conversion from precursors to the active 17β-estradiol. Prolactin and its receptor are
expressed in the hair follicle epithelium in a hair cycle dependent manner (FOITZIK et al. 2003).

Peptide growth factors are involved in the ER-signaling in the hair follicle. IGF-I stimulates hair growth in the absence of insulin in a dose-dependent manner in vitro and is therefore suggested to be an important physiological regulator of the hair follicle growth and the hair follicle cycle (PHILPOTT et al. 1994). The expression of IGF-I, but not of IGF-IR was regulated in female scalp hair follicles by E2 application in vitro (Fig. 3.9.1). This suggests a cross-talk between ER and IGF-I in the human hair follicle as reported from IGF-I and ERα in the uterus (KLOTZ et al. 2002) and supports the concept that ER stimulation affects the regulation of key growth factors.

To summarize the results of the human hair follicle in vitro experiment it was shown that profound locational and sex-dependent differences exist with respect to estrogenic effects on human scalp hair growth and ERβ-expression. Furthermore, the endogenous modulation within the hair follicle was proven using microdissected anagen VI scalp hair follicles in the absence of the sebaceous gland, a key compartment for steroid hormone synthesis and metabolism (ZOUBOULIS 2000). The ability of E2, melatonin and prolactin to regulate ERβ-expression in human hair follicles confirms the strong evidence for an interplay of ER and hormones in the human hair follicle. Cross-talk between the ERs and IGF-I was confirmed in E2 stimulated scalp hair follicles.

Additional to the mouse in vitro-studies, ERβ-deficient mice (BERKO) and age-matching wildtype animals were investigated regarding to alterations in the catagen development. ERα-deficient mice were not available for comparable studies as well as double-ER-deficient mice. The data have to be taken as preliminary because of severe breeding difficulties the number of investigated animals was very limited (three wildtype animals and six BERKO-mice). However, the hair follicle cycle in the catagen stage (p 19) showed no alterations in the BERKO mice compared to their wildtype controls, neither detected were histomorphological differences (Fig. 3.10.1, Fig. 3.10.3). Immunoreactivity of ERα was found to be expressed similar in both samples (Fig. 3.11.1). In BERKO mice the dermal thickness significantly increased
compared to age-matching wildtype controls in late catagen stage (Fig. 3.10.2). These results suggest an ERα-mediated pathway in hair follicle cycling as previously reported for the hair follicle (CHANDA et al. 2000; MOVERARE et al. 2002). Probably ERβ acts as a quencher for ERα as it has been shown that one of the main functions of ERβ is supposedly the repression or the replacement of ERα in the absence of ERα (LINDBERG et al. 2003). To further elucidate the exact function of ERβ in the hair follicle it would be necessary to have comparable skin samples from other ER-deficient mice and to perform in vitro-assays for modulatory effects of E2 and growth factors which are important in hair follicle cycling, i.e. IGF-I, VEGF, as shown in the uterus of BERKO mice for ERβ to be a modulator of ERα (WEIHUA et al. 2000). Because of the above mentioned breeding difficulties we were not able to get enough skin samples so far in order to perform all necessary investigations.

Finally the estrogen-responsive target genes in human hair follicles were explored (Table 3.12.1). The partially sex-dependent regulation confirms one hypothesis of this study: that hair follicle growth is differently regulated between males and females (Table 3.12.2). Just as the regulation of several primary E2-responsive genes, i.e. progesterone receptor protein or c-jun, and of hair growth related genes, i.e. BMP7, EPS8 [belongs to the epidermal growth factor receptor-pathway], CYR61 [homolog of insulin-like growth facto-binding protein], supports the concept of profound estrogenic effects in hair follicle cycling.

Some of the E2-regulated genes in the human hair follicles have been topics in previous studies: By investigating the epidermal differentiation in male mice it was reported that Filaggrin is highly expressed in wildtype animals and ERβ-deficient mice, but not in ERα and double KO mice, suggesting an ERα-mediated pathway of estrogenic regulation of hair follicle cycling (MOVERARE et al. 2002). Filaggrin was now detected to be down-regulated only in human male hair follicles, therefore it would be most interesting to repeat the above mentioned investigations in female mice in order to evaluate that sex-dependent difference is transferable to the murine system. For K6HF, a novel outer root sheath keratin type II (WINTER et al. 1998; WANG et al. 2003), we detected that the immunoreactivity in ERβ-deficient mice was
similar to the expression pattern in the wildtype animals (Fig. 3.12.1), suggesting again an ERα-mediated pathway in the murine hair follicle.

Recently a human osteoblast cell line for investigating the ER-isoform specific regulation of endogenous gene expression was created and after E2-stimulation a microarray was performed on the cells. Osteopontin which is down-regulated in male and female scalp hair follicles (Tab. 3.12.2), is supposedly mediated through both receptors in human osteoblasts, i.e. ERα and ERβ. The results were confirmed by real-time PCR (MONROE et al. 2003).

Furthermore, a novel highly conserved zinc-finger protein, Ini, has been just identified by screening an estrogen-induced rat myometrial expression library. Ini binds the proximal connexin 43 promoter and enhances its response to estrogen. Connexin 43 (gap junction protein) which was reported to lack in a special subset of cells in the bulge region (MATIC et al. 1998) and therefore probably involved in the hair follicle cycling was now found to be an estrogen-related gene in the human hair follicle and down-regulated in both male and female scalp hair follicles by E2 (Tab. 3.12.1).

By determining the role of ERß in vivo on global estrogen-regulated transcriptional activity in bone it was detected that ERß inhibits ERα-mediated gene transcription in the presence of ERα, whereas in the absence of ERα it can partially be replaced by ERß (LINDBERG et al. 2003). Those studies show the great importance and influence of gene expression profiles on contemporary research.

The evaluation of the ERs throughout the whole murine hair follicle cycle, the sex- and location dependent differences of ER-expression in the human scalp hair follicle, the list of target genes of E2 in the human hair follicles, these results need to be further elucidated. To extend the murine model, one could perform a vibrissae culture to investigate the site-dependent differences in the mouse as it was shown in the human model. It is noteworthy to emphasize, that the murine pelage hair follicle cannot be compared with the human scalp hair follicle, therefore one must be careful in drawing conclusions from the one model towards the other. Additional to the microarray, which urgently needs to be further confirmed by real-time PCR and expanded in view to immunohistochemical studies, an investigation of the sex-
dependent differences of ER-expression on the gene level in the human scalp hair follicles should be performed using real-time PCR.

Since 1996, when the second ER was cloned, it became clear that probably nothing regarding to estrogens and estrogen receptor-signaling pathways known so far was for sure and almost in every tissue other ER variants than the classical ERα were detected or even an ortholog, as previously reported from the mollusk *Aplysia* (SCHWABE and TEICHMANN 2004). This study shows once more the complexity of estrogens and estrogen receptors and the interplay with other hormones, growth factors and enzymes. Although it was possible to gain new insights of estrogens affecting the murine pelage hair follicle and the human frontotemporal scalp hair follicle cycling, open questions still remain:

1. What are the differences between male and female hair follicles with respect to aromatase activity (SAWAYA 1997; HOFFMANN et al. 2001; HOFFMANN et al. 2002)?
2. How do occipital and frontotemporal hair follicles, as well as various other integumental sites, differ from each other in this respect?
3. Is there any indication that $E_2$ exerts similarly “paradoxical, site-dependent” effects on human hair growth as androgens (JAHODA and REYNOLDS 1996)?
4. Which important regional differences in the extrafollicular estrogen metabolism of defined integumental sites must be taken into account when estrogens are administered topically (e.g. with respect to epidermal, sebaceous and dermal activities of key enzymes like aromatase, 17β-hydroxysteroid dehydrogenase or steroid sulfatase)?
5. How is the metabolism of topically applied estrogens in loco influenced by the choice of vehicle?
5. SUMMARY

Franziska Conrad: Role of Estrogen receptor signaling in the control of hair follicle cycling

The hair follicle (HF) underlies a life-long cyclic transformation and can serve as a perfect model system for dissecting the impact of steroid hormones such as estrogens on epithelial-mesenchymal interactions. Even though the HF is highly estrogen-sensitive, it is still unclear how exactly estrogens affect hair growth. In animals, estrogen (17-β-estradiol, E2) exhibits inhibitory effects on hair follicle growth and cycling, whereas in human scalp skin E2 is thought to prolong the growth phase of the hair cycle (anagen). Therefore, E2-modulation of hair growth must be addressed species-specific.

The aim of the current study was to show how the two major subtypes of estrogen receptors (ER), ERα and ERβ, are expressed throughout the murine hair cycle and in human scalp hair follicles. In addition it was studied whether ER-expression in the HF is regulated by recognized endogenous ER-modulators in vitro. Finally, new estrogen-modulated target genes in the hair follicle were explored.

In mice, ERα and ERβ expression was shown to be strikingly hair cycle-dependent, both on the protein and gene level. In murine skin organ culture, ER-expression of both subtypes decreased rapidly within a few hours, both on the protein and on the gene level. After modifying the model by shortening the length of the culture period (total 6 hours), an inhibitory effect of E2, prolactin and melatonin on ERβ-expression could be assessed, on RNA and, in case of melatonin, also on protein level.

These results were confirmed in microdissected, organ-cultured human scalp HFs: ERβ immunoreactivity, known to be predominant in human skin (compared to ERα), decreased after prolactin or melatonin treatment in cultured human female scalp HFs.

Furthermore, ERβ-expression in human scalp hair follicles showed surprisingly sex-dependent differences (predominant ERβ-expression in male scalp HF: matrix keratinocytes; in female HF: fibroblasts of the dermal papilla). Treatment with E2 modulates ERβ-expression in male scalp HFs. The effect of E2 on human hair shaft elongation in vitro was therefore investigated with regard to sex-differences. In female scalp hair follicles, E2 inhibited hair shaft elongation and hair matrix keratinocytes proliferation. In contrast, E2 stimulated both the hair shaft elongation of male frontotemporal scalp hair follicles and hair matrix keratinocytes proliferation in organ-culture.

Next, potential cross-talk between peptide growth factors and ER-signaling was investigated and significant changes of the immunofluorescent signals for IGF-I were detected in E2-treated human scalp HFs. This supports the emerging concept that ER stimulation possibly affects the expression of key growth factors.
Because pure ERα-deficient mice were currently not available, ERβ-deficient (BERKO) mice were investigated with respect to hair cycle abnormalities. Compared to the wild-type animals, BERKO mice showed no significant alteration in catagen development, but a significant increase of dermal thickness. ERβ showed similar immunoreactivity pattern in the skin of BERKO-mice and wild-type controls.

In order to detect novel, ER-regulated target genes in human HFs, E2 treated scalp HFs (male and female) were subjected to DNA-microarray. Several novel estrogen-related target genes were detected in this way including, e.g. K6HF (cytokeratin type II), filamin, filaggrin and fibronectin. In addition, sex-dependent differences in E2-mediated gene regulation were detected (e.g., for BMP7, EPS8 [belongs to the epidermal growth factor receptor-pathway], CYR61 [homolog of insulin-like growth factor-binding protein], FOS-like antigen2).

To summarize, these results suggest a crucial role of estrogens and their receptors in both murine and human hair growth and control, and call attention to previously underappreciated, critically important features of ER-mediated signaling within the skin, such as sex-dependent differences and endogenous modulation. These novel findings have to be taken into account in the investigation and treatment of estrogen-related hair growth disorders, and open new insights into the role of estrogens in hair follicle biology.
5. ZUSAMMENFASSUNG

Franziska Conrad: Die Rolle des Östrogenrezeptor-signaling in der Kontrolle des Haarzyklus


Im Mausmodell zeigte sich eine haarzyklus-abhängige Expression von ERα und ERß sowohl auf der Protein-, als auch auf der Genebene. In einer Maushautorgankultur sank die ER-Expression schon nach wenigen Stunden rapide ab, ebenfalls sowohl auf der Protein-, als auch auf der Genebene. Nach Optimierung der Kulturbedingungen durch eine drastische Verkürzung der Gesamtdauer (auf sechs Stunden), konnte ein hemmender Einfluss von E2, Prolaktin und Melatonin auf die ER-Expression beobachtet werden, im Falle von Melatonin war zusätzlich zu den verringerten mRNA-Mengen ebenfalls eine mindere Immunreaktivität zu verzeichnen.

Diese Ergebnisse konnten im Humanmodell bestätigt werden. Mittels mikrodissezierter menschlicher Kopfhauthaarfollikel, welche in Kultur verbracht wurden, konnte eine verminderte ERß Immunreaktivität nach Behandlung mit Prolaktin oder Melatonin in weiblichen HF festgestellt werden. Da ERß im Vergleich zu ERα im menschlichen HF überwiegt, wurden die Untersuchungen diesbezüglich verstärkt durchgeführt.
Erstaunlicherweise stellte sich heraus, dass ERß im menschlichen Kopfhauthaarfollikel geschlechtlich unterschiedlich exprimiert wird (im weiblichen HF liegt die Expression im Bereich der Dermalen Papillen Fibroblasten und der Matrixkeratinozyten, wohingegen im männlichen HF die meiste Expression in den Matrixkeratinozyten zu finden ist). Nach Behandlung mit E2 veränderte sich die ERß-Expression im männlichen Kopfhauthaarfollikel. Als nächstes wurde die Wirkung von E2 auf die Elongation des Haarschaftes in vitro im Hinblick auf geschlechtliche Unterschiede untersucht und es stellte sich heraus, dass E2 im weiblichen Follikel das Haarschaftwachstum und die Proliferationsrate der Matrixkeratinozyten hemmt und im männlichen HF sowohl das Wachstum des Haarshafts als auch die Proliferation der Matrixkeratinozyten deutlich stimuliert.


Da zur Zeit keine ERα-defizienten Mäuse zu erhalten sind, wurden ausschließlich ERß-defiziente (BERKO) Tiere hinsichtlich eventueller Haarzyklusveränderungen untersucht. Im Vergleich zu den Wildtypen gab es in den BERKO Mäusen keine Unregelmäßigkeiten im Verlauf der Regressionsphase des Haarfollikels (Catagen), aber es wurden signifikante Unterschiede in der Hautdickenmessung festgestellt. Immunhistochemische Darstellung von ERß in der Haut zeigte keinerlei Unterschiede zwischen den Wildtypen und den BERKO-Mäusen.


Zusammenfassend lässt sich sagen, dass die Ergebnisse dieser Studie die bedeutende Rolle der Östrogene und der Östrogenrezeptoren im menschlichen und tierischen Haarwachstum nachdrücklich suggerieren. Einige bislang eher unbedeutende Faktoren, wie z.B. die geschlechtlichen Unterschiede oder die Beeinflussbarkeit durch endogene Stoffe, erhalten auf Grundlage der hier präsentierten Ergebnisse eine höhere Gewichtung und sollten in zukünftige Forschungen dringend einbezogen werden. Durch die vorliegenden Ergebnisse eröffnen sich neue Perspektiven und Ansatzpunkte für weitergehende Untersuchungen über die Bedeutung von Östrogenen und der Östrogenrezeptoren in der Haarfollikelbiologie.
6. REFERENCES


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