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Characterisation of the estrogen receptors in the chicken *(gallus gallus)*

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Für meinen Papa…

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1 Introduction

1.1 The endocrine System

In multicellular organisms it is necessary to wisely coordinate different tissues and organs. This particular communication is among others, guarded by hormones. The classical definition says that hormones are chemical substances which are produced in endocrine cells and carried by the circulatory system (Starling, 1905). Arrived at their distant target cells, hormones bind specifically to their hormone receptors, which are present on the cell surface, the plasma membrane or found in the cytoplasm. These receptors get “switched on” by composing a complex with the hormone and initiate a plurality of various effects (reviewed by Tata, 2005).

1.2 Steroid hormones

Steroid hormones represent an ancient family of signaling molecules with diverse functions all over the body (Kumar, 1987). These hormones are essential for diverse physiological and cellular actions, such as growth, reproduction, sexual differentiation and play also a crucial role for the immune system, and have certain functions of the brain (O’Lone, 2004).

All steroid hormones are derived from cholesterol, an oil-based structure, which is an essential compound of the plasma membrane and carried in the bloodstream by lipoproteins. Because of their common origin, they all share a ring structure of three six membered rings and one five membered ring (Berg, 2002). Cholesterol gets into the body by food intake or is synthesized in the cells from acetyl-CoA via a

sophisticated metabolic pathway. For one cholesterol structure eighteen acetyl-CoA units are needed (reviewed by Mariëtte, 2013).
Modifications like clipping of the side chain or addition of hydroxyl functions from the apparently simply ring-structure of the basic structure of cholesterol, results in molecules with very divers operations – such as steroid hormones (reviewed by Mariëtte, 2013).
The class of steroid hormones can be categorized into fife groups: glucocorticoids, mineralcorticoids, androgens, progestins and estrogens (reviewed by Miller, 2013).

1.3 Estrogen – synthesis and effects

Estradiol-17β (E2) has been recovered in the 20s of the last century by Allen and Doisy (Allen, 1923). Since then, the female hormone has always been an interesting topic for scientist to understand how the hormone works, understand its functionality and also figure out the therapeutic use of it.
Nowadays it's known that the ancient female hormone is also important in men. Experiments with the estrogen receptor alpha (ERα) knockout (αERKO) mouse presented information that estrogens and there receptors are important for normal male fertility (Eddy, 1996 and Dupont, 2000). Also male bone health with bone resorption and maintaining bone formation is subjected to estrogens (Falahati-Nini, 2000). To give just two examples. Estrogen is principle synthesized by testosterone aromatization. This reaction is catalyzed by an enzyme called aromatase. This enzyme is a member of the cytochrome P450 superfamily (Evan, 2013). In premenopausal women mainly the ovaries, the corpus luteum and the placenta produce the estorgens (Cui, 2012). In postmenopausal the situation is different, the overies are wane to produce the hormone. Extragonadial organs, such as the aorthic smooth muscle cells and the vascular endothelium, adipose tissue, several brain areas, as well as chondrocytes and osteoblasts of the bones undertake the task of estrogen production (Nelson, 2001).

The extragonadial produced estrogen differs in some points. The estrogen which is produced in these organs acts, in contrast to the ovarian estrogen, in a paracrine or intracrine fashion (Labrie, 1997). The concentrations of estrogen in the local tissue are high and perform a biological impact (Simpson, 2003).

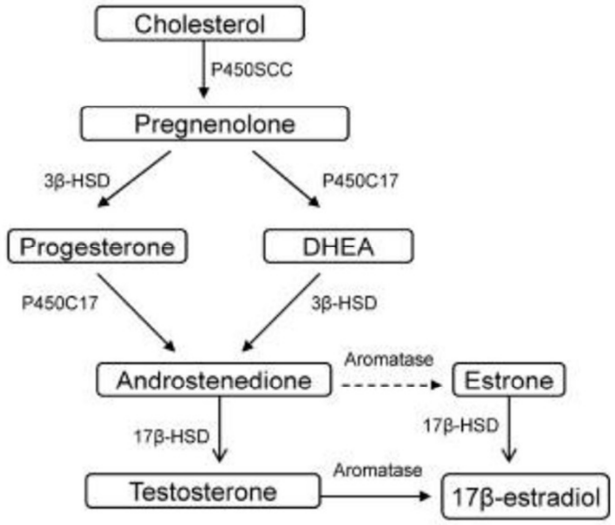


Figure 1: Estrogen synthesis (Cui, 2013)

Physiological estrogens exist in three major forms: estrone (E1), estradiol (E2 or 17β-estradiol) and estriol (E3). The most potent in the premenopausal period is estradiol. After menopause E1 gets a major role and is synthesized in adipose tissue. E3 produced in placenta during pregnancy, is the last potent of the three (reviewed Cui, 2012 and Tomas, 2013). Most estrogens in plasma are transported by binding to the sex hormone binding globulin (SHBG), only a few 2-3% are unbound. The “free hormone hypothesis” says that this free estrogens transverse the plasma membrane unspecific but freely, because of their small size and there fat-soluble character

(Adams, 2005). Estrogens bond to SHBG get actively internalized the low-density-lipoprotein receptor 2 (LRP2), which is located in clathrin–coated pits or it gets internalized by some other importers (Willnow, 2010).

1.4 Estrogen signaling

The produced estrogens regulated a plurality of effects in the body. When estrogen entered its target cell via diffusion or get actively transported inside by receptors like LRP2 (Hammes, 2005). Almost all effects are mediated through hormone-specific estrogen receptors (ERs). These proteins are acting ether in a genomic or non-genomic signalling way. Furthermore it is known that estrogen signals also in an ER-independent way, by interfering with enzymatic activities or non-sex steroid hormone receptors (Richardson, 2012).

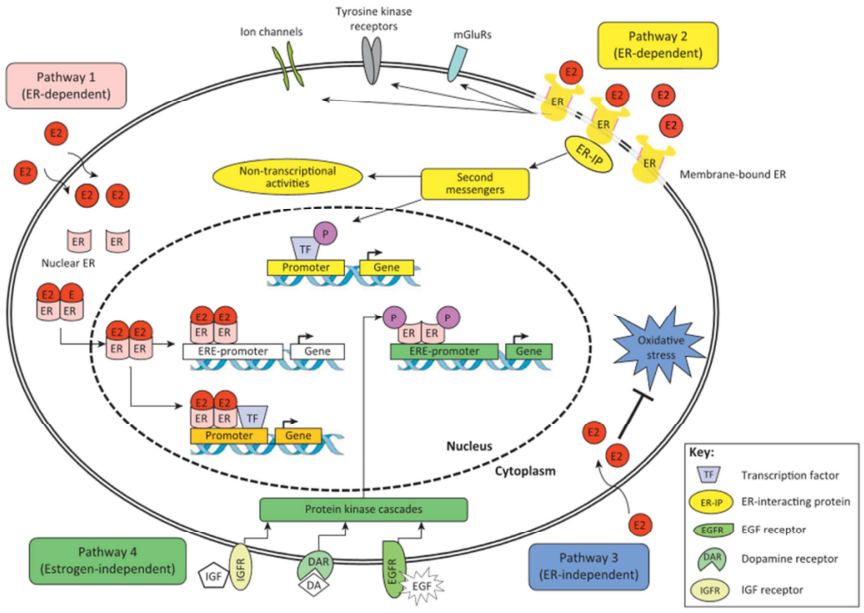


Figure2: Estrogen signalling pathways mediated by the ERs (Cui,2012)

The classical and non-classical, genomic ER dependent pathway

The classical, genomic pathway is mediated by estrogen receptors. These rectors are nuclear ligand-activated transcription factors that activating or repressing the target gene transcription (Couse, 1999). Once estrogen is bound to ER it induces conformational changes, this leads to dissociation from the heat shock protein 90 (Hsp 90), which enables the stability of the lone receptor, enhances the binding affinity and prevents from degradation under inactive circumstances (Lee, 2012). After dissociation the activated receptor forms functional hetero- or homodimers. (McDevitt, 2008). If homo- or heterodimerisation takes place, might be dependent on the relative expression level of the steroid receptors. It seems that in tissues where one form is predominant, homodimers are preferentially formed over heterodimers (Cowely, 1997). The dimer translocates into the nucleus and binds the DNA directly. To actively interfere in the transcription machinery of the cell, the dimer configuration facilitates a shape, which allows cofactors to promote (co-activator) or prevent (co-repressor) gene transcription (Hall, 2001). The cofactor proteins are also important for stabilisation of the formed pre-initiation complex and relieve the disruption of chromatin at the consensus sequence (Hall, 2001).

The first characterised co-activator was the steroid receptor coactivator-1 (SRC-1) (McKenna, 1999). Furthermore there is the possibility to be involved in gene transcription for ERs. The non-classical signalling pathway is characterised by binding other transcription factors, such as nuclear factor κB (NF- κB), activator protein 1 (AP-1), stimulating protein-1 (SP-1) or c-junk (Gottsch, 2009).

Interestingly ER subtypes are using this pathway with different goals. ERα stimulates AP-1 gene transcription indirectly and in contrast estrogen receptor beta (ERβ) repress transcription by the AP-1 site (Peach, 1997).

Membrane initiated estrogen signalling

Genomic signalling needs hours, thereby it was astonishing that in the group around Szego in the 1967s reported from physiological effects due to estrogen within 15 seconds (Szego, 1967). This effect is nowadays known as a membrane-initiated

estrogen signalling. By this pathway estrogen uses plasma membrane-associated ERs (mERs) for signalling. The rapid signal is transmitted via non-transcriptional transduction mechanisms through crosstalk with other membrane receptors or a plurality of cytoplasmatic signalling cascades gets activated. This estrogen signalling is important in the nervous system, the skeleton, the liver and other tissues (Chinmento, 2010). This pathway influences also insulin-like growth hormone receptors, the tyrosine kinase receptors, several growth factors and neurotransmitter receptors (Micevych, 2009).

Rapid estrogen signalling is also delivered by relative newly discovered membrane ERs such as G protein-coupled receptors like GRP30 and the ER-X protein. (Filardo, 2000 and Toran-Allerand, 2002). GRP30 was first subjected to the group of orphan receptors, because its ligands were unknown, but in the year 2000 the group of Filardo presented GRP30 as an estrogen-induced trans-activator for EGF receptor (Filardo, 2000) which increases adenyl cylase activity (Filardo, 2002). On the other hand there is debate about whether GRP30 is a new estrogen receptor or not (reviewed by Langer, 2010).

In 2002 the membrane protein ER-X was discovered in the brain and the uterus. ER-X is located at the caveolar-like microdomain (CLM) complexes. ER-X expression is up-regulated during development, in adults its expression is nearly not detected, expect after ischemic brain injury (Toram-Allerand, 2002).

The ER-independent pathway

Although most of estrogen processes are mediated trough estrogen receptors, there are studies presenting that estrogens perform antioxidative effects in an ER-independent way (Haas, 2012). Instead of binding ER, estrogen is signaling enzymatic reactions, or interacts with non-sex-steroid-hormone-receptors (non-SSHR), to protect the cell against damage. An example: estrogen prevents pro oxidant stress by inhibition of ROS (reactive oxygen species) release from damaged mitochondria, without get in touch with ERs (Richardson, 2012). The antioxidative feature is caused by the phenolic A ring in the molecule structure (Richardson, 2012).

But the estrogen receptor independent has also negative input to health conditions. Studies with ERα and ERβ knockout mice show that estrogen can foster breast cancer development, via an ER- independent signalling pathway (Yue, 2010).
In addition, PPARγ, a non-sex-hormone-nuclear-hormone-receptor gets also regulated by estrogen, without the assistance of ERs. Via PPARγ, estrogen is working in an anti-inflammatory way and prevents atheroprotective effects (Tiyenli, 2012).

ER-independent pathway – cross talk signalling

Studies show, that there is also the possibility to activate ER function without the assistance of classical ligands. Due to this pathway the activation is mostly achieved by phosphorylation of the estrogen receptor by protein kinases. Phosphorylation takes place, mostly by serine residues in the AF-1 region of the A/B domain (Tremblay, 1999; Lannignan, 2003).
It is reported that in mouse uterus ER shows the same biochemically characteristics after EGF or estrogen treatment. EGF mimiks estrogen and modulate the receptor (Ignar-Trowbridge, 1992).
The group around Schreihofer showed in the year 2001 that in cAMP activates ERs in an ligand independent way and that this is PKA dependent (Scheihofer, 2002). This reaction may be involved in the evolvement of tamoxifen resistance (Michealides, 2004). Interestingly fulvestrant, the pure ER antagonist represses this cross talk (Michalides, 2004).

1.5 Estrogen receptors – a little history

The estrogen receptors are ligand activated transcription factors, which mediate pleiotropic effects of the estrogen. In the year 1966 the ER was characterized for the

first time (Toft, 1966). Around 20 years later the receptor molecule was cloned and sequenced from MCF-7 human breast cancer cells (Walter, 1985) and one year later, the functional protein of ERα, was expressed in Chinese hamster ovary (CHO-K1) cells (Greene, 1986). For long time it was believed, that the functions of the hormone were mediated through a single ER, but in the year 1996 the group around Mosselman characterized a new estrogen receptor, named ERβ (Mosselman, 1996). The identification of a second ER subtype, set on a new dimension of the complexity of estrogen signaling pathways and the way how estrogens work in the body.
Both receptors are encoded by individual genes named ESR1 for ERα and ESR2 for ERβ. In addition there are several splice variants of both receptors in normal and diseased tissues (Pfeffer, 1995, and Petersen, 1998). Because splice variants are often expressed with wild-type receptors, their purpose in normal physiology or the question of any coherence with diseases is not answered know.

1.6 Structure and Domains

ERα and ERβ are classical steroid hormone receptors and belong to the superfamily of the nuclear hormone receptors (NR) (Mangelsdorf, 1995). Other nuclear receptors like receptors for other steroid hormones, retinoic acid, vitamin D and thyroid hormones are also elements of this family (Wheatherman, 1999).
NRs transduce extracellular signals into transcriptional response and express specific genes which are essential for development, growth and the physiology of the reproductive system (Vanden-Heuvel, 2009). One of the most common characteristics of the superfamily is that all share the same structure, composed of fife domains which serve specific functions (Beato, 1995). Starting from the N-terminus they are named A/B or N-terminal domain (NTD), C or DNA binding domain (DBD), D or hinge region, E or ligand binding domain (LBD) and at the C-terminal the F domain.

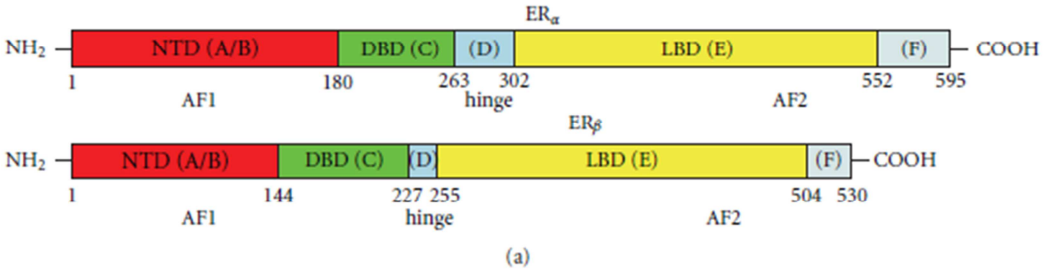


Figure 3: Illustration of the fife dominal structure of ERα and ERβ (Kumar, 2011)

A/B-region

The amino-terminal A/B region is hosting a transcriptional activation function (AF-1), which acts in a ligand independent way (Kumar, 2003). The receptor subtypes ERα and ERβ exhibit a sequence homology of few 17% and differ in size by 41 amino acids in this region (Zwart, 2010). Due to these facts it is suggested that the variability in the A/B region may be responsible for receptor-, species- and cell-type specific effects (Tora, 1989).The AF-1 site provides serine residues, which are targets for phosphorylation of inracellular signalling cascades like mitogen-activated protein kinases (MAPK) (Aranida, 2001).

C-region

The dimerised receptors bind the DNA in distinct sequence segments, known as estrogen response elements (ERE) upstream of their target genes (Laudent, 1992). EREs are characterised by a palindromic inverted repeat of 13 bp 5'-GGTCAnnnTGACC-3', were n is indicated as any nucleotides which flanking the palindromic half sides (Klein-Hitpass, 1988). Interestingly other steroid receptors bind to a sequence with another palindromic sequence – 5´-GGTACAnnnTGTTCT-`3 (Nelson, 1999). The DNA- binding domain involves two non-homologous zinc finger motifs (CI and CII) to bind to DNA major groove (Green, 1988). CI, the N-terminal zinc finger harbors the so called P-box, composed of six amino acids, to perform the

task of recognition of the ERE sequence on the DNA strand. The second zinc finger CII, include the D-box, the dimerization interface and is important for the stabilisation of the binding (Green, 1988 and Pettersson, 2001)

D-region

The D or hinge region is a flexible region which links the DBD to the E and F domains at the C-terminal. This region also involves nuclear localization signals (NSL) information (Robinson-Rechavi, 2003) which is exposed when a ligand is bond (Kuiper, 1996).

E-region

As a member of the nuclear hormone receptor superfamily, ERs also harbor the highly conformational dynamic E domain with its ligand binding function, it's necessary for dimerization and harbors also the hormone-dependent activation function (AF-2) (Mangelsdorf, 1995). The flexible secondary structure of the ligand binding domain is composed of 11 α-helices, folded into a three layered antiparallel sandwich (H1-H11) (Bronzozowski, 1997). The structure is completed with two small two-stranded antiparallel β-sheets (S1 and S2) and an additional α-helix (H12) flanking the helical structure (Brzozowski, 1997). The orientation of H12 gets influenced by the class of bond receptor ligands in the binding pocket. The ligand-dependent transcriptional activation function (AF-2) of the E-region is strongly influenced by Helix 12. An agonistic ligand turns the H12-structure into a transcriptionally active state, which serve the best conformation and shape for the leucine-rich LxxLL motifs of co-activators (reviewed by Heldring, 2007). In case of an antagonist is bond, H12 gets in a position a bit away from the ligand binding pocket (Shiau, 1998). Due to the H12 is crucial for the interplay with cofactors it seems that this helical structure determines whether a ligand woks in an agonistic or antagonistic way (Watanable, 2010).

F-region

At the very end of the carboxy-terminal of the amino acid from erstrogen receptors the F domain is located. This region was first described by the group around Montano by single-point mutation experiments. They found out, that the F domain is important for transcriptional activity of the ligands in an agonistic or antagonistic way (Montano, 1995).
Beyond that the F region might also play a crucial role for receptor dimerization in a cell type specific manner (Yang, 2008).

1.7 Diseases and treatments

Cancer

As already indicated, estrogens are essential for growth and differentiation, wherefore interruptions in this system lead to the promotion of unnatural cell proliferation which leads to cancer (Thomas, 2015). Breast cancer development is, among other factors, caused by estrogens (Thomas, 2015). Two hypotheses explain this link (Yue, 2005).The first hypothesis for the coherence of ERs and cancer development is that transcribed target genes are involved in cell proliferation. With an elevated cell proliferation, also the chance of accumulation of mutation, because of unidentified mistakes during DNA replication, is increased. Are these mutations located in genes encoding for DNA repair, apoptosis, cellular proliferation, and angiogenesis cancer development results. The second hypothesis says that derivate of estrogen have a genotoxic character and with this damaging the DNA, which also results in point mutations (Yue, 2005).

There may be an interesting difference between the task of tumorgenesis for ERα and ERβ. Elevated levels of ERα in breast tissue are indicated with a greater risk of neoplastic transformation of breast tissue in the initial phase, as well as progression (Ali, 2000). ERβ presents in return to the tumorgenic impact of ERα, seems to have a protective role for breast cancer development. The ERβ promotor is frequently methylated and thereby ERβ expression is repressed (Bardin, 2004 and Rody 2005). *In vitro* studies show that ERβ is modulating gene transcription in a tumorsuppressive way (Stettner, 2007), these results leads to the suggestion that ERβ loss could be an important step in tumorgenesis (Bardin, 2004).

Osteoporosis

Estrogens are also important for the skeletal homeostasis in male and female individuals (Manolagas, 2013). Osteoporosis is characterized by fragile bones; the decrease of density and bone mass implicates a higher risk of bone fractures. The role of estrogens is to reduce the bone resorption mediated by osteoclasts and boost new bone formation by osteoblasts (Deroo, 2006).

Mental health

Estrogens also have protective impact of neurodegenerative diseases like Parkinson disease (PD), Alzheimer disease (AD) and stroke (Simpkins, 2009). Postmenopausal women and man have the same risk of stroke (Lobo, 2009). Unlike premenopausal women, were strokes are not so often. This fact leads to the suggestion, that estrogen protects against strokes (Billeci, 2008) There are also reports that estrogen treatment lowers the risk of cardiovascular diseases (Grodstein, 2003), but the Heart and Estrogen Progestin Replacement study (HERS) demonstrated, that by women with coronary diseases hormone replacement therapy (HRT) is not lowering the risk of strokes (Simon, 2001). Some hypothesis like the ‘timing hypothesis’ try to explain this controversy (Grodstein, 2006 and Mendelsohn, 2005). Alzheimer disease is characterized by a loss of memory and other cognitive abilities. Cohort studies say

HRT lowers the risk of the onset of AD (Sherwin, 2003), but the protective impact may also related to the right timing of the beginning of the therapy (Pinkerton, 2005).

Cardiovascular diseases

For women the number one causes of death are cardiovascular diseases, with nearly 50% (Ansar, 1985). In general the risk for women is lower than for men, but the risk is increasing after menopause (Rossouw, 2002). This detail leads to the thought, that also in this case, estrogen may have an important impact (Baker, 2003). Decreased estrogen levels were responsible for the onset of atherosclerosis in women (Rossouw, 2002).

HRT seems to be the right treatment, but studies say that this therapy may have negative cardiovascular effects, like elevated risks of strokes or venous thromboembolisms (Farquhar, 2005). Estrogen receptor polymorphism may play also a role in these diseases. For example the ERα PvuII polymorphism is linked to myocardial infraction (Sheraman, 2003) and variations in blood pressure in men (Peter, 2005).

Obesity

Obesity is subjected by the WHO as abnormal fat accumulation which impacts health conditions. Estrogens are important for the localization and metabolism of white adipose tissue (WAT) (Deroo, 2006). In human WAT both estrogen receptor subtypes are expressed, but the mechanisms in detail, how WAT is regulated or linked to estrogens, is unclear. An interesting study with knockout mice from Naaz, shows that the two receptor types have converse effects (Naaz, 2002). It seems that ERα gain the volume of adipose tissue and ERβ lowers the general body and adipose weight (Naaz, 2002).

1.8 Ago- and antagonists

Due to the physiological functions of estrogens and its receptors in context of the development of diseases, quit a number of different ago-and antagonists were developed.

In women, one of the most common cancer types is breast cancer (Ganesh, 2010). In many cases this tumorgenisis is estrogen-dependent and therefore anti-estrogens were developed (Cole, 1971). Such an anti-estrogen is tamoxifen. It has an antagonistic effect in breast tissue, and works quite well to beat the tumor. But for example in uterine it works in an estrogen-like manner and thereby increases the risk of uterine cancer (van Leeuwen, 1994). Like other chemical substances tamoxifens anti- or agonistic effect is conditioned by the target cell. Both substances are members of the group of selective estrogen receptor modulators (SERMs). SERMs are chemical substances that lack of steroid structure, but there tertiary structure enables the binding of estrogen receptors (MacGregor, 1998 and Lawrence, 2003). The tissue specific repression or activation function of SERMs gives rise that the classic model is incomplete and that estrogen signalling is acting on a more complex level than expected (Jordan, 2001). This powerful pharmacology can be explained the following three phenomenons (Riggs, 2003). The first is the relative expression of each hormone receptor isoform is varying in target cells. It is known that estrogen receptor α is almost always an activator (Riggs, 2003). In contrast, ERβ has the ability to lower the transcriptional activity of estrogen, by composing a heterodimeric complex with ERα (Petterson, 1997). The co-localisation and subsequent heterodimerisation of the two receptors could alter the receptor activity refer to that of ER homodimers (Petterson, 2000; and Hall, 1999). Besides, experiments in mice with deleted ERα or ERβ, ERβ inhibit gene transcription when ERα is present, on the contrary if ERα is absence, estrogen receptor β partially replace it, so there is a “Ying-Yang” relationship between the two hormone receptors in mice (Lindberg, 2003). The second reason is, that each bond ligand forces a different estrogen receptor conformation (Brzozowski, 1997). SERMs require shapes which are

assumed to be intermediate forms between ago-or antagonistic ones (Riggs, 2003). The third reason is that estrogen signalling is dependent on co-regulators (Heldging, 2007). More than 20 such co-regulators are known and the expression level of them is various in estrogen target cells (McKenna, 1999). For example Tamoxifen recruits in breast tissue co-repressors and in the endometrium co-activators (Shang, 2002).
These ambivalent features of SERMs have also beneficial effects. For example Raloxifen shows antiestrogenetic functions in breast tissue but little estrogen like effects in uterus. In non-gonadial tissues like the cardiovascular system or in bones it has an agonistic impact (Francucci, 2005). Hence it is used for prevention and treatmend of postmenopausal osteoporosis (Cranney, 2005), but after latest findings raloxifen treatment should be avoid in patients with a risk of venous thromboembolism (D'Amelio, 2013).

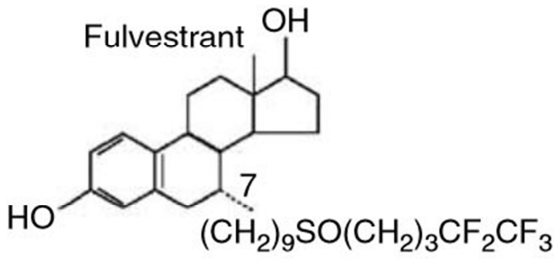


Figure 4: Chemical Structure of Fulvestrant (Osborne, 2004)

Because of the various side effects of SERMs, novel substances were developed to completely block ER action. Such a 'pure antagonist' is fulvestrant (Faslodex©, ICI 182780). Which serve an 89% higher binding affinity to ER over estradiol (Wakeling, 1987). It provides multiple features against ER. Fulvestrant inhibits nuclear-cytoplasm shuttling and thereby blocks ERs nuclear localisation (Farwell, 1990) and promote the degeneration of ER (Robertson, 2001). Due to this mechanism, different from SERMs, chemicals like fulvestrant are also called "selective estrogen receptor down-regulators (SERD)".

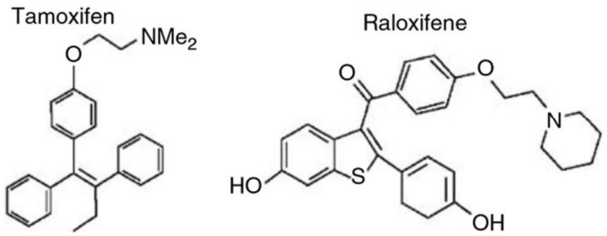


Figure 5: Chemical structure of Tamoxifen and Raloxifene (Osborne, 2004)

Subtype selective Ago-/Antagonists

ER- selective agonists have been developed to address specific biological functions ether to ERα or ERβ and may be also used for pharmacological properties.

The first reported ERβ agonist, Diarylpropionitrile (DPN), was invented by the group around Meyers in the year 2001. Although other selective agonists were developed, DPN is used most widely in rodent studies (Meyers, 2001).

PPT an ERα selective agonist with a 410 fold higher binding affinity to ERα than to ERβ, in contrast DPN has just a 70 fold higher binding affinity to ERβ than to ERα (Meyers, 2001 and Stauffer, 2000).

Besides the named ago- and antagonistic substances ER subtypes are also activated by a large number of xenoestrogenic molecules, like industrial chemicals, synthetic steroids, pesticides and phytoestrogens, which are incorporated by food intake. The group of Kuiper found out that the impact of phytoestrogens is significantly higher in comparison to industrial-derived estrogenic chemicals. Interestingly these effects are mediated mostly by estrogen receceptor β (Kuiper, 1998).

Phytoestrogens

Because of the different functions of estrogen receptor alpha and beta, it was interesting to invent subtype selective ago- and antagonists. Phytoestrogens were the first estrogen receptor ligands, which present such a moderate selective character (Barkhem, 1998). Phytoestrogens are plat-derived substances that mimic mammalian estrogens. In general phytoestrogens show a lower binding affinity than E2, they bind both receptor subtypes, but some of them bind ERβ preferred over ERα (Kuiper et al., 1998). Due to this binding character, and the information of different tissue distribution of the receptor subtypes, the tissue specific effects may explained (Ososki, 2003). Interestingly Phytoestrogens are known to lower biological activities of sex hormones, by inhibiting enzymes they need for hormone conversions and thereby reducing the risk of cancer (Adlercreutz, 1998). So phytoestrogens were tried to use instead of commercial hormonal replacement therapy strategies, but recent studies show there concerns (Ososkii, 2003). The most intensively studied phytoestrogen is genistein. Genistein show a 20 fold higher binding affinity to ERβ over ERα (Kuiper, 1997).

1.9 LRP2 - a short overview

The low-density lipoprotein related receptor protein 2 (LRP2), also called megalin or glycoprotein-330 (gp-330) is a protein at the apical surface of polarised epithelial cells (Kounnas, 1994) and works as receptor for various ligands, which are functioning in diverse cellular mechanisms (Birn, 2006). Initially it was identified as pathogenic autoantigen of Heymann nephritis (Kerjaschki, 1982) and is known the largest member of low density lipoprotein receptors (LRLR) which include other cell surface receptors like: LRP1, MEGF7/LRP4, LRP8/apolipoprotein E receptor 2, to name just a few of them (May, 2007). LRP2 is the largest member of this family, and is because of its size of 600 kDa also megalin called.

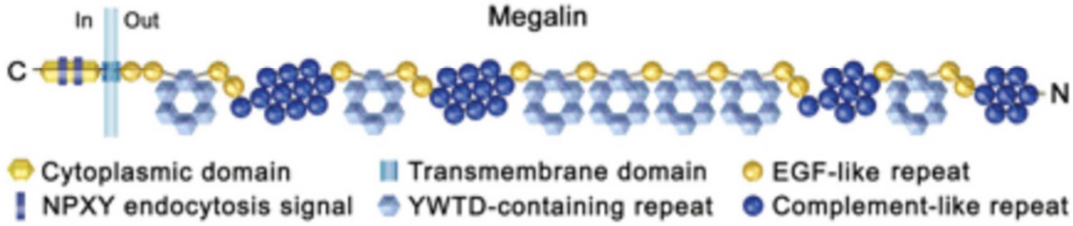


Figure 6: Schematic illustration of megalin (McCarthy, 2003)

It is composed of three distinct domains: a huge extracellular domain, a single trans-membrane domain and a small cytoplasmatic tail. The n-terminal located extracellular domain contains 17 EGF-like, 36 LDLR type A and 37 LDLR type B domains and a YWTP domain (Springer, 1998). The YWTP motif is essential for pH-dependent dissociation of bond ligands (Davis, 1987). The C-terminal tail contains three NPxY motifs with distinct functions. The flanking motifs are important for signalling activities and endocytosis, whereas the middle NPxY motif is functioning for the apical sorting and targeting (Takeda, 2003). The trans-membrane domain in the middle targets the molecule in cholesterol and glycospingolipid rich membrane areas (Marcolo, 2003).

LRP2 is located at the apical surface of polarized epithelial cells, clustered in clathrin-coated pits. First time LRP2 was found in the proximale tubule of the kidney. In the kidney LRP2 plays a role in the reuptake from macromolecules, such as lipids, carbohydrates, polyphenols or proteins). Without proteins like LRP2 these molecules would be lost in the urine (Leheste, 1999, and Christensen, 2001). Further studies showed that LRP2 is also located in lung (Yammani, 2001), endocrine glands, (Fischer, 2002) and gallbladder epithelium (Tsaroucha, 2008). Very surprisingly it was also found in sensory organs such as the inner ear (Köning, 2008).

LRP2 binds a variety of functional and structural different ligands, such as carrier proteins, lipoproteins, enzymes, vitamins and some more (Brin, 2006). Ca^{2+} plays a crucial role for ligand binding, in LRP2 and the whole LDL receptor family (Christinsen, 1992).

LRP2 is also involved in the bone metabolism and calcium homeostasis, which explains the fact that LRP2 knockout mice are suffering from rickets and low bone density (Nykjaer, 1999). In addition LRP2-knockout mice show malformations of the

forebrain, and most die immediately after birth, which gives evidence that the receptor is also important for the central nervous system (Willnow, 1996).

LRP2 is an essential importer for vitamins, bond vitamins get internalised, there carrier proteins are degraded in lysosoms and the vitamins are released into the cytosol where they can fulfil their functions. LRP2 is known to be crucial for the regulated uptake of estrogens and androgens, bond to the sex hormone binding protein (SHBG). With SHBG the cellular need of sex hormones can be managed and regulated, in contrast to the passive diffusion of sex hormones trough the plasma membrane (Hammes, 2005).

LRP2-/- knockout mice give evidence that LRP2 is essential for the cellular absorption of estrogens and other sex hormones in target cells. Knockout animals show insensitivity to sex hormones and also sex hormone treatment and are suffering poor genital maturation (Willnow, 2010).

2 Material & Methods

2.1 Cell Culture

2.1.1 Cell Culture – HEK 293

The abbreviation HEK stands for “Human Embryonic Kidney”. The cell line was invented in the year 1977 by Graham and Van der Eb. Human embryonic kidney cells cultures were transformed with sheared adenovirus 5 DNA (Graham, 1977).

HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (Sigma), in an atmosphere containing 5% CO_2 at 37°C. The medium was supplemented with 10% fetal calf serum (FCS) (SigmaAldrich), 2 mM L-glutamine (Gibco), 0.1 mg/ml streptomycin and 100 U/ml penicillin (Gibco).

2.1.2 Isolation of primary kidney cells

For primary chicken kidney epithelia cell culture, fresh kidneys from 2-3 days old chicks were isolated and pooled. First step was to wash the tissues several times in HBSS 6 (Sigma-Aldrich), to remove possible contaminations. Then kidneys were transferred into a fresh dish filled with a mixture of PBS (Sigma-Aldrich) and collagenase (Sigma) (1mg/ml) and were rigorously snipped. The resulting cell suspension was descanted into a fresh Erlenmayer flask and stirred at 37 °C for 30 minutes. For size exclusion cells were filtrated by a 70 µm nylon mesh (Falcon®) and transferred into a fresh 50 ml falcon for centrifugation (2 minutes at 300 g). The resulting supernatant was removed and the cell pellet was resuspended in cell culture medium (Mc Coy´s 5A, PromoCell), supplemented with 10 % fetal calf serum

(FCS), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100U/ml penicillin. Cells were cultivated in a humidified 95 % air 5 % CO_2 incubator.
Phenol red is supplemented to medias as a visual pH – indicator. Unfortunately phenol red has also the ability to mimic estrogen effects. To avoid this effect and bastardize the effects of the actual treatment, the media was changed into the phenol free version 24 hours ahead the treatment (Berhois, 1986).

2.2 Molecular Biological Methods: RNA/DNA

The used mature Derco-Brown (TETRA-SL) laying hens (n=3) and roosters (n=3) were ether among 30 - 40 weeks old or 10 days old, purchased from Diglas Co (Feuersbrunn, Austria). The birds were fed a commercial layer mash diet with free access to water and feed at 20 °C with a daily light period of 16 h. After cervical dislocation the organs of the animals were extracted surgically and immediately placed in liquid nitrogen, to insure the integrity of the tissues. The organs were stored at -20 °C. All animal procedures were approved by the “Animal Care and Use Committee” of the Medical University of Vienna.

2.2.1 Isolation of RNA/DNA

For RNA isolation 70-90 mg of chicken tissue was homogenized with 1 ml of TRI® Reagent (Sigma-Aldrich) using a potter or the ULTRA-TURRAX® T25 basic. The homogenized samples were incubated for 5 minutes at room temperature (RT). Afterwards 200 µl of chloroform were added. The tubes were shaken vigorously for 15 seconds and incubated for 3 minutes at RT. After incubation the samples were centrifuged at 12 000 x g for 15 minutes at 4 °C. After centrifugation, the mixture was separated into three different phases. The upper aqueous fractions, which contain the RNA, were transferred into a fresh Eppendorf tubes. For RNA precipitation 500 µl of isopropyl alcohol was added, mixed by vortex and incubated for 10 minutes at RT. Then the samples were centrifuged at 12 000 x g for 10 minutes at 4 °C. During centrifugation a pellet of RNA was formed. The supernatant was removed and the

pellets were washed with 1 ml of 75 % ethanol. After vortex the samples were centrifuged 7 500 x g for 5 minutes at 4 °C. At the end of the procedure, the ethanol was removed and the pellets were air-dried for a maximum of 10 minutes. Dry RNA pellets were solubilized in RNase-free water and incubated for 10 minutes at 60 °C. RNA concentration was measured by using Nanodrop 2000c Spectrometer. Samples were stored at -80 °C.

2.2.2 cDNA synthesis

For cDNA synthesis the SuperScript® II Reverse Transcriptase from Invitrogen™ was used. The following components were mixed together into a sterile Eppendorf tube and incubated for 5 minutes at 65 °C.

Mastermix	
1 µg	total RNA
1 µl	dNTP (10 mM each)
1 µl	Oligo $(dt)_{18}$ Primer (0,5 µg/µl)
x µl	H_2O up to a final volume of 12 µl

Then the samples were immediately cooled down on ice, 4 µl 5x First Strand Buffer and 2 µl 0,1 M DTT were added. Heating was continued for 52 minutes at 42 °C. After 2 minutes 1 µl SuperScript® II Reverse Transcriptase (200 U/µl) was added. In the end cDNA synthesis was stopped by a final incubation for 15 minutes at 70 °C.

2.2.3 Polymerase chain reaction (PCR)

To perform PCR, GoTaq ® Green Master Mix from Promega was used. A mastermix of the following components was blended.

Mastermix	
15,0 µl	GoTaq
1,5 µl	Forward Primer
1,5 µl	Reverse Primer
1,0 µl	cDNA
8,0 µl	H_2O

Used Primers	
Fw_ggERα	5´ GGAGTTACCCTGCTGCACCAG 3´
Rv_ggERα	5´GTACCTGCTGGCTGTGGTGA 3´
Fw_ggERβ	5´AAGTGAGACCACTGGACCCAG 3´
Rv_ggERβ	5´AGGATTCGATACCCACAGCGT 3´
Fw_ggβ-Actin	5´AGCTATGAACTCCCTGATGG 3´
Rv_ggβ-Actin	5´ATCTCCTTCTGCATCCTGTC 3´

To visualize the synthesised PCR products, a 1 % agarose gel containing 10 µl ethidium bromide (10 mg/ml) was made. PCR samples were mixed with 5x DNA loading dye and the gel slots were filled with 15 µl sample-loading-dye-mix and 6 µl DNA ladder (GeneRuler^{TM}, 1 kb Plus DNA Ladder). The gel runs for 40 minutes at 100 Volt.

2.2.4 Quantitative-Real-Time PCR (qPCR)

Quantitative real time PCR (qPCR) was used to determine the quantitative expression of ERα and ERβ at the transcript level. Therefore cDNA samples and the used primers were deluted 1:10. For each sample 8,2 µl H_20, 10 µl SYR, 0,4 µl of each primer and 1 µl cDNA were mixed and transferred into a 96-well plate appropriate for the light cycler. The 96-well plate was put into the Light Cycler® and the programm started. All samples were analyzed in triplicates and chicken beta-actin mRNA levels were measured as housekeeping genes and used for normalisation. The data was analysed by using excel.

2.3 Molecular Biological Methods: Proteins

2.3.1 Protein extraction - Tissues

Homogenisation buffer	
20 mM	Hepes
300 mM	Sucrose
150 mM	NaCl

Protein extracts were prepared from tissues frozen in liquid nitrogen. The homogenisation buffer (20 mM Hepes, 300 mM Sucrose, 150 mM NaCl) was added to each tissue sample (4 ml/ 1 g tissue) and homogenized with the ULTRA TURRAX® T25 Homogenizer three times for 20 seconds. The mixtures were centrifuged at 688 x g for 10 minutes at 4 °C. Afterwards supernatants were transferred into fresh centrifuge tubes and 1/20 volume of 20 % Triton X-100 was added. To mix all

components, tubes were vortexed and subsequently cooled down for 30 minutes on ice. For seperation of the membrane components and the inner cell mass, the samples were ultracentrifuged at 150 000 x g for one hour at 4°C (Optima^{TM} Ultracentrifuge, rotor: Beckmann TLA 100.4). The resulting supernatants, containing the proteins, were transferred into fresh Eppendorf tubes. The protein concentration was measured by using Bradford and a Nanodrop 2000c Spectrometer. Proteins were stored at -20 °C.

2.3.2 Protein extraction - cells

Lysis Buffe B

200 mM	TRIS
2 mM	$CaCl_2$
1,4 %	Triton X -100

pH 6,5

For cell lysis and protein extraction, cells got washed tree times with PBS. Afterwards 500 µl PBS were added to the cell layer of a 10 cm Ø tissue culture dish, scraped with a rubber policeman and transferred into a fresh Eppendorf tube. Cells got centrifuged at 2000 x g for 5 minutes (Sigma ® Laborzentrifugen). The arisen pellet got resuspended in 300 µl Lysis Buffer B and centrifuged at 50 000 x g for 1 hour at 4 °C (Optima TM Ultracentrifuge, Beckman). The resulting supernatant was transferred into fresh Eppendorf tubes. The protein concentration was measured by using Bradford and a Nanodrop 2000c Spectrometer. Proteins were stored at -20 °C.

2.3.3 SDS-Polyacrylamide gel electrophoresis (SDS – PAGE)

The components of the separating gel were mixed, poured into the glass cassette and were overlayed with isopropanol to get an even top of the gel. After 20 minutes the gel was fully polymerized and the components of the stacking gel were mixed together and poured over the stacking gel. A comb was inserted and the stacking gel was allowed to polymerize for at least 15 minutes. The electrophoresis camber was installed and filled with electrophoresis buffer. The gel was inserted into the chamber, comb was removed and the gel slots were filled with protein samples mixed with non-reducing loading buffer.
For size detection 10 µl protein ladder (Ladder-Precision Plus Protein® Unstained Standards, Bio-Rad) were loaded in one slot. The gel runs for 15 minutes at 120 volt. Afterwards the gel run till the bromphenolblue from the loading buffer reached the bottom of the gel at 180 volt. The gels were used for Coomassie Blue Staining or Western Blot.

Components of the SDS-PAGE gel (1mm)

	STACK	**SEPERAT**	
	4%	**10%**	**12%**
H_2O	1,525	2,025	1,675
1,5 M Tris pH 8,8 (ml)	-	1,25	1,25
0,5 M Tris pH 6,8 (ml)	0,625	-	-
30% PAA (ml)	0,325	1,65	2
10% SDS (µl)	25	50	50
10% APS (µl)	12,5	25	25
TEMED (µl)	5	5	5

2.3.4 Western Blotting

The seperated proteins were transferred on a nitrocellulose membrane, to make them accessible to antibody detection. Therefore a wet sandwich blot was performed. The gel was gently carryed over a transfer buffer wetted nitrocellulose membrande, 3

layers of wet Whatman paper and a wetted sponge on both sides makes the sandwich complete. The sandwich was moved into the blotting apparatus, filled with transfer buffer. The transfer was performed at 100 Volt (constant) for one hour in an icebox. After blotting was finished, the proteins on the membrane were stained with PonceauS for 2 to 3 minutes, to mark the ladder bands. To block non-specific binding sites, the membrane was incubated in TBS-T (TBS, 0,1 %Tween 100) containing 5% nonfat dry milk for one hour at room temperatur.

First and second antibodies for immunodetection, western blot

First antibody

Anti-mouse GAPDH	1: 20 000 in TBS-T containing 5 % nonfat dry milk
Anti-human ERα (Santa Cruz)	1: 50 in TBS-T containing 5 % nonfat dry milk
Anti-human ERβ (Santa Cruz)	1: 50 in TBS-T containing 5 % nonfat dry milk
Anti-chicken LRP2	1:250 in TBS-T containing 5 % nonfat dry milk

Second antibody

HRP-conjugated goat anti-rabbit IgG	1:50 000 in TBS-T containing 5 % nonfat dry milk
HRP-conjugated goat anti mouse IgG	1:20 000 in TBS-T containing 5 % nonfat dry milk

After blocking the membrane was incubated with the first antibody over night at 4 °C. Next day, the antibody was removed by shaking the membrane in TBS-T for fife minutes in a box, this step was repeated three times.The second antibody was added for one hour at room temperature. After removing the unbound antibodies with three times washing with TBS-T, the membrane was developed with enhanced chemoluminescence (ECL) (Pierce®ECL Western Blotting Substate, Thermo Scientific) and exposed to an X-ray film (ThermoFischer).

2.3.5 Immunoprecipitation & Cross Linking

PREPARATION OF THE BEADS

Reagents

Cross linking reagent:
Dimethyl pimelimidate (DMP)
Stock concentration 13 mg/ml DMP

Elution reagent:
1 M glycine (pH3)

Dilution buffer:
PBS+ 1 mg/ml BSA

Wash buffer:
0,2 M triethanolamine in PBS

Quenching buffer:
50 mM ethanolamine in PBS

For immunoprecipitation 0,2 g Protein A Sepharose^{TM} CL-4B beads were mixed with 1,5 ml PBS and centrifuged at 14 000 rpm for 1 minute at RT. The supernatant was decanted, 750 µl PBS were added and centrifuged again at the same conditions. After decanting the supernatant, 750 µl PBS were added and the beads were rolling end over end overnight at 4 °C. Next day the beads were centrifuged and washing buffer (1:1 of the volume of the bead slurry) was added. Beads were rolling end over end for 10 minutes at 4°C. Again the beads were centrifuged, 2 µg of the antibody solved in dilution buffer were added, this mixture was rolling end over end von 1 hour at 4 °C. After this time, beads were centrifuged and dilution buffer (1:1) was added and rotates for 5 minutes at 4 °C. To arrange the cross linking between beads and

antibodies, DMP was mixed 1:1 with wash buffer (pH 8-9), this mixture was added at the ratio of 1:1 to the bead slurry and rolling end over end for 30 minutes at RT. Then the beads where washed with wash buffer for 5 minutes rotation. This procedure was repeated three times. For quenching, quenching buffer, at 1:1 ration, was added rotated for 5 minutes at RT, centrifuged and washed with PBS (1:1). To remove unlinked antibodies beads were washed twice with 1 M glycine pH 3 for 10 minutes at RT. Finally the beads were washed three times with PBS.

IMMUNOPRECIPITATION

Approximately 120 µl bead slurry was mixed with 1 mg protein extract including protein inhibitor (25 x, Copmplete protease Inhibitor, Roche) and rolling end over end at 4 °C overnight. The samples were washed three times with PBS. Then 20 µl of non-reducing Laemmli-buffer was added and the samples were incubated for 5 minutes at 95 °C. After a final centrifugation step at 10 000 rpm for 1 minute, the supernatant, including the proteins of interest, were loaded on a SDS-Gel.

2.3.6 Coomassie Blue Staining

Reagents	
Coomassie blue staining reagent	
300 ml	glacial acetic acid
750 ml	isopropanol
0,862 g	Coomassie Blue R250
1950 ml	H_2O dd
Destaining reagent	
100 ml	acetic acid
300 ml	MeOH
700 ml	H_2O dd

After electrophoresis, the gel was gently removed from the glass plate and put into a plastic box filled with Coomassie Blue stain reagent. The gel was incubated with the reagent for 1 hour at RT with careful agitation. Afterward the Coomassie reagent was decanted and the gel was covered with destaining reagent till the bands were visual and the blue background was removed.

3 Results

3.1 Part 1: Tissue distribution

To explore the tissue distribution of the two ER subtypes in *Gallus gallus* and to determine the allocation is alike in other species PCR, qPCR, Western blot and immunoprecipitation were used.

To investigate an age related expression profile, tissues of chickens in two age groups (40 weeks and 10 days old) were sacrificed. Beyond that, I was also interested in possible distribution differences between sexes, therefore male and female animals of each age group have been analysed.

3.1.1 ERα and ERβ mRNA tissue specific distribution

To determine the relative distribution of the two estrogen receptors α and β mRNA at the transcript level, PCR was used.
Therefor total mRNA extracts form *gallus gallus* tissues were extracted and reverse transcribed into cDNA. For amplification of the PCR products, chicken-specific primers were used, as described in Material and Methods, Section 2.2.3.

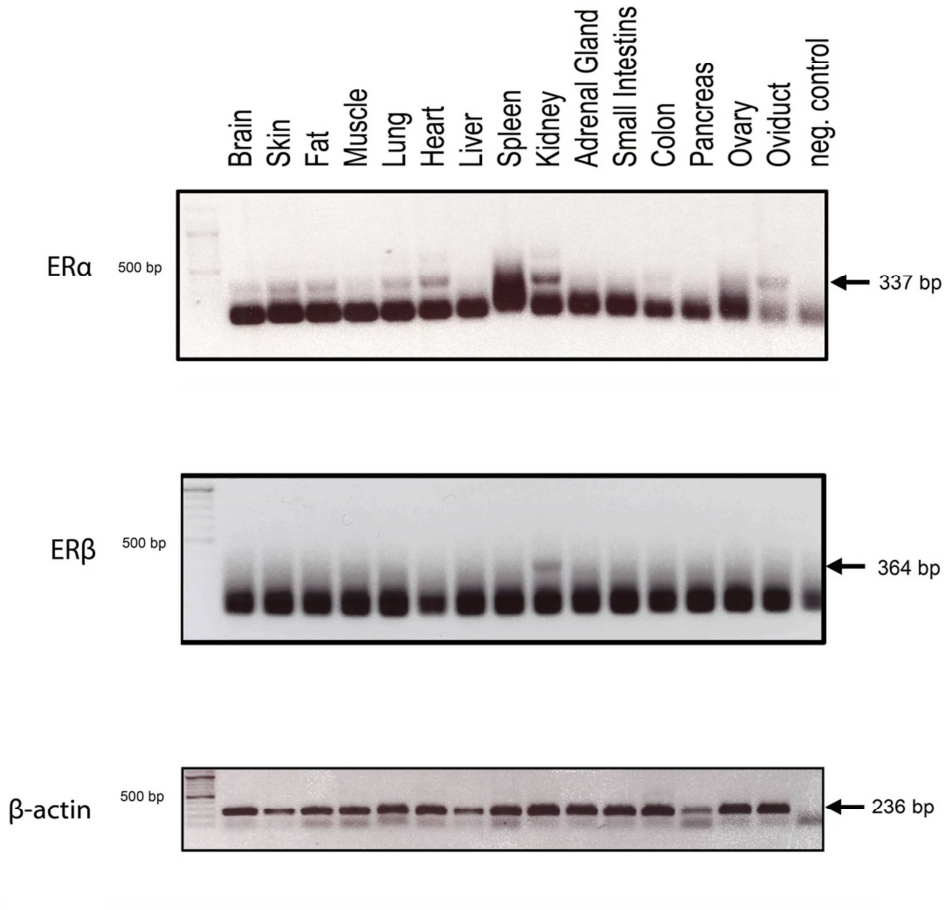


Figure 7: Tissue distribution of galline ERα and ERβ transcript in female chick (40 weeks old): For PCR, cDNAs from laying hen organs were used as templates. Specific 337 bp fragments for ERα and 364 bp fragments for ERβ were amplificated. For loading control a 236 bp fragment for β-actin was amplified.

As shown in Figure 7 ERα mRNA is expressed in high levels in heart, spleen and kidney of the laying hen. Brain, skin, adipose tissue, lung, colon, ovary and the oviduct show a moderate receptor expression. In the other tissues the receptor was not detectable. Surprisingly ERβ mRNA was found only in a single organ, the kidney.

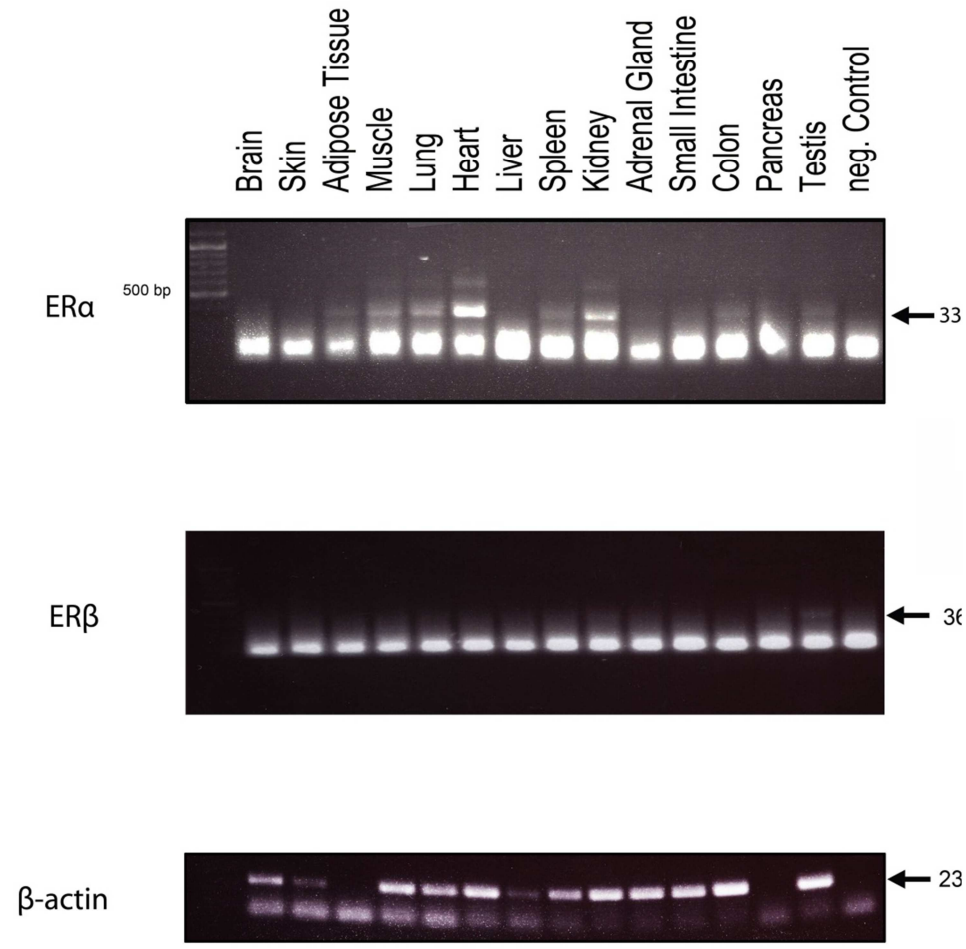


Figure 8: Tissue distribution of galline ERα and ERβ transcript in male chick (40 weeks old): For PCR, cDNAs from rooster organs were used as templates. Specific 337bp fragments for ERα and 364bp fragments for ERβ were amplificated. For loading control a 236bp fragment forβ-actin was amplified

In roosters (see Figure 8) ERα mRNA is present on a moderate expression level in adipose tissue, muscle, lung, spleen, adrenal gland, colon and testis. A much higher presence is shown in the tissues of the heart and the kidney. PCR experiments show ERβ mRNA in the testis and the kidney of the 40 weeks old rooster. Unfortunately the band in the lane for the kidney is hardly to see.

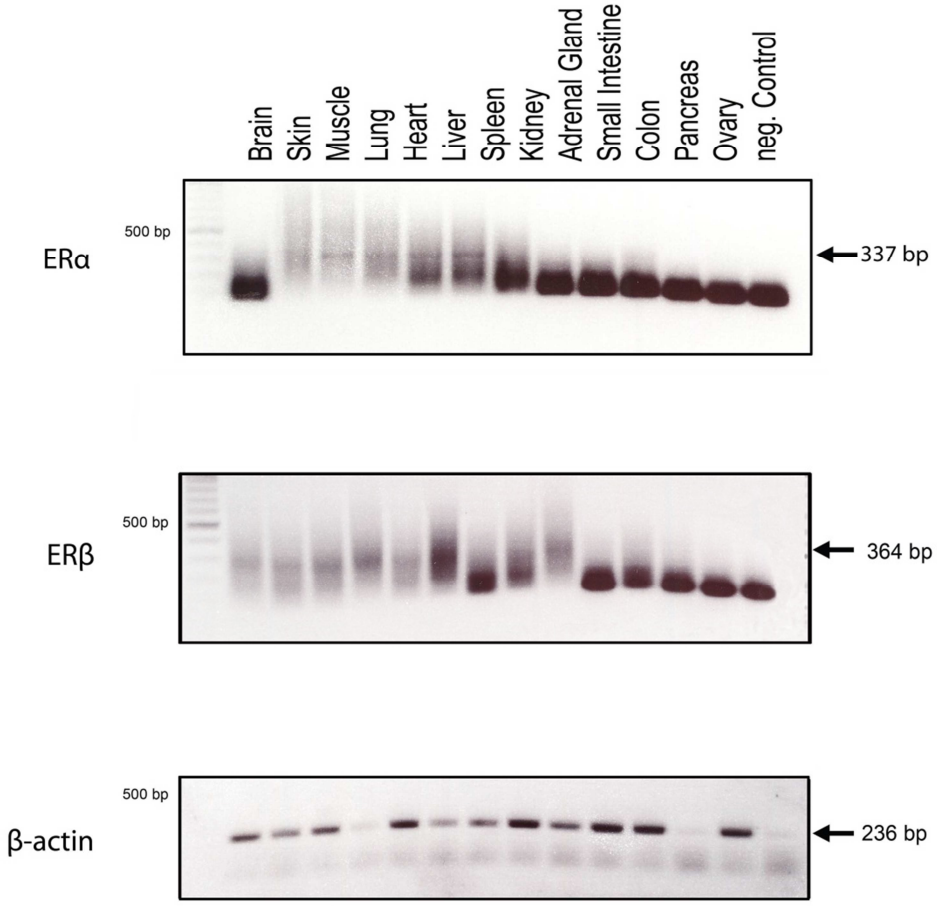


Figure 9: Tissue distribution of galline ERα and ERβ transcript in female chick (10 days old): For PCR, cDNAs from young chicken organs were used as templates. Specific 337 bp fragments for ERα and 364 bp fragments for ERβ were amplificated. For loading control a 236bp fragment forβ-actin was amplified

To investigate if there are age related differences in the tissue expression of the two estrogen receptor subtypes also 10 days old female and male chickens were tested. Figure 9 illustrates the transcript levels in 10 days old female chicken. After PCR experiments ERα mRNA was detectable in skin, muscle, lung, liver and also in a slight amount in the colon. ERβ mRNA was traceable in brain, skin, muscle, lung, heart and adrenal gland and in huge portions also in the liver.

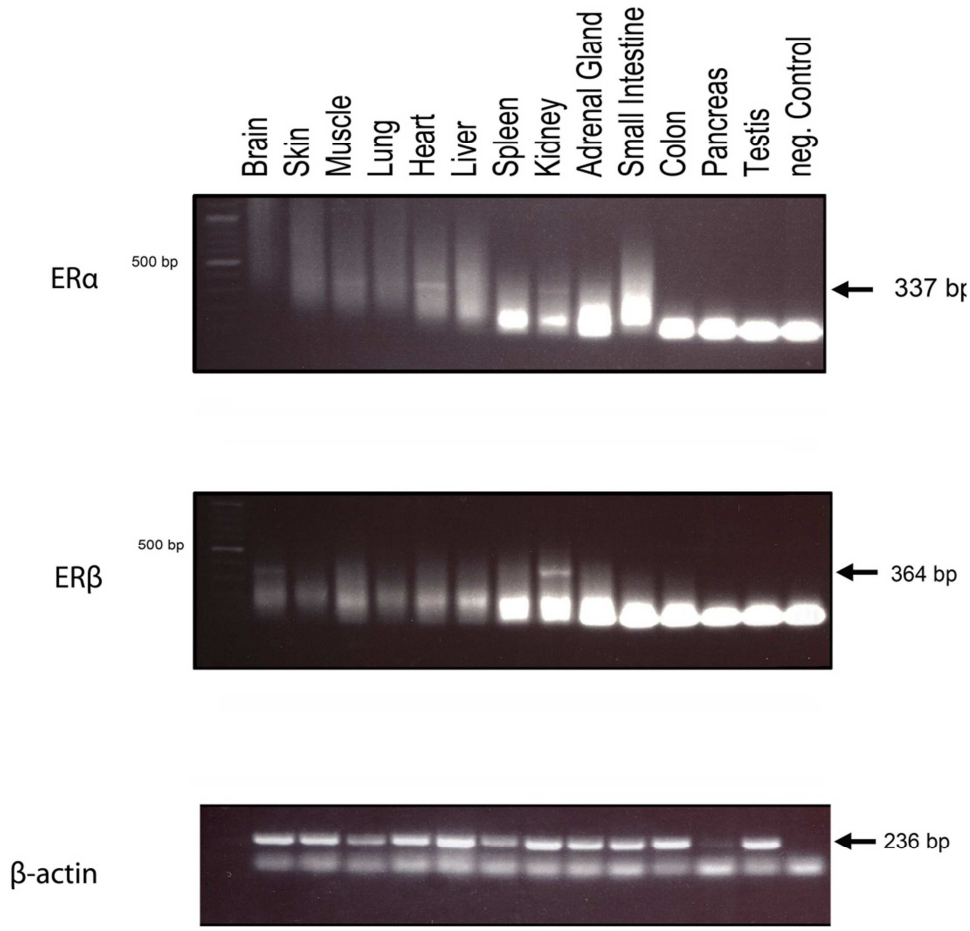


Figure 10: Tissue distribution of galline ERα and ERβ transcript in male chick (10 days old): For PCR, cDNAs from young chicken organs were used as templates. Specific 337 bp fragments for ERα and 364 bp fragments for ERβ were amplificated. For loading control a 236 bp fragment forβ-actin was amplified

Figure 10 demonstrate the relative mRNA tissue distribution of the receptor subtypes. ERα is detected by using PCR in muscle, heart and kidney whereas. ERβ mRNA was discovered in the brain and the kidney of the 10 days old male chicken, by using PCR.

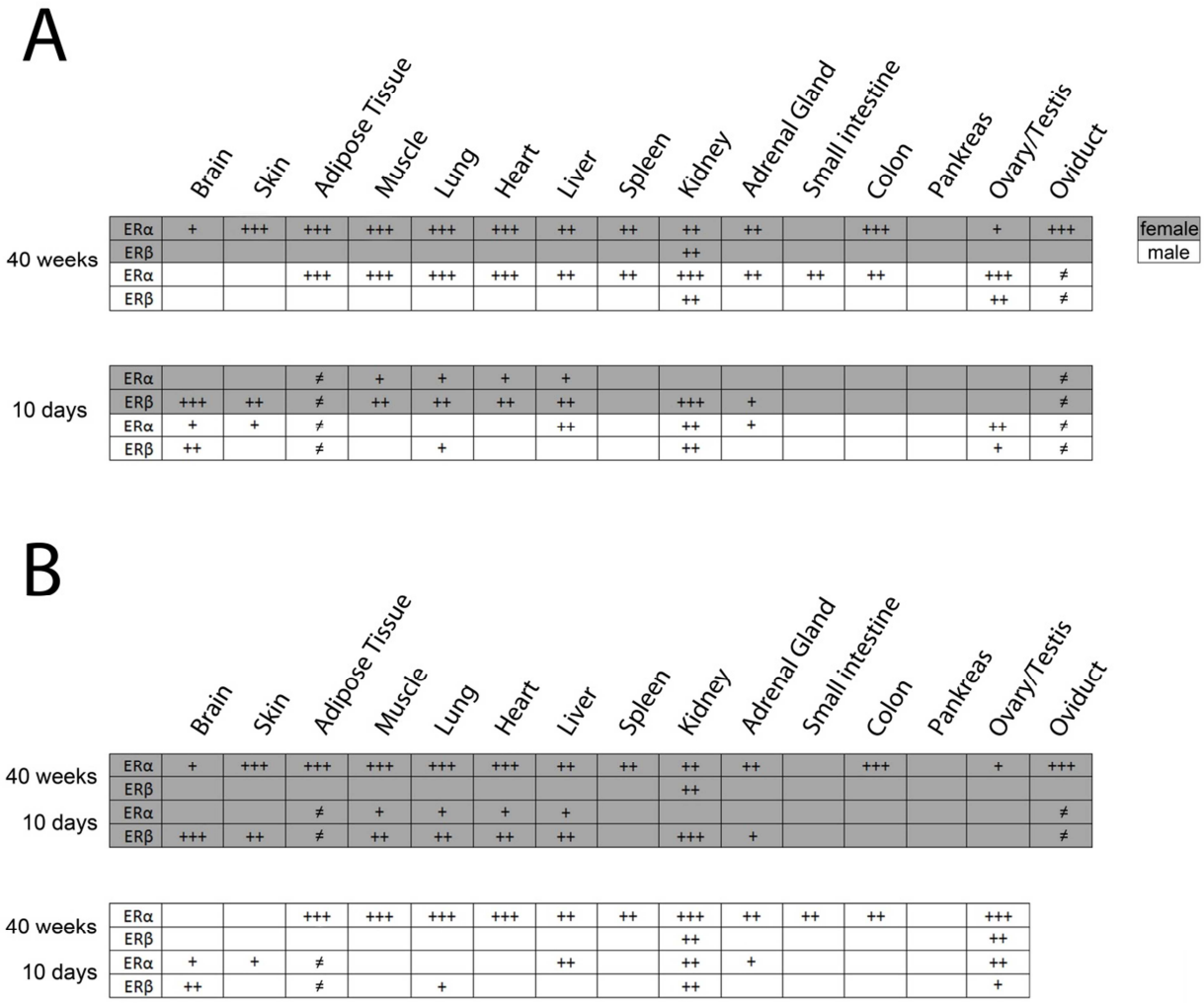


Figure 11: ERα and ERβ mRNA tissue distribution – comparison between age and sex groups: Collected PCR results from all tested animals, gray shaded boxes – female, blank boxes – male, +++ – positive for > 70%, ++ – positive for >40%, + – one of three tested animals ≠ - tissue was not tested. **A** highlights the differences between female and male in each age group and, **B** points out expression differences in the same sex between adult and young animals.

For the investigation of the tissue specific mRNA expression profile of ERα and ERβ in chicken three animals of each tested group, were sacrificed and several PCR studies were done. To give an impression of the whole data and some individual

results, caused by the method or distinct expression profiles in some individuals the following table was created (see figure 11).

Figure 11A shows that the expression profile of ERα and ERβ mRNA is in female and male chicks more or less the same. ERα mRNA is expressed in nearly every tested tissue whereas ERβ mRNA is found just in the kidney of both sexes and in the testes. In 10 day old chickens the tissue expression profile is more different between sexes. In female 10 day old chickens the co-expression of ERα and β mRNA in muscle, lung, heart and liver is eye-catching. In 10 day old male chickens, co-expression is just found in brain and the testis. In figure 11B the differences between ages in the same sex are highlighted. In this type of illustration it is interesting to see, that ERα mRNA seems to play an important role in many tissues of the 40 weeks old female chicken, but not in the tested younger age group. In 10 day old female chickens ERα mRNA is expressed just in one tested animal in muscle, lung, heart and liver. In exchange to the low ERα mRNA expression ERβ mRNA gains more importance. Also in 40 weeks old roosters ERβ mRNA plays a more important role than ERβ mRNA. But in contrast to the female 10 day old chickens ERβ mRNA is less expressed.

Due to PCR analysis deliver just semiquantitative results, the next demand was the exploration of the tissue distribution of estrogen receptor alpha and beta in both sexes and age groups in a quantitative manner. Therefore qPCR was used. By this technique a fluorescent dye intercalates in the amplified double-stranded DNA. The fluorescent signals are detected and the collected data gets analyzed by statistical methods.

For the relative quantification of the raw data delivered by qPCR analysis internal controls are essential to avoid any corruption of the final results, because of variations of the DNA quantities. Therefore housekeeping genes are used. Unfortunately it wasn't possible to find a gene which is expressed in the same level in all tested chicken tissues. Tested housekeeping genes were β-actin (unequal tissue expression is also shown in the figures presenting the PCR results (see figure 1- 4), RS 17 and GAPDH (results not shown).

3.1.2 ERα and ER β tissue distribution at the protein level

After experiments about the relative expression of ERα and ERβ mRNA levels, it was interesting if the transcripts were also translated in the corresponding proteins. Also on the protein level the tissue distribution was tested in 10 days and 40 weeks old female and male chickens, to investigate if there exists an age or gender related expression.

To get an insight in the tissue distribution on the protein level, protein extracts from each chicken tissue were prepared and loaded on an SDS-PAGE. This procedure was followed by Western blotting and incubation of the blotted membranes with corresponding antibodies.

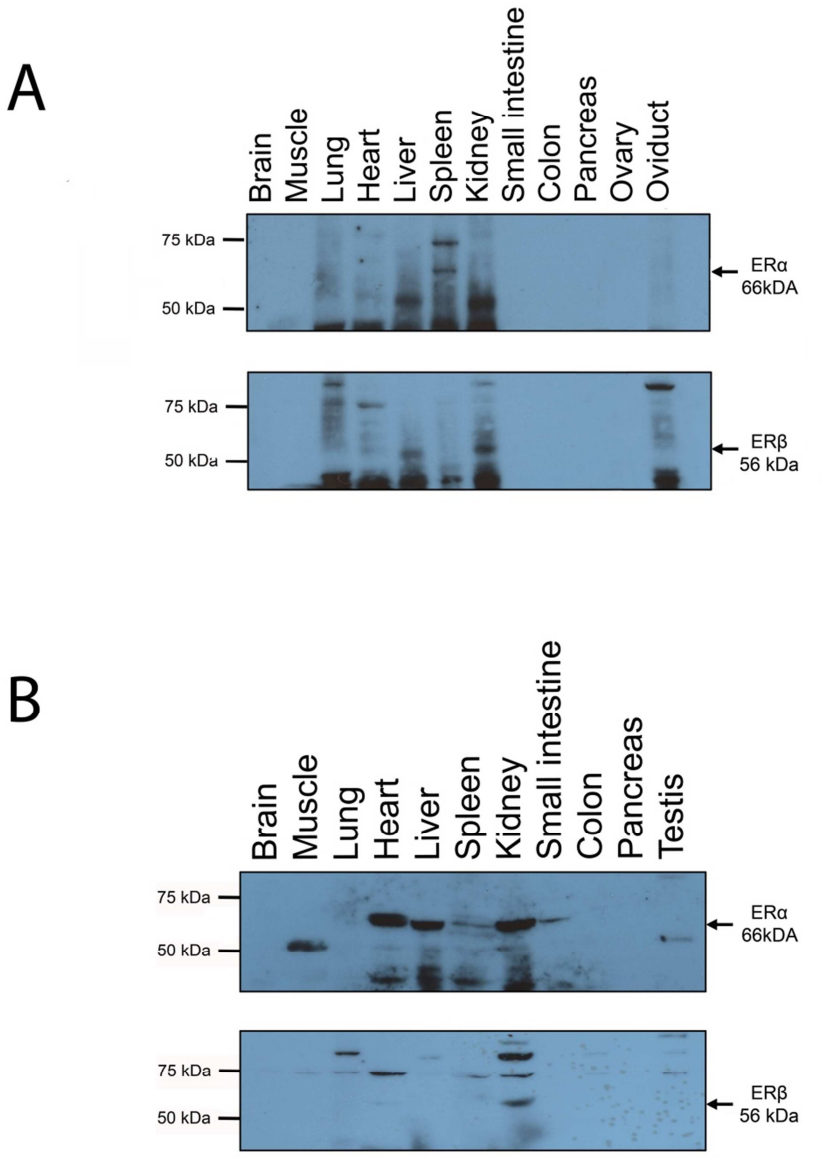


Figure 12: Tissue distribution of galline ERα and ERβ protein levels in 40 weeks old female and male chicken. For SDS-PAGE under non-reducing conditions and followed Western blot analysis, protein extracts of tissues (100 µg protein/lane) were used. For SDS-PAGE a 10 % gel was prepared and Western blot membranes were incubated with an anti huERα and anti huERβ antibody (1:50). A indicates the Western blot results for the female and B for the male chicken

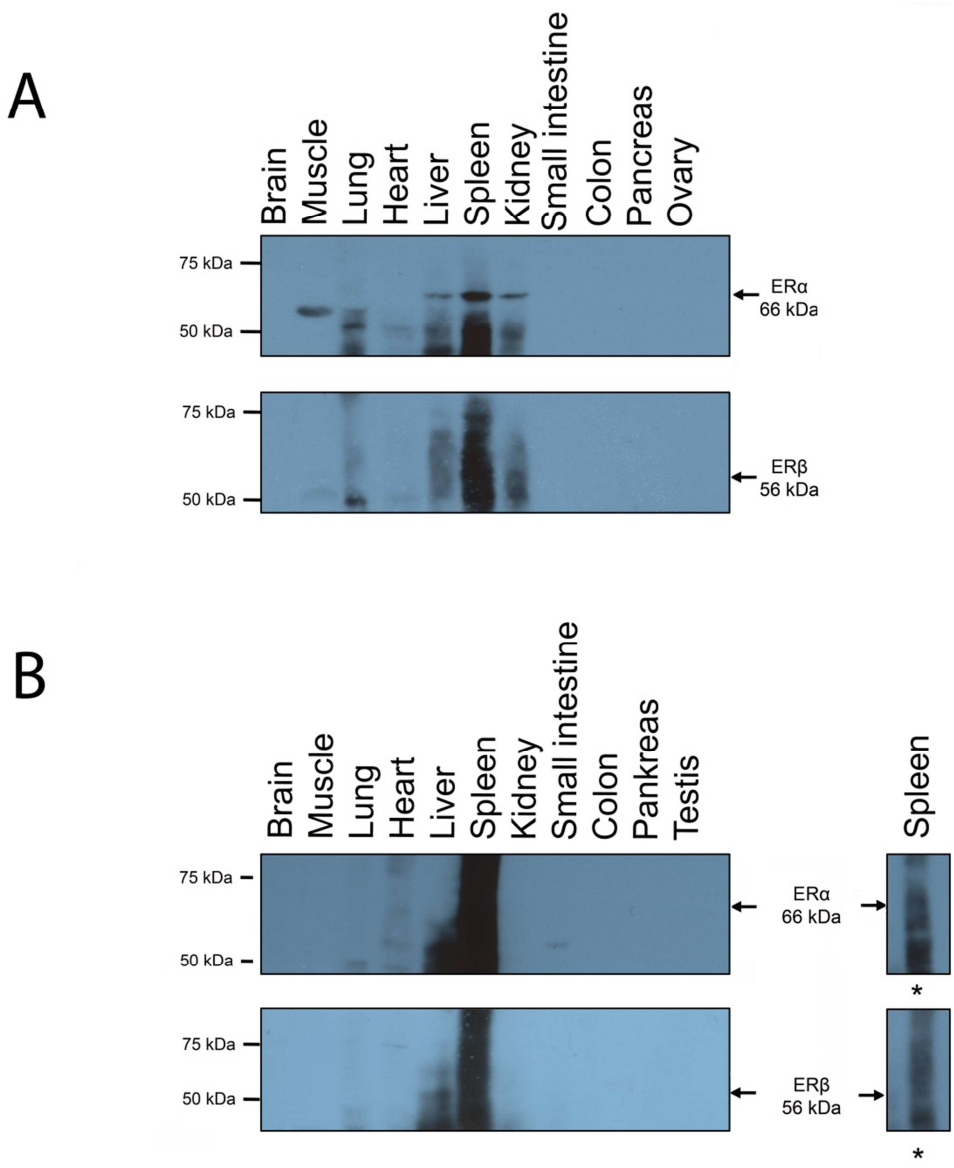


Figure 13 Tissue distribution of galline ERα and ERβ protein levels in 10days old female and male chicken. For SDS-PAGE under non-reducing conditions and followed Western blot analysis, protein extracts of tissues (100 µg protein/lane) were used. For SDS-PAGE a 10 % gel was prepared and Western blot membranes were incubated with an anti huERα and anti huERβ antibody (1:50). A shows Western blot results for the female and B for the male chicken.* indicates a lower incubation time.

Unfortunately there are no commercial anti chicken antibodies for the proteins of interest available. The used antibodies are specific for human proteins, but according

to the manufacturer (Santa Cruz) they should cross-react with chicken proteins as well. Like figure 12 illustrates the used antibodies bond very unspecific to the separated proteins, which make it difficult to clearly identify the proteins of interest. With best knowledge the bands for ERα and ERβ are indicated in the predicted highs. In the 40 weeks old female chicken ERα was found only in the spleen and ERβ was present in the heart, liver and kidney and very light band was visible in the lane for the oviduct (figure 12A). The Western blot for the tissue expression in the 40 weeks old male chicken, heart, spleen, liver and kidney were positive for ERα. Only the kidney showed ERβ signals (see figure 12B).
The protein tissue profile of 10 day old chickens showed positive signals for ERα and ERβ in spleen and kidney in case of the female chicken. ERα was also detected in the liver. It is know that in chicken a 61 kDa ERα isoform is existing. If the used antibody binds also the isoform, the muscle would be also positive (see figure 13A).
Western blots for the male 10 day old chicken were very unsatisfactory. Figure 13B showed ERα signals in the heart and spleen and ERβ was detected in the liver and the spleen.

Western blots experiments showed in general no (data not shown) or unspecific signals. To bypass this problem I tried to achieve better results by using Sepharose A beads, cross-linked with the antibodies of ERα or ERβ. These beads were mixed with the protein extracts form each tissue. The eluat of this procedure, containing ERα or ERβ proteins, was loaded on a SDS-PAGE. Separated proteins were stained with Coomassie Blue dye.

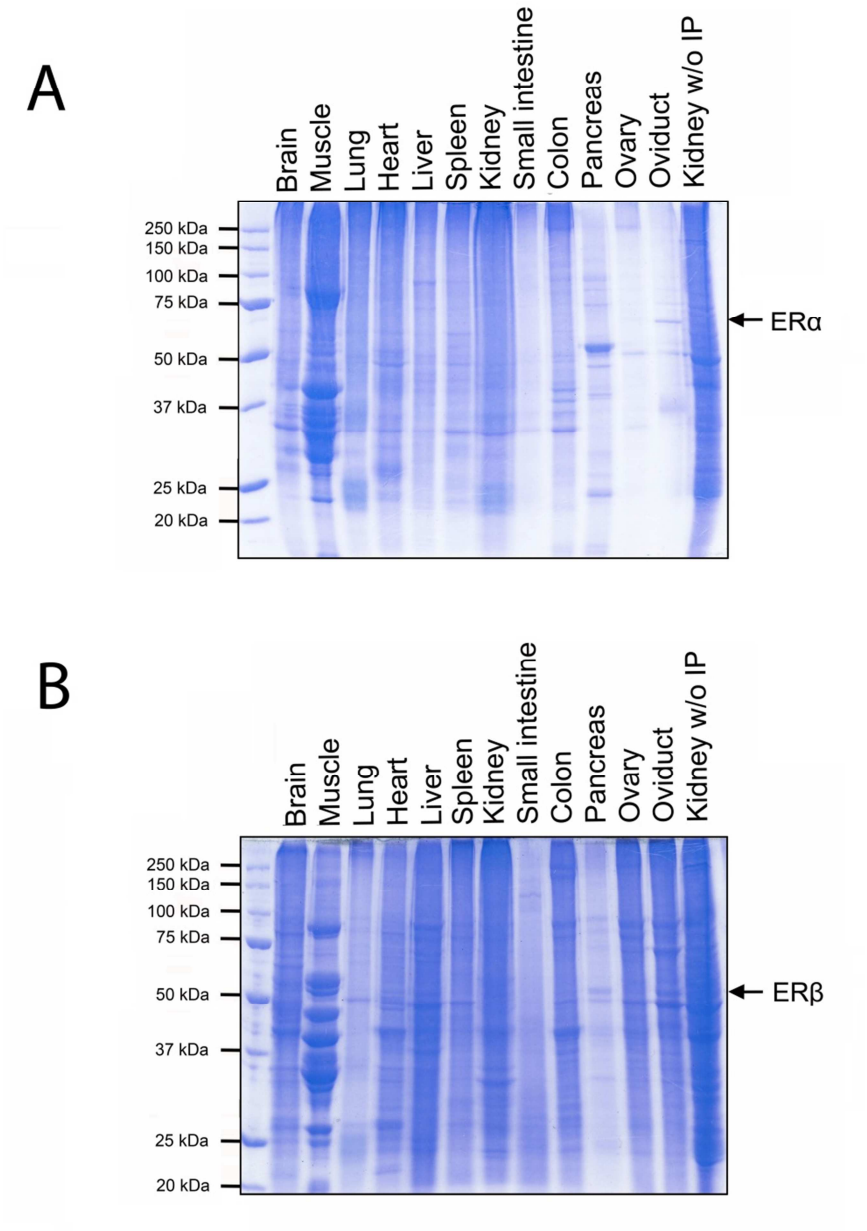


Figure 14: Tissue distribution of galline ERα and ERβ protein levels in the laying hen. For IP, Protein A Sepharose beads were cross linked with the corresponding antibodies and mixed with 1 mg protein/tissue. The eluate of the immunological clean-up was loaded on a SDS-PAGE (10 %) and subsequently stained with Coomassie Blue. **A** indicates IP with an anti ERα antibody, **B** indicates an IP for ERβ.

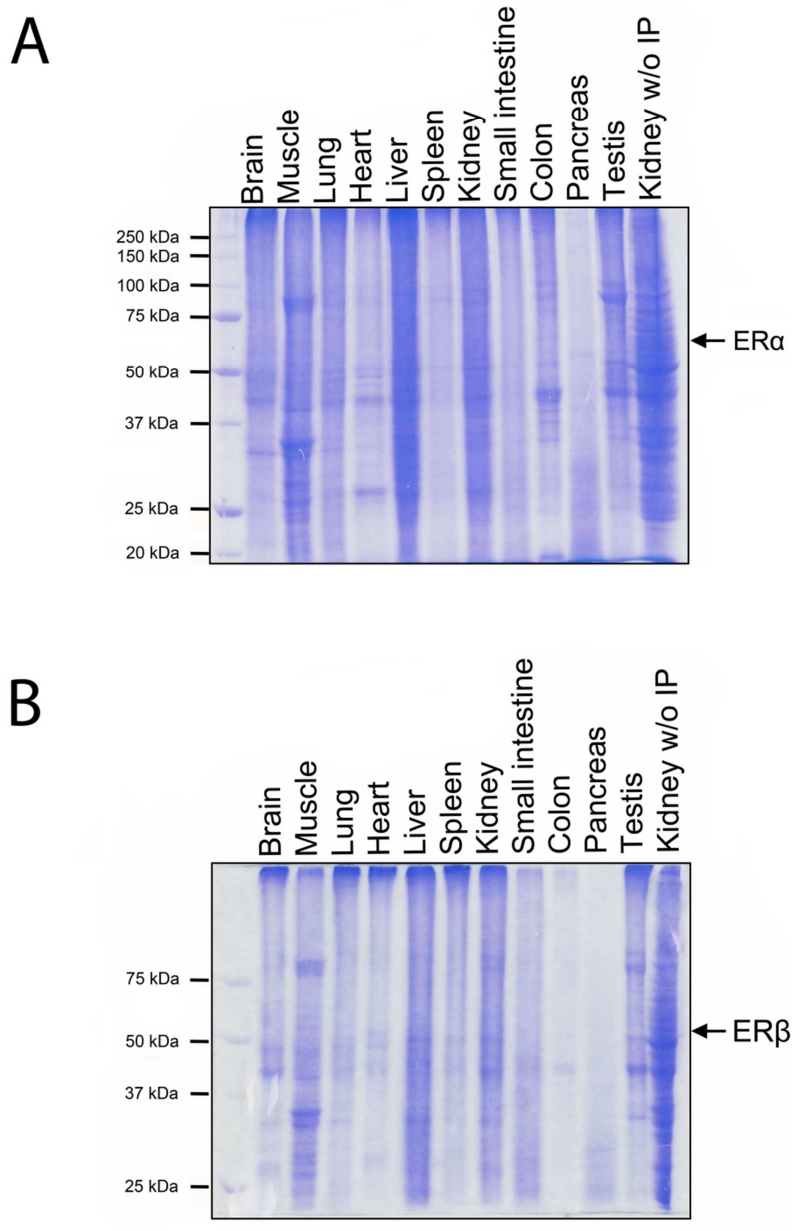
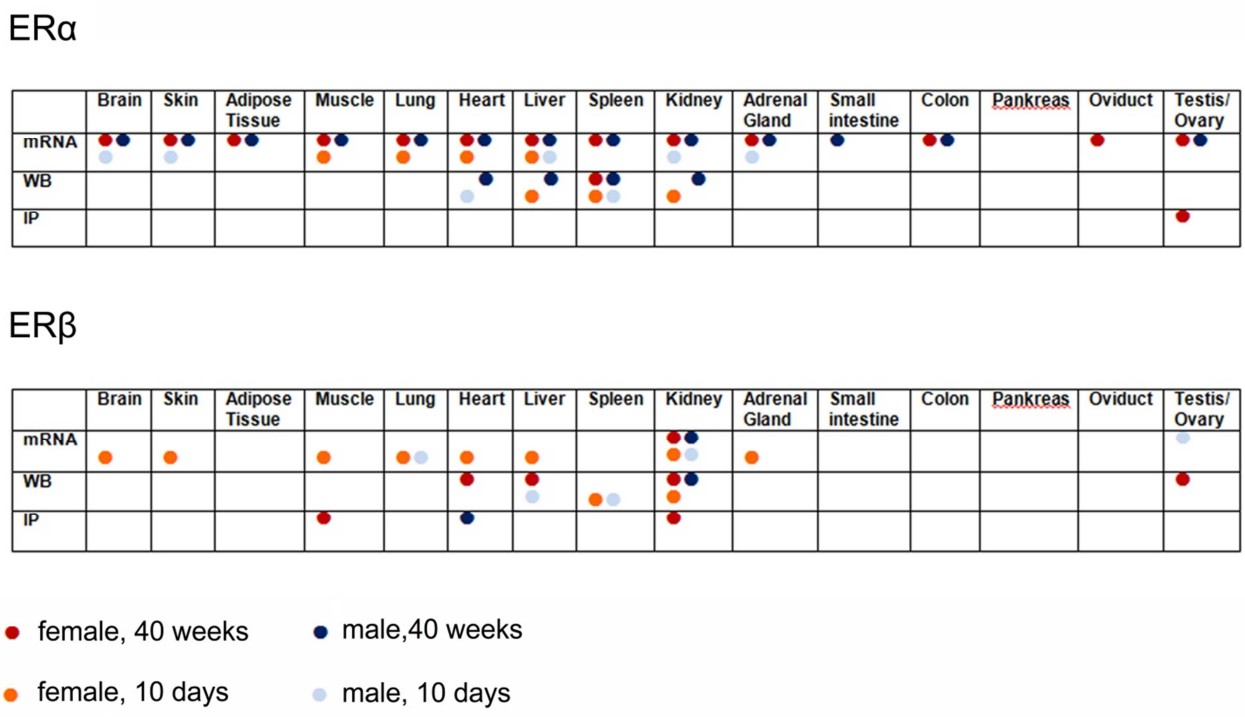


Figure 15: Tissue distribution of galline ERα and ERβ protein levels in the rooster. For IP, Protein A Sepharose beads were cross linked with the corresponding antibodies and mixed with 1 mg protein/tissue. The eluate of the immunological clean-up was loaded on a SDS-PAGE (10 %) and subsequently stained with Coomassie blue. **A** indicates IP with an anti ERα antibody, **B** indicates an IP for ERβ.

With this method signals in the high of 66 kDa, where ERα should be, were only detected in the oviduct. In this case, the antibody has also an affinity to the smaller isoform (61 kDa), as well the brain, heart, lung and liver and the colon would be positive for ERα (see figure 14A). According to figure 14B it seemed that ERβ is expressed in muscle and the kidney. In roosters ERα was not detectable in any tissue. ERβ might be in the tissues of the muscle, lung, heart and testis (see figure 15).

With the specific cleaning-up for ERα or ERβ with Protein A Sepharose beads it was



also not able to get a clearer impression of the specific protein expression profile. Due to this fact IP wasn't done for 10 days old chickens

Figure 16: Summary of the tissue expression pattern study.

Figure 16 is a complete collection of the results of the study of the tissue expression pattern of ERα and ERβin *gallus gallus.*

On a superficial look, the first eye catching result is that ERα is more expressed than the beta subtype. On a closer look one can see that this is true in case of the mature chicken, but not in case of the immature chicken. It seems that there is an age-related expression of ERα and ERβ.
On a sex specific few, it looks that the expression pattern of both ERs is more or less the same in mature chicken. In premature chickens this conformity is not shown. Here ERβ is more prominent in female and ERα in the male.

3.2 PART 2: Are the ERs involved in the LRP2 up-regulation due to estrogen?

Part 1 focused the interest on the tissue distribution of the estrogen receptor subtypes α and β in immature and mature chickens, part 2 is dealing with the investigation of a possible connection between the membrane protein and an essential importer of hormones - LRP2, and the estrogen receptors.

3.2.1 Effects of estrogen and RAP on LRP2 mRNA, ERα mRNA and ERβ mRNA

The first interest was to test the effect of different doses of estrogen and RAP of the mRNA expression of LRP2, ERα and ERβ in *in vitro*. Therefor the human embryonic kidney cell line – HEK-293 and a primary cell culture of kidney cells from three day old chickens (PKE) were cultivated and treated with the indicated substances.
The impact of the treatment was analysed by using quantitative PCR.

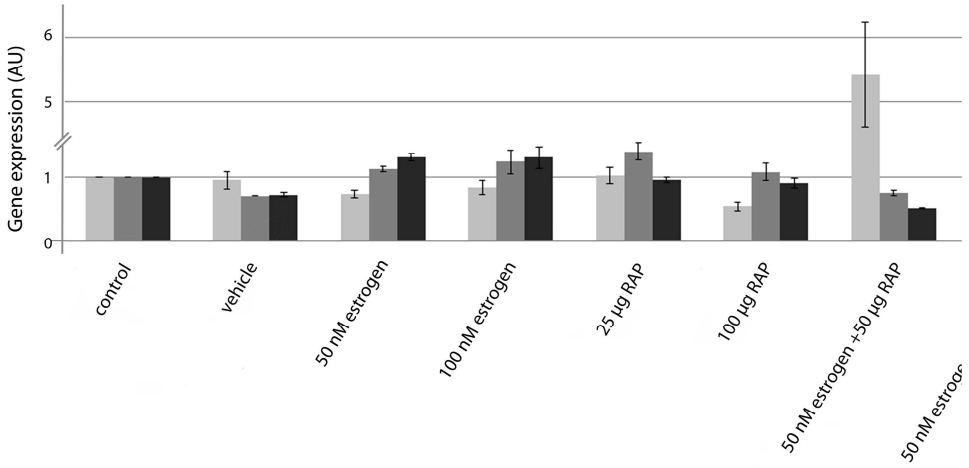


Figure 17: ERα, ERβ and LRP2 transcript levels in HEK–293 cells, after treatment with estradiol and RAP. Transcript levels were analyzed by using qPCR. The cultivated cells were harvested after 24h treatment. The numbers in the figure are indicating the following treatments.. Gene expression is denoted in arbitrary units (AU).

Figure 17 shows that LRP2 mRNA expression is slightly elevated by the treatment with estrogen, but there is no significant difference between the two doses. As one might expect, RAP treatment decreases the LRP2 mRNA level in a dose dependent manner, the higher the dose, the lower the expression. Interestingly, the LRP2 mRNA expression is lowest by a combination of estrogen and RAP.

ERα mRNA values decrease after estrogen and also after RAP treatment. The combined treatment of the two tested substances ERα mRNA seemed to be elevated. Notably the mRNA of ERβ was affected different, by the same treatment. The values were increased after RAP and estrogen treatment. Low doses of estrogen and high doses of RAP have the same impact like a single estrogen dose. Just in case of a low estrogen and RAP dose the ERβ mRNA expression is dropped.

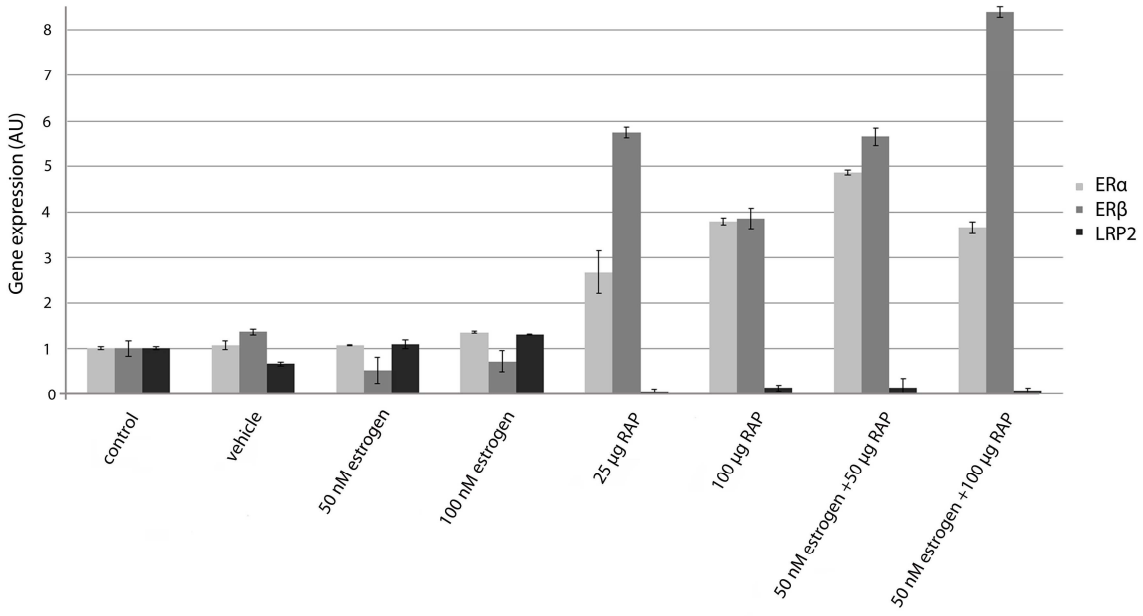


Figure 18: ERα, ERβ and LRP2 transcript levels in PKE cells, after treatment with estradiol and RAP. Transcript levels were analyzed by using qPCR. The cultivated cells were harvested after 24 h treatment. Gene expression is denoted in arbitrary units (AU).

LRP2 was affected by the different treatments in the same way in PKE cell, like in HEK–293 cells. Estrogen increased the expression, RAP and the combination of estrogen and RAP decreases it, but seemed that RAP has a greater impact in the reduction of the LRP2 mRNA, compared to HEK–293 cells. Very interestingly ERα mRNA is elevated after estrogen treatment in a dose dependent way, the more estrogen, the higher the value. In contrast ERβ was down-regulated after estrogen treatment. RAP and estrogen strongly promote the mRNA expression of both receptor subtypes (see figure 18).

3.2.2 Effects of estrogen and RAP on LRP2, ERα and ERβ in the protein level

The next task was to check if the changes after the treatments are also reflected on the protein level. Therefore protein extracts from the treated cells were made and SDS-PAGE and Western blot was performed.

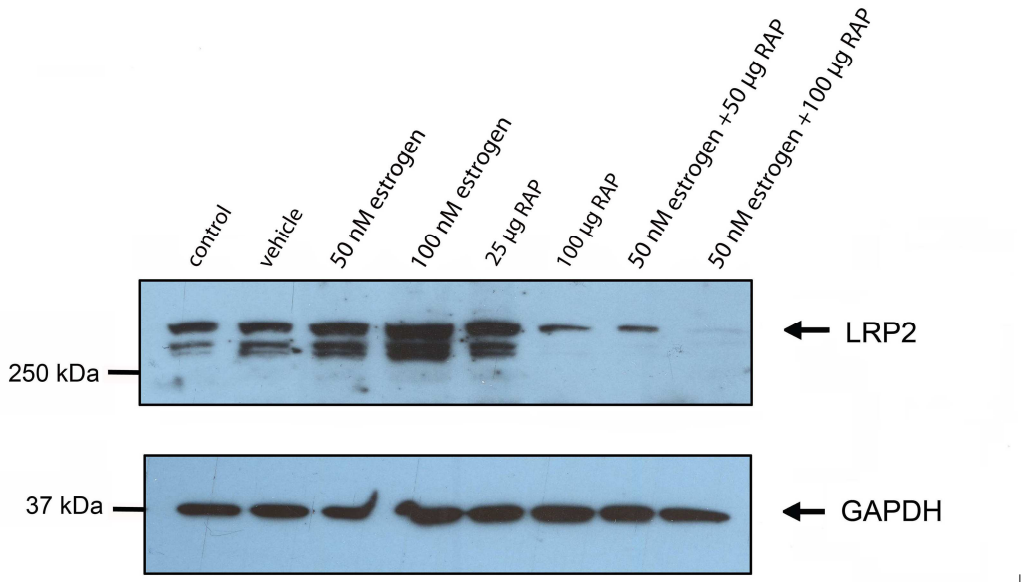


Figure 19: LRP2 protein expression in HEK cells after treatment with different doses of estrogen and RAP. For SDS-PAGE under non-reducing conditions and followed Western blot analysis, protein extracts of tissues (100 µg protein/lane) were used. For SDS-PAGE a 10 % gel was prepared and Western blot membranes were incubated with a ggLRP2 antibody (1:250). For loading control the housekeeping gene GAPDH was used.

Figure 19 showed very nicely that the results on the transcript level were reflected on protein stage. Estrogen increases LRP2 expression and RAP and a combination of estrogen and RAP leading to a decrease of the LRP2 protein.

ERα and ERβ wasn't able to detect by Western blotting techniques. Also the loading of the maximum of the protein extracts and the use of high concentrations of the antibody didn't enable any detectable signal, as well as the use of different antibodies. It is possible that the estrogen receptors are very low expressed in HEK -

293 cells and consequently hard to detect by Western blotting. Due to this idea in the next steps co-immunoprecipitation was used. This method was used for the next cellular treatment to clean-up the protein extracts for the proteins of interest (Figure20).

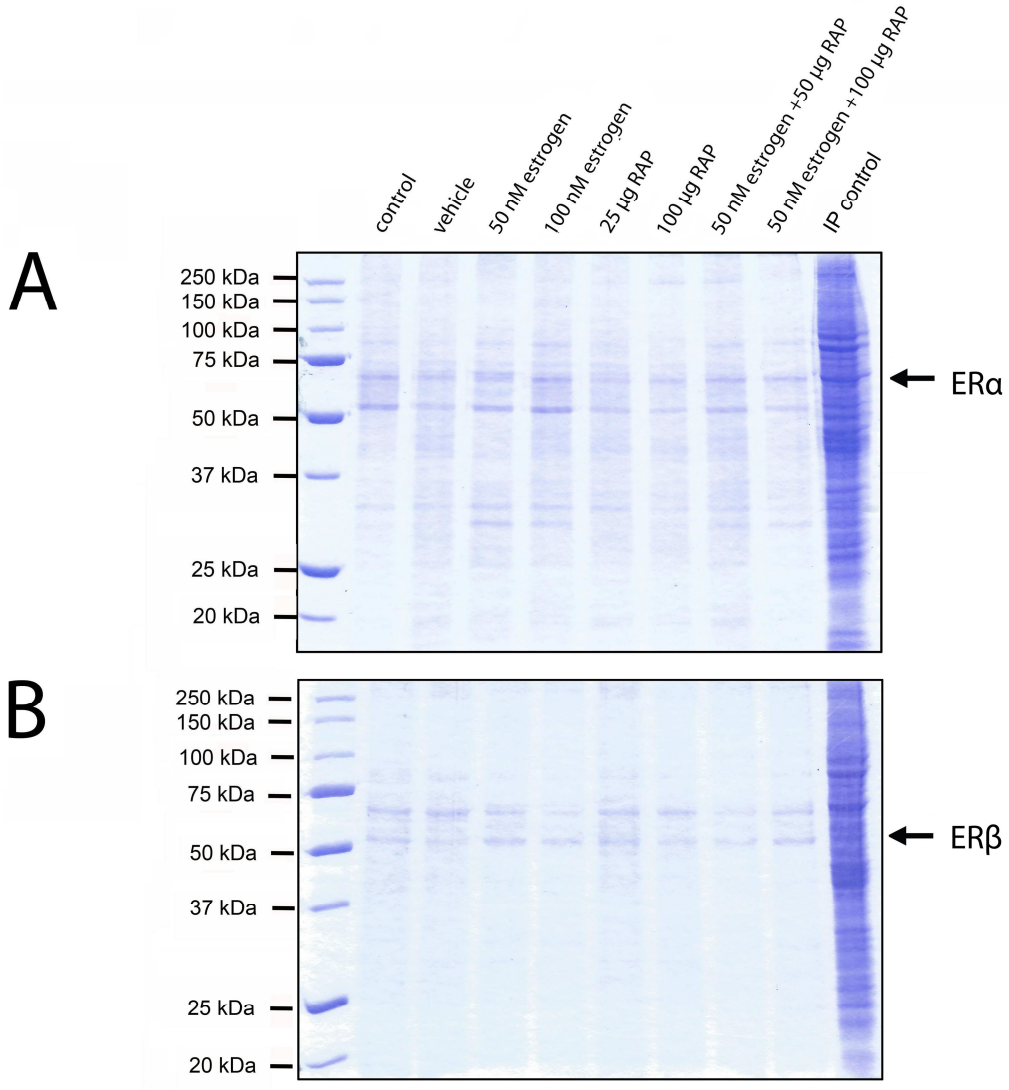


Figure 20: The impact of estrogen, RAP and ICI treatment on the protein levels of ERα and ERβ in HEK–293 cells. Cells were harvested after 24h treatment, whereby ICI 182780 was given one hour before harvesting. For IP Sepharose A beads were cross-linked with anti huERα or anti huERβ antibodies, beads were mixed with 1 mg protein extract/sample. Purificated proteins were loaded on a 10 % SDS-PAGE and stained with Coomassie Blue.

3.2.3 Does the antiestrogen ICI effect LRP2 expression?

After the experiments showed that estrogen treatment elevates the LRP2 expression, I wanted to examine this effect from another point of view.
For this purpose ICI 182780, also named Fulvestrant or Faslodex ® was used because of the following predicted functions on both estrogen receptors: 1) the competitive binding to ERs, so they can't bind estrogen, 2) the receptors get down-regulated through the binding and 3) the binding causes changes in the receptor surface, which change the binding affinity to cofactors. As positive control of the HEK–293 cell treatment with ICI 182780 the two estrogen receptors were validated. Therefore an IP with the collected protein extracts from the treated cells was done, followed by SDS-PAGE and Coomassie Blue staining.

Figure 20 indicates that the treatment worked for both receptor subtypes. ERα as well as ERβ is less expressed in samples treated with the inhibitor. Furthermore it seems that due to the use of IP heterodimers between the estrogen subtypes are cleaned up, because in case of the use of ERα as well as ERβ antibodies for the IP both receptor subtypes show presence in on the stained membranes. In addition it seems that ERα is expressed in higher doses in HEK–293 cells than ERβ, or the purification wasn't as successful in case of ERβ, compared to ERα.

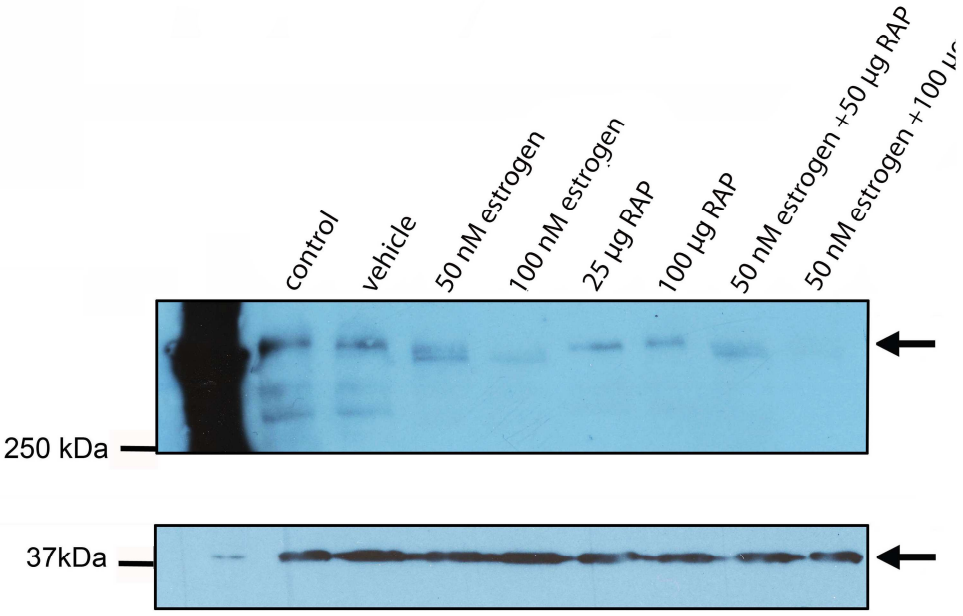


Figure 21: The impact of estrogen, RAP and ICI treatment on the protein levels of LRP2 in HEK-293 cells. For SDS-PAGE under non-reducing conditions and followed Western blot analysis, protein extracts of cultivated HEK cells were (100 µg protein/lane) were used. To detect LRP2 anti huLRP2 (1:250) and for housekeeping gene detection anti huGAPDH (1:20 000) was used. Cells were harvested after 24 h treatment, whereby ICI 182780 was given one hour before harvesting.

The Western blot with antibodies against LRP2 in figure 21 shows that ICI 182780 also decreased the level of LRP2 protein. Furthermore it prevented the up regulation of the protein after estrogen treatment and it enhanced the negative effect of RAP. LRP2 was not detectable in cells which were treated with all three substances (ICI 182780, estrogen and RAP).

4 Discussion

Normal reproductive, physiological and developmental processes in animate beings are dependent on steroid hormones such as estrogens (Harris, 2006). Estrogens play an important role in different stages of the female development. Nowadays it is known that the ancient female hormone is also an important factor of male health - even male fertility is affected by estrogens (Eddy, 1996 and Dupont, 2000). Most actions of estrogens are coordinated and modulated by the very complex work of the estrogen receptors α and β (Pearce, Jordon, 2004; Petterson, 2000). Due to the mediating function of the signals form estrogens in their target genes, the receptor subtypes should be present and detectable in them. In various tissues of humans, rats, mice and other species, the existence of ERα and ERβ is proved (Stenberg, 2002) In *gallus gallus* little is known about their tissue distribution. According to current knowledge ERs are found in various tissues of the galline body like the ovaries and testis, the kidney and the brain (Gonzales-Moran, 2014; Gonzales-Moran, 2008; Hansen, 2003 and Griffin, 2001).

But very little is known about other tissues or organic systems in *gallus gallus*. Due to this knowledge gap the first part of this study deals with the analysis of the tissue distribution of the two estrogen receptor subtypes in mature chickens. Another interesting question was if there might be a discrepancy of the expression pattern between male and female ones.

The results of the analysis on the transcript level showed very nicely that the expression of ERα mRNA is quite similar in both sexes. Very unsurprisingly male and female chickens produce the receptor in their sexual organs. The publication from Hrabia says that the alpha receptor plays a predominant role in the chicken ovary and that the beta receptor is also expressed, but in much lower quantities (Hrabia, 2008). Maybe ERβ was also present in the tested ovary tissue, but in too low concentrations to detect it with the used system. Roosters and chickens, both have estrogen receptor α mRNA in their heart, liver, spleen, kidney, adrenal gland and the colon. These results harmonize with the findings of Kuiper and Harvery by their

proposed effects of ERs in the electrolyte and fluid household (Kuiper, 1997 and Harvery, 2001). Very surprising all tested testis were positive for ERα mRNA but just one tested chicken produced it in its ovary. A possible explanation for this result might be the asymmetric expression of the estrogen receptors in order to the asymmetric development of the left and the right chicken ovary (Gonzales-Moran, 2014), maybe most of the tissue samples were taken coincidently from the less developed ovary. After PCR tests with the taken primers for ERα and ERβ, it seems that ERβ mRNA has a reduced impact on the mediation of estrogen signals in chickens. ERβ mRNA was only detected in the kidney of both sexes and the sexual organs. In comparison with the findings from a group around Kuiper in the year 1997, which analysed the tissue distribution of ERα mRNA and ERβ mRNA in rat, it is very surprising that ERβ mRNA is present just in two tissues (Kuiper, 1997).

The group around Hansen in the year 2003, which found out that ERα is expressed in an age dependent manner (Hansen, 2003). This knowledge leads to investigate the tissue distribution of 10 day old chickens. In this age class, ERα mRNA and ERβ mRNA were both expressed in muscle, lung, heart and liver. In addition ERβ mRNA was also found in the kidney and the adrenal gland. In 10 day old male chicks both receptors were detected in brain, kidney and testis. ERα mRNA was also found in skin, liver and adrenal gland. After these results the first eye catching difference is that ERβ mRNA is expressed in more tissues of the younger chicken compared to the results from 40 weeks old chickens. It seems that ERβ mRNA has a greater role in mediating the actions of estrogen in the young chicken compared to the tested 40 weeks old animals.

Furthermore it is very interesting that in the 10 day old chickens ERα mRNA and ERβ mRNA are present together in some tissues (female: muscle, lung, heart and liver; male: brain, kidney and testis). The co-localitsation of both receptors, allows the forming of heterodimers, which have distinct functions from homodimers (Petterson, et al., 2000). If heterodimeristation takes place or not is an exciting question addressed on further studies. The presence of specific mRNA levels in a tissue is no evidence that these ribonucleic molecules are also translated in functional proteins. That’s why the next step was to detect the proteins of ERα and ERβ by Western blotting. Unfortunately the Western blot analysis results in no band, why the

maximum amount of proteins was loaded on the gels and very high concentrations of the antibodies were used. This results in a total smear or in multiple bands, which made a clear discussion hard.

In female 40 weeks old chicken, proteins of ERα were detected only in the spleen and ERβ was found in the tissues of heart, liver, kidney and the oviduct. This result was very surprising compared to the PCR results. On one hand because ERα protein was found only in one tissue and also because of the results for ERβ, the proteins were detected in tissues were the mRNA was not. The Western blot results of the 40 weeks old roosters harmonize better with the PCR results, ERα was detected in the heart, spleen, liver and the kidney, were also mRNA was present. And also the results for beta matched perfect (found in the kidney).

Western blotting results in 10 day old chickens were very unsatisfactory and don't match fine with PCR results. To bypass this problem it was tried to directly extract proteins of ERα and ERβ by Sepharose-A-beats linked to specific antibodies for the protein of interest. Unfortunately this procedure was also not crowned with success.

The number of unspecific bands and the low outcome of the different test on the protein level, might be due to the factor that the used antibodies for both estrogen receptors were anti human. Very small group of scientists address their questions on ERα or ERβ in chicken models, because of this fact it was not possible to find commercial produced antibodies for the proteins of interest especial for chickens.

Further test, set with self-produced antibodies against the two chicken estrogen receptors might be essential to reply this question more precise. A specific antibody production is a time-consuming procedure, that's why it was not possible to do this in the period of the master thesis.

The second aim of this study was to get a closer insight in the relationship between LRP2 and estrogen. Further studies have shown that estrogen has a great impact of chicken gens which are involved in the metabolic pathway. Estrogen especially triggers the up-regulation of LRP2 in the galline kidney at transcript as well as the protein level (Plieschnig, 2012).

The activation of the transcription might be achieved by a ligand-dependent interaction between estrogen receptor alpha and Sp1 (stimulating protein-1) (Li, 2001).

The treatment of two different cell types (cultivated primary chicken cells and HEK-293 cells), show sensitivity against estrogen. The results present that estrogen treatment triggers in both cell types an increased LRP2 level. Interestingly in HEK-293 cells, the elevation seems to be independent due to the doses of estrogen. In contrast to the cultivated primary chicken cells, were the regulation of LRP2 might be function in a dose-dependent manner.

Surprisingly the effect of estrogen due to its receptors is in reverse in the cell types. Whereas in HEK-293 cells ERα is down- and ERβ is up-regulated in contrast primary cells show the opposite result. Maybe this is an effect due to the different cellular environment of the two different cell types.

To see if the up-regulation of LRP2 is due to estrogen is mediated directly by the estrogen receptors, the receptors were blocked by the antiestrogen ICI 182780. The result of this study shows that in cells which were previously treated with the antagonist, LRP2 is less expressed. This result gives reason to confirm the impact of ERs in this case.

Unfortunately it is not possible to distinguish between the effects of each receptor.

Is estrogen receptor alpha or beta essential for the up-regulation of LRP2 or is it a heterodimer molecule of both, which is doing all the work? This questions give reason for further exciting studies in this area of scientific research

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6 Abbreviations

AF-1	activation function 1
AP1	activator protein 1
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CHO-K1	chinese hamster ovary
CLM	caveolar like microdomain
DNA	desoxyribonucleic acid
dNTP	deoxynucleotidetriphosphate
DPN	diarylpropionitrile
E1	estrone
E2	erstradiol-17β
E3	erstriol
ECL	enhanced chemoluminescence
EGF	epidermal grothfactor
ERE	estrogen response element
ERs	estrogen receptors
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ESR1	estrogen receptor alpha gene
HEK	human embryonic kidney
HERS	heart and estrogen replacement study
HRT	hormone replacement study
Hsp90	heat shock protein 90
huER	human estrogen receptor
IP	immmunoprecipitation
kDa	kilodalton
LBD	ligand binding domain

LDLR	low dendity lipoprotein receptor
LRP1	low density lipoprotein receptor-related protein 1
MAPK	mitogen-activated protein
MCF-7	michigan cancer foundation-7
µg	microgram
µl	microliter
ml	mililiter
ml	millliliter
mM	milimonar
mRNA	messenger RNA
NF- ϰB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear location signal
NTD	n-terminal domain
PCR	polychianreaction
PKE	primary kidney epitheilia cell
PPARγ	peroxisome-proliferator-activated receprot
PPT	4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol
qPCR	quantitative PCR
RAP	receptor associated protein
RNA	ribonucleic acid
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate – polyacracrylamide gel electrophoresis
SERMS	selective estrogen-receptor modulator
SP1	specific protein 1
WAT	white adipose tissue
αERKO	alpha estrogen receptor knockout

7 Abstract

Estrogens influence a variety of physiological processes in the whole body, of female and male individuals. The steroidhormone is mediating many effects of the cellular growth, development and the maintenance of diverse tissues. It's mainly produced in the genital organs, from there the hormones reach the target cells due to passive diffusion or active transport.

Estrogen activates the estrogen receptors ERα and ERβ. For many years just one ER receptor was known (ERα). The discovery of the second receptor (ERβ), made it even harder to understand the known and the new effects. The complexity of the estrogen effects is shown by the diverse choices of signal pathways. The information is delivered via the classical pathway by dimerization and interaction with the DNA another option is to perform a cross-talk with transcriptionfactors, to name just two of the choices.

This study is divided into two sections. The first part deals with the tissue specific expression pattern of the two receptor subtypes in *gallus gallus*. On a sex specific point of view, the experiments showed that in mature chickens (40 weeks) both sexes have more or less, the same expression pattern of the ERs. Interestingly this conformity was not mirrored in younger chickens (10 days). In the younger chick ERα was more prominent in male and ERβ in female. Besides the mentioned age specific discrepancies, it was shown that in mature chickens particularly ERα was produced and in younger chickens ERβ plays a greater role. All these findings are supporting the complexity of the functions of estrogen mediated by estrogen receptors.

The second part was forcing the connection between estrogen and LRP2. LRP2 is a receptor protein, which is among other things, responsible for the intake of molecules like: vitamins, lipoproteins and hormones like estrogens. Earlier studies showed that the LRR2 quantity decreases after estrogen donation. If ERs are somehow partitioning in this signalling way, was figured out by the inhibition of the receptors. The inhibition of the two receptors wasn't leading to a LRP2 boost, no enrichment

was detectable. Due to this experiment it is shown that ERα and ERβ, together or just one of them is/are essential for the syntheses of LRP2 in connection with estrogen.

8 Kurzfassung

Das Östrogen, beeinflusst eine Reihe von physiologischen Prozessen im ganzen Körper, weiblicher sowie auch männlicher Natur. Das Steroidhormon mediiert viele Effekte wie zelluläres Wachstum, die Entwicklung und die Aufrechterhaltung verschiedenster Gewebestrukturen. Produziert wird das Sexhormon vor allem in den Geschlechtsorganen und gelangt von dort aus durch passive Diffusion oder aktiven Transport zu seinen Zielzellen.

Die Wirkung des Östrogens wird durch die Östrogenrezeptoren (ERα und ERβ) auf das zelluläre bzw. molekulare Umfeld weitergeleitet. Viele Jahre war nur ein Typus dieses Rezeptors bekannt - ERα. Durch die Entdeckung eines weiteren, folglich ERβ genannt, wurde das wenig bekannte Wirkspektrum der Östrogenrezeptoren um ein vielfaches diffiziler. Die Komplexität der Östrogenwirkung zeigt sich auch durch die verschiedenen Wege seiner Rezeptoren. Die Informationen werden entweder auf klassische Weise durch die Bindung an die DNA weitergegeben oder es kommt zu einem sogenannten „cross-talk“ mit anderen Transkriptionsfaktoren, um nur zwei der möglichen Varianten zu nennen.

Diese Studie gliedert sich in zwei Teile. Im ersten Teil wird das gewebespezifische Expressionsmuster der beiden Rezeptoren ERα und ERβ in *Gallus gallus* erläutert.

Geschlechtsspezifisch betrachtet zeigte sich in reifen Tieren (40 Wochen) annähernd gleiche Expressionsmuster beider Rezeptoren. Interessanterweise war diese Übereinstimmung in Jungtieren (10 Tage) nicht zu finden. Hier war in männlichen ERα und in den weiblichen ERβ dominierend. Neben den genannten Altersabhängigen Unterschieden, zeigte sich auch, dass in älteren Tieren v.a. ERα produziert wird und in jüngeren Tieren ERβ die dominierende Rolle einnimmt. Alle diese Unterschiede bekräftigen die komplexe Funktionsweise der östrogen-mediierten Rezeptormoleküle.

Der zweite Teil beschäftigt sich mit dem Zusammenspiel von Östrogen und LRP2, einem Rezeptorprotein das unter anderem auch für die Aufnahme von unterschiedlichstem Molekülen wie z.B.: Vitaminen, Lipoproteine und Hormonen wie Östrogen verantwortlich ist. Frühere Studien zeigten dass LRP2 durch Östrogengabe vermehrt gebildet wird. Ob in diesen Syntheseweg auch die klassischen Östrogenrezeptoren involviert sind wurde im Experiment durch die Inhibierung dieser untersucht. Nach Hemmung beider Rezeptoren konnte keine LRP2 Anreicherung mehr festgestellt werden. Daraus lässt sich schließen, dass ERα und ERβ für die LRP2 Synthese im Zusammenhang mit Östrogen, wichtige Faktoren darstellen

9 Curriculum vitae

Personal Data

Name	**Barbara Walder**
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Work experience

September 2014 to present	**Baxalta- Quality Control**
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October 2006 to January 2008	**University of Innsbruck, AUSTRIA** Diploma Studies of Architecture
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