Physiological and pathological aspects of the ligand-activated transcription factors: AhR and ERβ

A Dissertation Presented to the Department of Biology and Biochemistry University of Houston

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

By

Ryan Butler

May 2013

Physiological and pathological aspects of the ligand-activated transcription factors: AhR and ERβ

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Abstract

Ligand-activated transcription factors are a diverse group of proteins that are involved a variety of physiological processes. The purpose of these studies was to investigate the aryl hydrocarbon receptor (AhR) knockout mouse and the effects of hormone replacement therapy on the postmenopausal breast in order to better understand the functions of the ligand-activated transcription factors AhR and estrogen receptor β (ERβ).

In the first part of these studies, we investigated the AhR knockout mouse in order to further elucidate the physiological functions of AhR. These mice developed stones in their urinary bladders composed of 100% uric acid while the serum uric acid levels remained normal. We determined these stones were formed by the breakdown of DNA into uric acid from a large number of dying cells. These mice also develop fibrosis of their ventral prostates and a phenotype in the immune system similar to chronic myeloid leukemia.

The second part of these studies was to investigate the effects of hormone replacement therapy on the histology and expression of various ligand-activated transcription factors in the postmenopausal breast. We treated postmenopausal women with estrogen or estrogen-progesterone therapy for 3 months and took biopsies of the breasts before and after treatment. Expression of several proteins including AhR, ERα, and ERβ were unchanged after treatment while expression of progesterone receptors was increased. Proliferation and breast density were unchanged by treatment in these breasts;

however, we show possible mechanisms leading to proliferation and development of density in non-cancerous breast tissue.

In conclusion, these studies revealed a role for AhR in prostatic development, uric acid formation, and leukemia, and have provided new information on the safety of short-term use of HRT.

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Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ABL	Abelson murine leukemia viral oncogene homolog 1
ADA	Adenosine deaminase
AF	Activation function
AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor
ALH	Atypical lobular hyperplasia
AML	Acute myeloid leukemia
AP-1	Activator protein 1
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
Bax	Bcl-2-associated X protein
BCR	Breakpoint cluster region
BCRP	Breast cancer resistance protein
bHLH	Basic helix-loop-helix
BPH	Benign prostatic hyperplasia
BrdU	5-bromo-2-deoxyuridine
cAMP	Cyclic adenosine monophosphate
Cyp	Cytochrome P450
CML	Chronic myeloid leukemia
CNS	Central nervous system

DAB	Diaminobenzidine
DHT	Dihydrotestosterone
DI	Deionized
DNA	Deoxyribonucleic acid
E2	17β-estradiol
EDTA	Ethylenediaminetetraacetic acid
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response element
FICZ	6-formylindolo[3,2-b]carbazole
Foxp3	Forkhead box P3
Glut 9	Glucose transporter 9
GST	Glutathione-S-transferase
H&E	Hematoxylin and esoin
HAH	Halogenated aromatic hydrocarbon
HDL	High density lipoprotein
HER2	Human epithelial growth factor receptor 2
Hif-1β	Hypoxia inducible factor-1β
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
HSC	Hematopoetic stem cell
Hsp	Heat shock protein

LDL	Low density lipoprotein
NA	Norethindrone acetate
NADPH	Nicotinamide adenine dinucleotide phosphate
NPT1	Novel putative transporter 1
OAT 2	Organic anion transporter 2
PAH	Polycyclic aromatic hydrocarbon
p-ALAT	Plasma alanine transaminase
PARP	Poly ADP-ribose polymerase
PAS	PER/ARNT/SIM
p-ASAT	Plasma aspartate transaminase
PBS	Phosphate-buffered saline
PR	Progesterone receptor
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SERM	Selective estrogen receptor modulator
T4	Thyroxin
TAD	Transcriptional activation domain
TCDD	2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor beta
Th17	T-helper 17 cells
TRAMP	Transgenic adenocarcinoma of the mouse prostate
Treg	T-regulatory cells

TSH	Thyroid stimulating hormone
UDH	Usual ductal hyperplasia
Urat 1	Urate transporter 1
UGT1A1	Uridine diphosphate glucuronosyltransferase 1
WT	Wild-type
XAP	X-associated protein
XRE	Xenobiotic response element
YSM	Years since menopause

1. General Introduction

1.1 Ligand-activated transcription factors

Ligand-activated transcription factors are intracellular proteins which are activated upon binding of various, very selective small molecules. Receptor activation leads to transcription of genes which are targets of the specific receptors. The importance of these receptors in normal physiological processes is evident from the spectrum of diseases that arise when receptor signaling is disrupted. These receptors are very important drug targets due to the ability of chemists to develop small molecular weight agonists or antagonists (Overington, Al-Lazikani et al. 2006).

The basic helix-loop-helix/ PER/ ARNT/ SIM (bHLH/PAS) family is an important group of transcription factors which include the aryl hydrocarbon receptor, hypoxia-inducible factor 1α, the single-minded proteins and others. These proteins share a similar structure which includes a basic helix-loop-helix DNA binding domain as well as a PAS domain responsible for dimerization and ligand binding (Kewley, Whitelaw et al. 2004). Members of this family are responsible for cellular functions such as response to stress, proliferation, differentiation, circadian rhythm, and many others (Kewley, Whitelaw et al. 2004). bHLH/ PAS proteins can be activated in many different ways including small-molecule ligand binding, cellular stress (such as hypoxia), and the binding of other proteins.

Nuclear receptors are a large superfamily of ligand-activated transcription factors located in the nuclei of numerous different types of cells (Abbouni, Elhariry et al. 2004;

Huang, Chandra et al. 2010). This group of molecules includes receptors for: steroid hormones (estrogen, androgen, progesterone, glucocorticoids, and mineralocorticoids); vitamins and non steroidal hormones (thyroid, vitamin D, and vitamin A); cholesterol metabolites (oxysterols and bile acids); fatty acids and various hormone metabolites. Several members of this family are orphan receptors because their specific agonists have not been discovered. All nuclear receptors share a similar structure with specific domains involved in ligand binding, DNA binding, co-repressor binding, and co-activator binding.

Most of the research covered in this thesis focuses on estrogen receptor beta (a nuclear receptor with a zinc finger DNA-binding domain) and the aryl hydrocarbon receptor (a transcription factor with a basic helix-loop-helix (bHLH) DNA-binding domain) and their connections to different diseases.

1.2 The aryl hydrocarbon receptor

1.2.1 AhR structure and mechanism of action

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor whose gene, in humans, is located on chromosome 7 (Abel and Haarmann-Stemmann 2010) and encodes a protein of 96 kDa (Burbach, Poland et al. 1992). The AhR, along with other members of this transcription factor family, has a bHLH domain at the N-terminus of the protein which is responsible for DNA binding (figure 1) (Murre, Bain et al. 1994; Gu, Hogenesch et al. 2000). AhR also has a PER/ARNT/SIM (PAS) domain harboring dimerization and ligand binding domains and a nuclear localization signal. At

the C-terminal of the receptor is the Transcriptional activation domain (TAD), responsible for binding of various co-activators (Ramadoss and Perdew 2005).

The AhR binds to many environmental contaminants including the polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH). The receptor is also activated by dietary compounds and some endogenous ligands (Beischlag, Luis Morales et al. 2008). When not bound to its ligands, AhR is located in the cytoplasm where it is bound to a dimer of Hsp 90 together with p23 and XAP 2 (Whitelaw, McGuire et al. 1995; Nair, Toran et al. 1996; Meyer, Petrulis et al. 2000).

In the cytoplasm, this complex of proteins bound to AhR helps stabilize it and prevent it from entering the nucleus (Johnson and Toft 1994). Upon ligand binding, AhR dissociates from these proteins, heterodimerizes with the AhR nuclear translocator (ARNT or Hif-1β), enters the nucleus, binds to xenobiotic response elements (XRE) and activates transcription of target genes (Reyes, Reisz-Porszasz et al. 1992).

There is a long list of AhR-target genes with a wide variety of functions in many types of cells. The most well studied AhR-regulated genes belong to the cytochrome P450 family 1 family of enzymes. Cyp1A1, 1A2 and 1B1 are all involved in metabolism of various environmental toxins including PAHs and heterocyclic amines (Nebert, Dalton et al. 2004). Other AhR induced-genes have functions in metabolism, drug transport, cell proliferation, and apoptosis; table 1 contains a list of many of these genes.

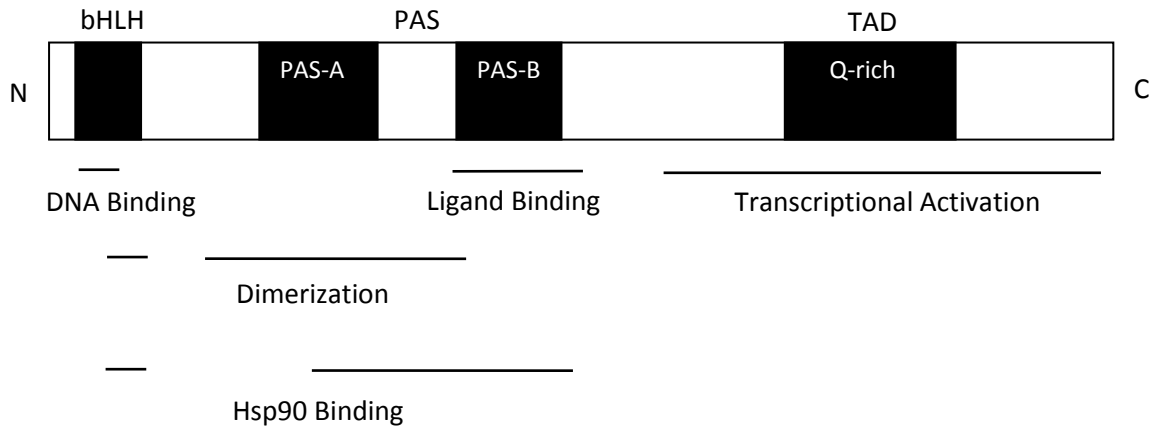


Figure 1. The structure of the aryl hydrocarbon receptor. The bHLH domain is responsible for DNA binding and is located on the N-terminus. The PAS domain is involved in ligand binding and binding to other proteins. The TAD region of AhR is responsible for transcriptional activation through binding to co-activators.

Through its activity as an E3 ubiquitin ligase AhR is responsible for degradation of several proteins (Ohtake, Fujii-Kuriyama et al. 2009). The most well-studied example of AhR ubiquitin ligase activity is its interaction with the estrogen receptor α. Once it has bound to ligand and associated with ARNT, AhR can bind to ERα and recruit the cullin 4B complex (a complex of proteins that causes ubiquitination) which then ubiquitinates ERα (Ohtake, Baba et al. 2008). Ubiquitinated ERα is then degraded by proteasomes. Through a similar mechanism, AhR also acts as an E3 ubiquitin ligase which inactivates androgen receptor, β-catenin, and possibly ERβ (Kawajiri, Kobayashi et al. 2009; Ohtake, Fujii-Kuriyama et al. 2011).

The AhR can be negatively regulated by repression from AhRR (an AhR target gene) or by proteasomal degradation. AhRR has a similar structure to AhR but does not require ligand to heterodimerize with ARNT (Hahn, Allan et al. 2009). The AhRR/ ARNT complex enters the nucleus, binds to the XRE and attracts co-repressors, thus preventing transcription of target genes (Hahn, Allan et al. 2009). AhR can be degraded by proteasomes in certain cells after it is exported from the nucleus (Davarinos and Pollenz 1999).

Table 1: AhR targets and their functions

AhR-target protein	Function	Reference
Cyp1A1	Metabolism of different compounds, including PAHs	(Nebert, Dalton et al. 2004)
Cyp1A2	Metabolism of drugs, PAHs, N-heterocyclic amines and other compounds	(Nebert, Dalton et al. 2004)
Cyp1B1	Metabolism of drugs, PAHs, N-heterocyclic amines and other compounds	(Nebert, Dalton et al. 2004)
GST-Ya	Metabolism of xenobiotic compounds	(Pimental, Liang et al. 1993)
NADPH quinone reductase	Metabolism of xenobiotic compounds	(Favreau and Pickett 1991)
Aldehyde dehydrogenase	Metabolism of xenobiotic compounds	(Lindros, Oinonen et al. 1998)
UGT1A1	Metabolism of xenobiotic compounds	(Togawa, Shinkai et al. 2008)
Xanthine oxidase	Purine metabolism	(Sugihara, Kitamura et al. 2001)
ADP ribose polymerase	Purine metabolism	(Lin, Lin et al. 2007)
Adenosine deaminase	Purine metabolism	(Muralidhara, Matsumura et al. 1994)
BCRP or ABCG2	Drug transport	(Ebert, Seidel et al. 2005)
AhRR	Repression of AhR	(Baba, Mimura et al. 2001)
p27kip1	Cell cycle arrest	(Kolluri, Weiss et al. 1999)
Bax	Apoptosis	(Detmar, Rabaglino et al. 2006)
Fas	Apoptosis	(Park, Mitchell et al. 2005)

1.2.2 Ligands of AhR and their effects

As mentioned previously, ligands for AhR have a variety of chemical structures, however the most potent AhR ligands are environmental contaminants such as halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) (Vanden Heuvel and Lucier 1993). These chemicals can be produced by the burning of organic materials and can be found in cigarette smoke and grilled foods and are released into the environment by various industrial processes (Lioy and Greenberg 1990). A consequence of activation of AhR is induction of cytochrome P450 enzymes and increased metabolism of PAHs, such as benzo-a-pyrene, into nucleophilic derivatives which form covalent adducts with DNA, leading to mutations and cancer (Shimada and Fujii-Kuriyama 2004) (Conney 1982).

TCDD, AhR's most potent agonist, is an HAH and is considered highly toxic to humans. Most of what is known about the effects of TCDD in humans comes from studying instances of accidental exposure. During the Vietnam War the United States used Agent Orange as a defoliant which was later discovered to have contained TCDD as a contaminant (Schecter, Dai et al. 1995). Millions of Vietnamese people were exposed to Agent Orange and it has been reported to have caused birth defects, increased miscarriages, and chloroacne (Ngo, Taylor et al. 2006). Some studies suggest Agent Orange exposure also increases the incidence of cancer but results are inconclusive (Boffetta, Mundt et al. 2011). There was also a major incident in Seveso, Italy in 1976 where large amounts of TCDD were accidently released into the air by an industrial plant (Baccarelli, Pesatori et al. 2004). It was reported this exposure resulted in an increased

proportion of female births and possibly increased cancer rates; however the effects of this incident are still under investigation (Eskenazi, Mocarelli et al. 2003; Warner, Mocarelli et al. 2011). Another high-profile case of TCDD exposure was when the Ukrainian president Viktor Yushchenko was poisoned by the substance, resulting in the formation of chloroacne (Saurat, Kaya et al. 2012).

Many AhR ligands do not produce the strong adverse effects that the PAHs and HAHs do. There are also some endogenous compounds which can act as AhR ligands such as cAMP, kynurenine, 6-formylindolo[3,2-b]carbazole (FICZ) and bilirubin (Sinal and Bend 1997; Bergander, Wahlstrom et al. 2003; Oesch-Bartlomowicz, Huelster et al. 2005; Opitz, Litzenburger et al. 2011). There are some compounds found in cruciferous vegetables, such as quercetin and curcumin, which are thought to offer health benefits by affecting the metabolism of FICZ which then activates AhR (Heath-Pagliuso, Rogers et al. 1998; Mohammadi-Bardbori, Bengtsson et al. 2012).The full role that the endogenous AhR ligands play in normal physiological functions, however, is still under intense investigation.

1.2.3 AhR knockout mouse models

Although much is known about the role of AhR in mediating the toxic effects of environmental chemicals, its normal physiological roles are less understood. Mice lacking the AhR gene have been produced in order to elucidate the physiological functions of AhR. Three strains of AhR-null mice have been generated independently by three different labs, each by deleting different parts of the bHLH DNA-binding domain

(exons 1 and 2). The AhR -/- mice developed in the lab of Gonzales lack exon 1 of the gene and the mice only had a 50% survival rate immediately after birth but were fertile (Fernandez-Salguero, Pineau et al. 1995). These mice also had fewer lymphocytes in the spleen and lymph nodes, decreased liver size and fibrosis of the bile ducts (Fernandez-Salguero, Pineau et al. 1995). AhR -/- mice developed in the lab of Bradfield had exon 2 of the gene removed and showed no increased mortality, no changes in the number of lymphocytes, however they did have fibrosis and fatty build-up of the liver (Schmidt, Su et al. 1996). Finally, mice developed in the lab of Fujii-Kuriyama by removing exon 1 of AhR were seemingly healthy at birth but had reduced fertility and smaller seminal vesicles as they aged (Baba, Shima et al. 2008). All of the knockout mice appeared to develop cardiac hypertrophy, higher incidence of rectal prolapse and resistance to the toxicity of PAHs and HAHs (Shimizu, Nakatsuru et al. 2000). The phenotypes that develop when AhR is deleted in mice demonstrate that this receptor has an important role in development and normal physiological processes. Much still remains to be studied in the AhR-knockout mice and the reasons for the varying phenotypes of the different strains remain to be understood.

1.2.4 The role of AhR in the immune system

AhR is expressed in almost all types of immune cells and one of the most well-known functions of AhR is in the development and regulation of the immune system. Treatment with TCDD was long known to cause immunosuppressive effects, suggesting an important role for AhR in the immune system (Kerkvliet and Brauner 1990; Esser 1994; Moos and Kerkvliet 1995). Numerous studies on the role of AhR in the immune

system indicate it is important in the activation of regulatory T-cells (Treg) (Gandhi, Kumar et al. 2010). Treg cells are inhibitory cells which act as suppressors to other types of cells in the immune system in order to control immune response (Yuan and Malek 2012). Activation of Treg cells is the primary mechanism through which liganded AhR suppresses the immune system. It is not well understood how AhR activates Treg cells but some studies have shown that AhR induces expression of Foxp3, an important protein in certain types of Tregs (Marshall and Kerkvliet 2010). AhR is also known to induce differentiation of Th17 cells; immune cells that are normally found in the GI tract and skin (Marshall and Kerkvliet 2010). Different AhR ligands have differing effects on Th17 cells since TCDD was found to suppress Th17 cells while another AhR ligand, FICZ, can cause them to increase (Quintana, Basso et al. 2008). Also, through AhR, PAHs are known to induce apoptosis in monocytic cells, as well as inhibit their differentiation into macrophages (Shin, Bae et al. 2000; van Grevenynghe, Rion et al. 2003). The ability of AhR to affect different types of immune cells and the fact that its different ligands have varying effects on these cells, demonstrate the complexities of AhR signaling.

AhR is needed for differentiation of hematopoietic stem cells (HSCs) and is down-regulated during HSC proliferation (Singh, Wyman et al. 2009). Treatment of bone marrow cells with TCDD causes a decrease in lymphoid progenitors and an increase in myeloid progenitors with an overall decrease in immature-bone marrow cell growth (Singh, Wyman et al. 2009). In the bone marrow of AhR knockout mice there is an altered HSC profile with a greater number of proliferating immature cells (Singh, Garrett et al. 2011). TCDD was shown to inhibit HSC migration *in vivo* and also alter some of

the genes important in HSC development and function (Casado, Singh et al. 2011). However, the molecular mechanisms by which AhR controls HSC differentiation remain to be fully understood.

1.3 Estrogen receptor β

1.3.1 ERβ structure and mechanism of action

ERβ is one of two the estrogen receptors which are members of the nuclear receptor superfamily. ERα was discovered by Elwood Jensen in 1950s and 45 years later ERβ was discovered by Jan-Åke Gustafsson (Kuiper, Enmark et al. 1996). Until the discovery of ERβ, it was thought that estradiol exerted all of its effects through a single estrogen receptor. We now know that ERα and ERβ mediate different and often opposing functions of estradiol.

The ERβ gene is located on chromosome 14q in humans and encodes a full length protein of 530 amino acids with a molecular weight of 59 kDa (Enmark, Pelto-Huikko et al. 1997). ERα is encoded by a different gene located on chromosome 6q. It is composed of 595 amino acids with a molecular weight of 67kDa (Menasce, White et al. 1993). In humans, there are several splice variants of ERβ (ERβ2, ERβ4, and ERβ5) which do not bind to estradiol but can form heterodimers with ERβ and ERα (Leung, Mak et al. 2006). ERβ2 is of particular interest because of its possible role in the progression of certain cancers and has been subject to growing interest in recent years (Taylor, Martin-Hirsch et al. 2010). Mice possess a different ERβ splice variant from humans, known as ERβ-ins

(or mERβ2), which creates difficulties in studying these splice variants using animal models (Zhao, Toresson et al. 2005).

ERβ and ERα have a similar structure which they share with all members of the nuclear receptor superfamily (figure 2). The AF-1 (activation function) domain at the N-terminus is responsible for transcriptional activation in ERα but it is much shorter in ERβ and is not thought to be important in its transcriptional activity (Nilsson, Makela et al. 2001). The DNA binding domain is the most conserved domain between ERα and β, and is responsible for the receptor's binding to DNA (Enmark, Pelto-Huikko et al. 1997) (Ogawa, Inoue et al. 1998). The ligand binding domain is located on the C-terminus of ERβ and is responsible for the binding of various agonists and antagonists to the receptor (Ogawa, Inoue et al. 1998; Katzenellenbogen, Choi et al. 2000). The C-terminus also has a small AF-2 domain which is important in transcriptional activation. Upon ligand binding to ERβ, there is a conformational change in the ligand binding domain which exposes a region of the AF-2 to allow co-activators to bind (Brzozowski, Pike et al. 1997). Selective estrogen receptor modulators (SERMs), can have tissue/cell selective effects depending on the co-activator expression profiles of the cells (Barkhem, Carlsson et al. 1998). The binding of some antagonists completely block the binding of co-activators to ER, inhibiting the receptor from activating its target genes (Pike, Brzozowski et al. 2001). There is one ER antagonist known as ICI which stimulates degradation of the receptor.

Several ERβ ligands have been identified: some are synthesized in the body, some are plant derived and some have been synthesized by chemists. The most well-known agonist of both estrogen receptors is 17β-estradiol (E2). E2 is a steroid hormone derived from testosterone and is synthesized in the ovaries and (in smaller amounts) in the testes. Estrone and estriol, the metabolites of E2, are also known to bind to the estrogen receptors but are very weak agonists (Kuiper, Carlsson et al. 1997). 5α-Androstane-3β, 17β-diol (3β-adiol), a metabolite of dihydrotestosterone (DHT), is also known to be an agonist of ERβ (Lund, Hinds et al. 2006; Sugiyama, Andersson et al. 2009). The physiological roles of 3β-adiol are still under investigation and are of particular interest because of its higher affinity for ERβ than ERα (Pak, Chung et al. 2005). Phytoestrogens are estrogens produced by plants. They are abundant in the diet and can be agonists or antagonists of ER (Moutsatsou 2007). Synthetic estrogens of very diverse chemical structures have been synthesized by chemists. They can be agonists, antagonists or mixed agonists/ antagonists (SERM) in different tissues (Barkhem, Carlsson et al. 1998; Katzenellenbogen, Choi et al. 2000). Pharmaceutical companies have synthesized ERβ-selective ligands and tested for their effects in diseases such as cholangiocarcinoma, rheumatoid arthritis (Follettie, Pinard et al. 2006), and prostatic disease (McPherson, Hussain et al. 2010; Nilsson and Gustafsson 2011).

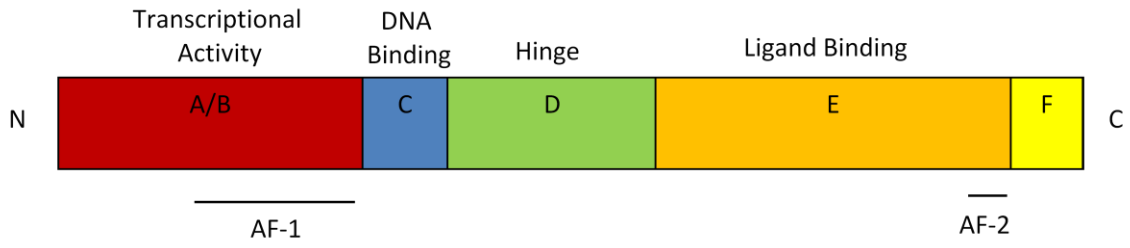


Figure 2. The basic structure of estrogen receptor β. The N-terminus of ERβ consists of the A/B domain which contains the AF-1 domain which is involved in transcriptional activity in those nuclear receptors with long N-terminal domains but is not active in ERβ. The C- domain is involved in the binding of the receptor to DNA. The D domain, or the hinge region, separates the DNA binding domain and the ligand-binding domain. The E domain is responsible for ligand binding and contains the AF-2 domain which is important in transcriptional activation. The F domain on the C-terminus is involved in ligand recognition and also binding of co-activators/ co-repressors.

There are several distinct mechanisms through which ERβ can exert it effects on transcription. The most well-known mechanism is direct transcription which involves dimerization of ERβ molecules following ligand binding. The ERβ homodimer then binds to the estrogen response element (ERE) on the DNA, recruits co-activators and promotes transcription of estrogen target genes (Pace, Taylor et al. 1997). In an alternate pathway, ERβ is tethered to other transcription factors, such as Fos/Jun (AP-1) and SP-1, which then bind to their own specific response elements (Kushner, Agard et al. 2000; Safe 2001). ERβ can be activated in the absence of ligands by phosphorylation (Joel, Traish et al. 1998). ERβ signaling can also occur through a lesser known, rapid, non-genomic mechanism in which ligand binding causes it to interact with other proteins in the cell. These rapid effects of ERβ have been known to lead to changes in the cell such as increasing nitric oxide levels or activating kinases (Song and Santen 2006; Warner and Gustafsson 2006). Many of the non-direct ERβ signaling pathways are still under investigation and remain to be fully understood.

1.3.2 The role of ERβ in physiology and disease

ERβ is widely expressed in most tissues across the body including the immune system, prostate, mammary gland, adipose tissue, cardiovascular system, digestive system, central nervous system, skeletal and smooth muscle, white and brown adipose tissue, lung, ovary, and uterus (Kuiper, Carlsson et al. 1997; Nilsson, Makela et al. 2001). ERβ is not expressed in the heart. ERβ is important in the development and physiology of these tissues (Nilsson, Makela et al. 2001; Heldring, Pike et al. 2007; Sugiyama, Barros et al. 2010). The different transcriptional pathways used by ERβ together with its

expression in many tissues each with different patterns of co activators and co repressors mean that there are tissue specific actions of ERβ. ERβ knockout mice have aided greatly in the understanding of ERβ functions. Some of the most unexpected characteristics of ERβ -/- mice are infertility of females (Weihua, Saji et al. 2000), deafness (Simonoska, Stenberg et al. 2009), hypertension (Forster, Kietz et al. 2004), myeloid leukemia (Shim, Wang et al. 2003), and hyperplasia in the epithelium of ventral prostate (Warner, Nilsson et al. 1999).

1.3.2.1 ERβ in the breast

Estrogen signaling through ERα and ERβ is important in both physiological and pathological changes in the breast. ERβ is abundant in normal, resting breast epithelium where it has an anti-proliferative function (Saji, Jensen et al. 2000). In ERβ knockout mice there is increased proliferation and decreased expression of adhesion molecules in the mammary gland epithelium (Forster, Makela et al. 2002). During development and progression of breast cancer, ERβ expression is usually decreased or absent from the epithelium while ERα expression increases (Roger, Sahla et al. 2001; Bardin, Boulle et al. 2004). ERα is known to mediate the proliferative effects of E2 in the breast epithelium and its presence in breast cancer is the factor which determines the type of pharmacological intervention which will be used to treat the disease (Maehle, Collett et al. 2009). Common breast cancer treatments involve the blocking of ERα activation by inhibition of estrogen synthesis (aromatase inhibitors) or selective antagonism of ERα in the breast with the SERMS, tamoxifen or raloxifen (Shanle and Xu 2010). *In vitro* studies have demonstrated an anti-proliferative and anti-metastatic role for ERβ in breast cancer

cell-lines (Lazennec, Bresson et al. 2001; Sotoca, van den Berg et al. 2008). Interestingly, ERβ2 is expressed in breast cancer where it seems to be correlated with poor prognosis. The mechanism behind this correlation is still under investigation (Esslimani-Sahla, Kramar et al. 2005) (Dey, Jonsson et al. 2012).

1.3.2.2 ERβ in the immune system

ERβ is highly expressed in immune cells and is an important regulator of the immune system (Koehler, Helguero et al. 2005). Studies using ERβ knockout mice have shown that these mice develop a severe myeloproliferative disorder similar to chronic myeloid leukemia (Shim, Wang et al. 2003). ERβ is required to help control the immune system by decreasing proliferation and regulating differentiation of hematopoietic progenitor cells. ERβ also has an anti-inflammatory function in microglia of the brain and in Th17 T cells (Saijo, Collier et al. 2011). Treatment with ERβ agonists, such as 3β-adiol, represses genes which lead to pro-inflammatory responses and can slow disease progression in mouse models of multiple sclerosis (Tiwari-Woodruff and Voskuhl 2009; Saijo, Collier et al. 2011). Several other studies on ERβ agonists *in vivo* and *in vitro* have demonstrated anti-inflammatory activity of ERβ in macrophages and leukocytes (Stygar, Masironi et al. 2007; Subramanian and Shaha 2009). From its profile of actions in the immune system, there is hope that ERβ-selective ligands could prove to be useful therapeutics in the treatment of autoimmune diseases.

1.3.2.3 ERβ in the central nervous system

ERβ has important functions in the development and function of the central nervous system. ERβ is first expressed in most the rodent brain at embryonic day 12.5 (E12.5) and becomes important in neuronal migration beginning at E14.5 (Wang, Andersson et al. 2003). Since estradiol is not present in the brain at this early stage, there may be another ERβ ligand present in the neonatal brain (3β-adiol is a possibility) or the receptor may be activated in a non-ligand dependent fashion such as by phosphorylation. ERβ expression increases in the fetal brain up to day E18.5, when it is expressed in most areas, and continues to be highly expressed until postnatal day 7 (P7) (Sugiyama, Andersson et al. 2009). Expression of ERβ declines after P7 and it is generally only detected in the hypothalamic area and the dorsal raphe during adult life (Sugiyama, Andersson et al. 2009). Studies using ERβ knockout mice have revealed roles for ERβ in controlling aggression, anxiety and feeding behavior (Ogawa, Lubahn et al. 1997; Nomura, Durbak et al. 2002). Ongoing studies using mouse models have also highlighted ERβ-selective ligands as a possible treatment for depression (Hughes, Liu et al. 2008).

1.3.2.4 Prostatic diseases and the role ERβ

The prostate is a small, walnut-shaped organ located near the bladder and surrounding the urethra in males. In humans, the prostate secretes alkaline fluid into the semen which is thought to help sperm survival; however, the functions of the prostate are not fully understood. Cancer of the prostate is the second leading cause of cancer deaths in men in the United States with an estimated 241,740 new diagnoses and 28,170 deaths

in 2012 (Siegel, Naishadham et al. 2012).The mean age for diagnosis of prostate cancer in the U.S. is 67 and the disease rarely occurs in men under 50 (Siegel, DeSantis et al. 2012). Despite the large number of men affected by prostate cancer, improved methods for early detection and surgery have led to a relatively high survival rate. Androgen receptor (AR) is key to normal prostatic development as well as prostate cancer (Tamburrino, Salvianti et al. 2012). The endogenous androgens, testosterone and dihydrotestosterone (DHT), bind to AR in the prostate and cause both proliferation and differentiation. The blocking of AR and androgen ablation represent the first line of treatment of prostate cancer (Hamilton and Freedland 2011). Radical prostatectomy, or surgical removal of the prostate, is another treatment of more advanced prostate cancer and is performed in order to prevent malignant cells from metastasizing (Fradet 2012). However, androgen ablation inevitably leads to a much more dangerous form of the disease (castration-resistant prostate cancer) that continues to grow in the absence androgens. There is no effective treatment for castrate-resistant prostate cancer (Loriot, Zoubeidi et al. 2012). New and more effective ways of treating prostate cancer are under intense investigation.

Another very common disease of the prostate is benign prostatic hyperplasia (BPH). BPH is a non-malignant growth of the prostate caused by proliferation of the prostatic epithelial and stromal cells and occurs in the majority of men over 50 (Roehrborn 2008). Due to their anatomical position, enlarged prostates often cause pressure on the urinary bladder and constriction of the urethra which make urination urges more frequent and urination itself more difficult. Drugs which block the α1-

adrenergic receptor and cause the prostatic muscles to relax are the most common treatment for BPH (Lepor 2011). BPH can also be treated with 5α-reductase inhibitors in order to block the synthesis of DHT and decrease proliferation in the prostate (Andriole, Bruchovsky et al. 2004).

ERβ is also an important nuclear receptor in both prostatic development and disease and studies in recent years have demonstrated ERβ's importance in BPH and prostate cancer (Ellem and Risbridger 2009; Hartman, Strom et al. 2012). ERβ is the primary estrogen receptor expressed in the prostate and is normally present in high levels (Heldring, Pike et al. 2007). ERβ is thought to have an anti-proliferative and pro-apoptotic function in the prostate since mice lacking the receptor suffer from prostatic hyperplasia (Imamov, Morani et al. 2004). Prostate cancer cell lines have decreased proliferation and increased apoptosis when they are expressing ERβ (Dey, Jonsson et al. 2012). Also, ERβ levels in clinical prostate cancer samples decrease with increasing Gleason grade and ERβ is usually absent from prostate cancer cells in culture (Horvath, Henshall et al. 2001; Bardin, Boulle et al. 2004). Due to ERβ's growth-inhibitory function in the prostate, selective ERβ ligands may be useful pharmaceuticals for the treatment of prostate cancer and BPH in the future.

1.4 Human diseases related to elevated uric acid

When cells die, their DNA needs to be broken down by various enzymes in the liver to be reutilized or excreted. Purines, including adenine and guanine, are broken down through a complex process involving many enzymes into uric acid in man and

allantoin in mice (Moriwaki, Yamamoto et al. 1999). The lack of the enzyme responsible for the synthesis of allantoin, urate oxidase (uricase), in humans means that the end product of purine metabolism is uric acid (Moriwaki, Yamamoto et al. 1999). Uric acid can cause disease if its levels are elevated.

Urinary bladder stones can be very painful and are usually composed of uric acid, calcium or magnesium ammonium phosphate. Bladder stones can sometimes develop if the urine is too concentrated, often caused by BPH preventing the bladder from voiding completely (Philippou, Moraitis et al. 2012). A disorder in the nervous system known as neurogenic bladder, where the bladder cannot void completely, can also lead to the development of stones (Stein, Schroder et al. 2012). Diet can also play a role in the development of urinary stones with dehydration, vitamin deficiency and high oxalate consumption to be considered risk factors (Philippou, Moraitis et al. 2012). Other risk factors include low urine pH, the introduction of foreign bodies into the bladder, and infections; all of which can cause urine solutes to precipitate and form stones (Philippou, Moraitis et al. 2012). An increase in uric acid throughout the entire body is also a risk factor for urinary bladder stones and there are several medical conditions in which this occurs.

Gout is a painful disease caused when very high levels of uric acid in the body (hyperuricemia) precipitate as crystals in the joints. This disease is normally caused by the ingestion of too many “rich foods” containing high levels of purines which eventually leads to very high levels of uric acid in the body (Eggebeen 2007). Other risk factors for gout include: high alcohol consumption, obesity, enzymatic defects in purine metabolism,

recent surgery and diuretic therapy (Eggebeen 2007). Mutations or deficiencies in the uric acid transporters in the kidneys can cause alterations in blood and urine uric acid levels which can also lead to gout (Schlesinger 2004). People suffering from gout can also develop urate stones in their kidneys and urinary bladder (Hasegawa, Fuller et al. 2012). Treatment of gout and hyperuricemia in general usually involve drugs that can inhibit xanthine oxidase; an enzyme in the purine degradation pathway which catalyzes conversion of xanthine to urate (Burns and Wortmann 2011). Another common treatment for gout is the use of recombinant urate oxidase, to convert uric acid to allantoin (Garay, El-Gewely et al. 2012).

Lesch-Nyhan disease is another disorder caused by elevated levels of uric acid in the body. A mutation in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene causes this disease to occur. HPRT is an important enzyme in the purine degradation pathway and without it, uric acid levels will become elevated (Nyhan, O'Neill et al. 1993). Hyperuricemia is so severe in Lesch-Nyhan patients that they develop severe gout as well as neurological disorders which result in severe mental retardation and self-mutilating behavior (Torres and Puig 2007). There is no effective treatment for this disease which develops in the early years of life and patients succumb in their teens or twenties (Nyhan, O'Neill et al. 1993).

Tumor lysis syndrome occurs in certain types of cancer when large numbers of cells die in a short period of time, releasing their products into the body. This condition usually occurs in hematologic malignancies such as leukemia but can also occur when a large number of cells die in a solid tumor (Firwana, Hasan et al. 2012). The various

components of the dying cells are released into the body, including purines, which are broken down into uric acid (Sirelkhatim, Sejnova et al. 2008). Acute hyperuricemia caused by tumor lysis syndrome can cause stones to form in the kidney and urinary bladder and may even lead to organ failure (Ribeiro and Pui 2003). Treatment for hyperuricemia in tumor lysis syndrome is similar to treatment of gout (Ribeiro and Pui 2003).

1.5 Chronic myeloid leukemia

Leukemia is a malignancy of immune cells in the blood or bone marrow. In the US, there were an estimated 47,150 new cases and 23,540 deaths from all types of leukemia for 2012 (Siegel, Naishadham et al. 2012). There are several different types of leukemia which are classified according to the type of cells affected and which way they are affected. Leukemia is usually classified as acute: referring to the build-up of more immature cells of a certain type, or chronic: the increase in number of more mature cells. The four most common types of leukemia are acute lymphocytic, chronic lymphocytic, acute myeloid (AML), and chronic myeloid (CML).

Both acute and chronic myeloid (or myelogenous) leukemia are caused by abnormal growth of the myeloid cells. Myeloid cells are any cells which are derived from a myeloid precursor and include different types of granulocytes, erythrocytes, megakaryocytes, macrophages and mast cells. In 2012, approximately 5,430 new cases of CML were diagnosed and 610 deaths resulted from the disease in the United States (Siegel, Naishadham et al. 2012). The first stage of CML usually consists of the chronic

stage which can last several years and is characterized by the increase in normal myeloid progenitor cells (Maru 2012). The accelerated phase of CML follows and is a more severe increase in myeloid cells in the bone marrow and throughout the body (Faderl, Kantarjian et al. 1999). The blast crisis (or acute stage) is the final stage of CML in which a large number of myeloid progenitors proliferate with abnormal differentiation; this stage usually has a very low survival rate (Faderl, Talpaz et al. 1999). Symptoms of CML include abdominal pain caused by splenomegaly, anemia, joint pain, and increased susceptibility to infections (Jabbour and Kantarjian 2012). CML accounts for approximately 15% of leukemia cases and is most common in middle-aged to older-aged patients with more cases occurring in men (Faderl, Talpaz et al. 1999).

CML is usually caused by mutation known as the Philadelphia chromosome which leads to the fusion of the BCR and ABL genes (Heisterkamp, Stephenson et al. 1983). The BCR-ABL protein is a constitutively active protein kinase which causes increased proliferation in myeloid progenitor cells, leading to CML. The Philadelphia chromosome mutation is present in approximately 90% of CML cases but can also be present in other forms of leukemia such as acute lymphocytic and AML (Talpaz, Shah et al. 2006; Reddy and Aggarwal 2012). Tyrosine kinase inhibitors, such as Imatinib, Nilotinib, Desatinib, and Ponatinib, have proven to be successful in treating CML with 5-year survival rates close to 90% (Reddy and Aggarwal 2012). ERβ also has a potential role in CML as evidenced by a CML-like pathology in ERβ knockout mice (Shim, Wang et al. 2003).

1.6 Menopause and hormone replacement therapy

Menopause refers to the period of a woman's life when ovulation permanently ceases and reproduction is no longer possible (Nelson 2008). The time of onset of menopause can vary greatly in different individuals but usually occurs in women in their mid-40s to early-50s. The unpleasant symptoms of menopause can continue for an average of about 4 years (Burger, Dudley et al. 2002). These symptoms of menopause are caused by the lack of estrogen (Nelson 2008). Since estrogen is important in the regulation of body temperature, a reduction of estrogen during and after menopause leads frequent "hot flashes" (Santen, Allred et al. 2010). Estrogen also has an important role in bone remodeling, therefore, osteoporosis (a disease in which bone mass is reduced) is a common problem for women after menopause. Other symptoms of menopause include cardiovascular disease, depression, vaginal dryness, and sleep disorders (Freeman, Sammel et al. 2007). The symptoms of menopause can significantly affect the quality of life of women, leading to a great deal of interest in treatments for menopausal symptoms.

The most common treatment for postmenopausal symptoms is known as hormone replacement therapy (HRT). Since most postmenopausal symptoms occur due to a lack of estrogen in the body, treatment of women with estrogen is known to significantly decrease these symptoms (Freedman 2002). Progesterone has been commonly used in combination with estrogen therapy in order to decrease the risk of uterine cancer (since progesterone inhibits the proliferative effects of estrogen in the uterus). In 2002, a controversial study by the Women's Health Initiative (WHI) came to the conclusion that estrogen and progesterone combination therapy increased the risk of breast cancer

(Rossouw, Anderson et al. 2002; Chlebowski, Hendrix et al. 2003; McTiernan, Martin et al. 2005). A year later, the "Million Women Study" published similar results (Beral 2003). Since these studies were published, there has been a significant decrease in HRT across the world (Verkooijen, Bouchardy et al. 2009). The value of these studies has been forcefully debated, because of questionable analysis of data and choice of subjects for the study. Many endocrinologists have argued that breast cancer is not significantly increased by HRT (Gompel, Rozenberg et al. 2008; Pines 2008; Bluming and Tavris 2009). While much remains to be investigated regarding HRT and its side effects in the clinical setting, much more information is needed to understand the hormonal regulation in the normal postmenopausal breast.

As was discussed in section 1.3.2.1, ERα is the estrogen receptor which modulates the pro-proliferative actions of E2 and is often elevated in breast cancer (Roger, Sahla et al. 2001; Bardin, Boulle et al. 2004). ERβ is usually at high levels in the normal breast and is responsible for the anti-proliferative actions of E2 (Bardin, Boulle et al. 2004). Since estrogen signaling is modulated through these two estrogen receptors, there needs to be a better understanding of the mechanism through which HRT increases breast cancer risk (if any). This need was what prompted our studies on the effects of HRT on ERα and ERβ, the progesterone receptors (PRA and PRB) and their involvement in the reported increased breast cancer risk.

1.7 Dissertation rationale and aims

Many common human diseases are a result of abnormalities in signaling of nuclear receptors. These include: cancer of the prostate and breast, rickets, Addison's disease, thyroiditis, and goiters (Betterle and Morlin 2011; Brent 2012; Yoshida and Stern 2012). Most of these diseases stem from the receptors for the well known steroid hormones and vitamin D. Diseases associated with the more recently discovered receptors and their ligands are just beginning to be unveiled and these revelations are likely to shed more light on diseases whose etiologies are still mysterious. The overall aim of the studies in this dissertation was to investigate cross talk between AhR and ERβ and whether AhR influences the physiological function of ERβ and/or contributes to development of pathologies. Our organs of choice were the prostate, breast and immune system; all of which express both ERβ and AhR.

The aryl hydrocarbon receptor is well known for being a transcription factor activated by numerous environmental contaminants such as HAHs and PAHs. The toxic effects of the more potent AhR ligands have been investigated and debated for decades. Human exposure to chemicals such as TCDD and benzo-a-pyrene is considered dangerous and has been linked to cancer, immunosuppression, and birth defects (Shimada and Fujii-Kuriyama 2004; Ngo, Taylor et al. 2006). Some of the dangerous effects of these chemicals have been attributed to their activation of AhR and, in some cases, also to increases in AhR-mediated induction of oxidative enzymes which convert inactive chemicals to their genotoxic metabolites. However, many other compounds such as indoles, cAMP, bilirubin and others which are present endogenously or in the diet can

activate AhR but do not elicit the same toxic effects as PAHs. During the past 2 decades, researchers have used knockout mouse models to help better understand the role of AhR in normal physiology. While much has been learned about the roles of AhR, much remains to be understood. Since AhR is known to interact with ERα and modulate estrogen signaling, we were particularly interested in the interaction between AhR and ERβ.

The molecular mechanisms through which HRT causes changes in the breast, leading to the increased cancer risk remains to be investigated. Observations of changes in the expression of various nuclear receptors important in regulating breast proliferation (ERα, ERβ, PRA, PRB) would be required to understand this mechanism. Breast biopsy samples are necessary to measure changes in the nuclear receptors in the breast following HRT as well as to determine which cells and in what pattern the cells are proliferating. Obtaining breast biopsies from healthy women is difficult since most people do not volunteer to undergo a painful procedure when it is not necessary. Therefore, very few studies have been able to study the effects of HRT on the histology of the normal breast, expression of nuclear receptors or proliferation. The aim of this study was to use breast biopsy from women undergoing HRT to investigate its effects on the histology and nuclear receptor expression profiles, breast density and proliferation.

The questions we planned to address in these studies were: (1) whether AhR was involved in the development of prostatic disease; (2) whether AhR influenced development of diseases of the immune system or disrupted estrogen signaling in the

immune system; and (3) whether hormone replacement therapy affected expression of nuclear receptors such as ERβ or AhR in the postmenopausal breast.

2. Materials and Methods

2.1 Mouse models

AhR-deficient mice were generated in the laboratory of Dr. Fujii-Kuriyama (Tokyo) by using a homologous recombination as previously described (Shimizu, Nakatsuru et al. 2000). Briefly, Male AhR (−/−) mice were back-crossed to C57BL/6J AhR (+/+) females to give rise to heterozygotes. The AhR (+/−) mice were interbred to yield AhR (+/+), AhR (+/−), and AhR (−/−) mice. Among 100 offspring obtained from heterozygous matings, the relative frequencies of AhR (+/+), AhR (+/−), and AhR (−/−) mice were approximately 1:2:1, as expected from Mendelian law. Mice were shipped to the Huddinge animal facility at the Karolinska Institute (Sweden) and University of Houston (Houston, TX) and cleansed into the system. Heterozygous littermates were used as controls when wild-type mice were not available (AhR +/- mice are phenotypically similar to wild-type mice).

ERβ knockout mice were generated by replacing exon 3 of the ERβ gene with a copy of the neomycin-resistance gene as previously described (Krege, Hodgin et al. 1998).

2.2 Human clinical studies

Sixty apparently healthy postmenopausal women, with at least one year since last menstruation, FSH levels above 30 IU/ml and at least 12 months since taking HT, volunteered for the study. They were between 48 to 72 (61.9±5.5) years of age and their years since menopause (YSM) were 1-22 (11.9±5.5). The women received written and

verbal information on the purpose and procedures of the study, and informed consent was obtained. The women were computer-randomized into two groups, group A, daily oral intake 1 mg estradiol (E2) and group B, daily oral intake 1 mg estradiol (E2) and 0.5 mg norethindrone acetate (NA). Both drugs are marketed by Novo Nordisk Scandinavia AB, Sweden. The duration of treatment was three months. BL recruited the volunteers. The information which she received was blinded for all the other authors until the analysis was complete.

Before and after the three-month treatment with E2 or E2/NA the women had a routine medical examination involving blood pressure measurement, analyses of circulating lipoproteins, kidney, liver, and thyroid function as well as hematological status. A gynecological examination including Pap smear and vaginal ultrasound with measurements of endometrial thickness and uterine size was also performed.

Mammography and a middle needle biopsy of breast tissue were conducted using ultrasound to identify glandular tissue as previously described (Cheng, Wilczek et al. 2007). Brigitte Wilczek and colleague performed all the mammographies, ultrasound recordings, and biopsies, by noting and marking where the first biopsy was obtained and then obtaining the second biopsy at the same location. The size of the biopsy was always 14 Gauche with a Bard biopsy-gun.

Weekly records of vasomotor symptoms were collected. The women registered number of hot flashes and night sweats per day as well as intensity of these vasomotor symptoms once weekly before and during treatment. Intensity of the climacteric

symptoms was scored by the women on a five-step self-rating scale adapted from the climacteric symptoms rating scale developed by Collins and Landgren (Collins and Landgren 1997). During the study, the women were encouraged to lead normal lives with no changes in dietary habits, alcohol consumption, or physical activity.

Blood sampling for lipoproteins was performed after one night of fasting. Lipoprotein levels were measured at the Biochemical Central Laboratory of Karolinska University Hospital Huddinge using standard methods.

Mammograms of the mediolateral oblique projection and cranio-caudal projection of both the right and left breast were obtained. Women were examined at baseline and after 3 months of treatment. Mammographic density of all films was classified according to Wolfe[26] into four categories: N1: breast with parenchyma composed primarily of fat with at most a few fibrous connective tissue strands. P1: Breast with a prominent ductal pattern in up to one-quarter of the breast volume. P2: Breast with a prominent ductal pattern in more than one quarter of the volume. DY: Breast with an extremely dense parenchyma, which usually denotes connective tissue hyperplasia.

In addition to the Wolfe classification, for each individual woman, all films were classified according to a percentage scale with 5 categories of the amount of dense breast parenchyma in relation to the whole breast volume. The five categories were: 1 (0-20%), 2 (21-40%), 3 (41-60%), 4 (61-80%), 5 (81-100%). In addition, more discrete differences were recorded as approximately 10%. The films were analyzed by an independent radiologist specialized in mammography and blinded to treatments and were

interpreted on two different occasions with different distribution of women. The results of both viewings were similar.

2.3 Tissue preparation

All mice used in these studies were euthanized in a chamber filled with CO2 before their tissues were harvested. Internal organs were examined. All tissues to be used for histological examination were removed, placed in 4% buffered paraformaldehyde overnight and thereafter switched to 70% ethanol. They were then dehydrated in graded ethanol concentrations using a Thermo Tissue Processor Excelsior ES and paraffin embedded using a Thermo Tissue Embedder. 5 μm-thick sections were cut using a Thermo microtome 355S and placed on glass slides (Tru Scientific) at 40° C, overnight, to allow the tissue to adhere before histological examination.

Bones harvested from mice for histological examination were first decalcified in EDTA buffer before tissue processing and embedding. First the fixed bone was washed three times in PBS for 30 min, followed by three washes in D.I. water for 30 min and then added to 5% EDTA buffer pH 6.5 with agitation. Decalcification time depended in the size of the bone. If the bone was not decalcified after 3 days, the buffer was replaced and the bone was left until it was fully decalcified. The bone was then washed 3 times in D.I. water for 30 min and processed and embedded using the same procedure as for other tissues.

Tissues that were to be used for Western blot or qPCR analysis were removed and flash-frozen in liquid nitrogen before storage at -80° C.

2.4 Histological techniques

2.4.1 Immunohistochemistry

Paraffin-embedded sections were de-waxed in xylene followed by rehydration in graded concentrations of ethanol. Antigen retrieval was achieved by placing slides in 97° C, 10 mM citrate buffer (pH 6.0) for 5-10 min. Endogenous peroxidase was quenched by a 30 min incubation in 1% H_2O_2 in 50% methanol followed by blocking of unspecific protein binding with 3% BSA for 10 min. Sections were incubated with their primary antibody. Corresponding HRP polymer solution (Bio care medical, Concord, CA) was added for 30 min at room temperature. The slides were developed using the DAB method (Dako, Denmark) and counterstained with hemotoxylin. Tunel staining was done using the *in situ* cell death detection kit, fluorescein from Roche (Carpinteria, CA).

Primary antibodies used for ERα (1D5), AR (clone AR441) and Ki67 (Mib-1, M7240) were from DAKO, Denmark. Monoclonal antibodies against PR (PGR-312) and PR-B (clone san27) were obtained from Novocastra, UK. The polyclonal rabbit anti-human HER 2 (neu) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Chicken anti-human ERβ polyclonal antibody (IgY) and sheep anti-human ERβcx were made in our lab. Dilutions of ERβ, ERβcx, PR-312, PR-B and Ki67 antibodies were 1:100; dilution of the HER 2 antibody was 1:200; dilutions of ERα and AR antibodies were 1:30. Rabbit anti-E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) dilution was 1:200; rat anti-F4/80 (BD Pharmingen, San Diego, CA) dilution was 1:50; rabbit

anti-pSmad2/3 (Millipore, Billerica, MA) used was 1:2000; rabbit anti-myeloperoxidase (Abcam, Cambridge, MA) dilution was 1:200.

2.4.2 Hematoxylin and eosin staining

Hematoxylin and eosin staining was performed by incubating deparaffinized and rehydrated tissue sections for 5 s in eosin solution and 1 min in Mayers hematoxylin solution (Sigma-Aldrich, St. Louis, MO).

2.4.3 Grocott's methenamine silver stain

Slides were deparaffinized, rehydrated, and then placed in periodic acid for 5 min. They were then incubated in methenamine silver solution for 50 min at 60° C and rinsed in D.I. water. Slides were then placed in gold chloride for 1 min, rinsed in D.I. water, placed in sodium thiosulfate for 1 min, rinsed, placed in fast green solution for 1 min, dehydrated, and mounted.

2.4.4 Masson's trichrome stain

All reagents for Masson's trichrome stain were purchased from electron microscopy sciences and the protocol used was suggested by the company. Slides were deparaffinzed, rehydrated and placed in Bouin's fixative at 56° C for 1 h. Slides were then rinsed in D.I. water and placed in Weigert's iron hematoxylin solution for 5 min, then rinsed in running water for 10 min. Slides were placed in Biebrich scarlet-acid fuchsin for 15 min and then rinsed. Then slides were added to phosphomolybdate-phosphotungstic acid for 15 min and stained with aniline blue solution for 15 min. After rinsing in D.I.

water, the staining was developed by incubating in 1 % acetic acid for 5 min. The slides were then rehydrated and mounted.

2.4.5 Choroacetate esterase stain

The kit for chloroacetate esterase staining from Sigma was used and the protocol included in this kit was used. Slides were deparaffinized, placed in 100 % ethanol and then allowed to dry. Slides were then placed in a staining solution which contains 2 ml sodium nitrate, 2 ml fast red violet, 80 ml of D.I. water, 10 ml trizmal buffer, and 2 ml naphthol AS-D chloroacetate solution at 37° C for 15 min. Slides were then rinsed with DI water, places in Gill's hematoxylin for 2 min, rinsed again and mounted.

2.4.6 BrdU staining

Mice to be stained with BrdU were first injected with 100 mg/kg of BrdU in sterile PBS subcutaneously, 3 times in 12-h intervals before sacrifice. Tissues were routinely fixed and processed for histology as previously described. Slides were placed in 97° C citrate buffer for 15 min and then placed in 2 M HCl for 25 min at room temperature. Slides were then washed in PBS for 30 min and placed in 0.1 M borate buffer (pH 9.0) for 5 min. Slides were washed in PBS, placed in 0.5% triton x-100 for 10 min and washed again in PBS. Endogenous peroxidase was blocked by incubation in 1% H2O2 for 30 min and then washing in PBS. The mouse primary antibody to BrdU (BD Pharmingen, San Diego) was diluted in 3% BSA in PBS and incubated overnight at room temperature. The slides were then treated and developed for color with the DAB method as described in section 2.4.1.

2.4.7 Transmission electron microscopy

Bladder tissues used for transmission electron microscopy were fixed in 2% glutaraldehyde in 80 mM sodium cacodylate buffer at 330 mOsm/kg and given to Dr. Alan Burns at the University of Houston, Optometry department. Dr. Burns' lab performed the tissue processing and provided the images.

2.5 Real-time qPCR

RNA was extracted from homogenized kidney and liver tissues from six, 6-month-old wild-type and AhR -/- mice (n=6) using the Qiagen (Valencia, CA) RNeasy mini kit followed by cDNA synthesis using Invitrogen's (Carlsbad, CA) SuperScript II RT method. Real time qPCR was performed using Sybr Green for detection with an Applied Biosystems (Carlsbad, CA) 7500 fast qPCR machine and each sample was plated in triplicate. Primers used in this study are given in (Table 2).

2.6 Western blot

Homogenized tissue protein concentration was measured with a Thermo Nanodrop. Equal protein amounts were loaded onto a 4-20 % Tris gel and run for 1.5 h at 100 V in Laemmli running buffer (4% SDS, 1% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris HCl). Protein was then transferred onto a PVDF membrane for 1 h at 0.26 A in 1X Tris-glycine buffer. The membrane was then blocked using 5 % milk in 0.1 % NP40 in PBS for 30 min and incubated in primary antibody at 4˚C overnight. The membrane was then washed in 0.1 % NP40 in PBS twice for 15 min each, incubated in corresponding secondary antibody and washed again.

Table 2. Primers used in real-time qPCR in this study

Target Gene	Forward Primer	Reverse Primer
Urat 1	5’-TTC TTC TGG CCG TCT CCA TC-3’	5’-CGT GGC GTT GGA CTC TGT AAG-3’
Glut 9	5’-GCC CAC GCT ACC TTC TCT TTG-3’	5’-AAC CAG ATC GCA TTG AGT CCA-3’
NPT1	5’-TCT GTT CCT TCC GGT ATG GAC -3’	5’-AGA ACT GAG AAT AAG CCC TTG GA-3’
OAT2	5’-CAA CTG CGG AAT CTG GTG CT-3’	5’-ATC AGG CAG GGC ACA ATG ATG-3’
HPRT	5’-TCA GTC AAC GGG GGA CAT AAA-3’	5’-GGG GCT GTA CTG CTT AAC CAG-3’
ABCG2	5’-GAA CTC CAG AGC CGT TAG GAC-3’	5’-CAG AAT AGC ATT AAG GCC AGG TT-3’
Urate Oxidase	5’-GAA GTG GAA TTT GTC CGA ACT GG-3’	5’-CGA AGT TGC CAC CTC TTT GAT-3’
XDH	5’-ATG ACG AGG ACA ACG GTA GAT-3’	5’-TCA TAC TTG GAG ATC ATC ACG GT-3’
ADA	5’-ACC CGC ATT CAA CAA ACC CA-3’	5’-AGG GCG ATG CCT CTC TTC T-3’

Proteins were visualized using the Amersham ECL kit and membrane was exposed to film and placed in an AGFA film developer.

2.7 Microscopy and image analysis

Hisological sections were analyzed using an Olympus BX51 microscope with a DP72 camera for bright field images and an XM10 camera for fluorescent images. Microscopic sections were analyzed using Cell Sense Dimension software.

Two pathologists from Baylor College of Medicine (Houston, TX) were consulted as independent evaluators of the various slides in these studies. Dr Roger Price evaluated some of the mouse tissues (bladders and prostates) and Dr Alejandro Contreras evaluated the human breast samples.

2.8 Mouse blood, urine, and stone analysis

Urine was analyzed at Taconic, Albany NY. Uric acid, potassium, creatinine, sodium, nitrate, chloride, total protein, calcium, osmolality, and volume were measured.

The composition of the bladder stones was analysed at the Urolithiasis Laboratory, Houston TX.

2.9 Statistical analysis

The slides for each study were examined independently by at least three researchers. Scores were written down and the three reports were compared. The staining was clear and all three made similar observations. In this study we compared the breast of each woman before and after HT. The percentage of cells which had stained positive for

each marker was determined for each slide and the data are shown as means ± SD for each group. Statistical differences between groups were analyzed with Student's t-test and Paired-sample t-test using SPSS (SPSS Inc., Chicago, IL). A value of $P<0.05$ was considered significant.

3. The Aryl Hydrocarbon Receptor, Estrogen Receptor β, and the Prostate

3.1 Introduction

AhR is expressed in the prostate and several recent studies have identified roles for AhR in prostatic development and cancer. Studies of U.S. soldiers exposed to TCDD through Agent Orange in the Vietnam War have demonstrated that these people suffer from an increased risk of prostate cancer (Akhtar, Garabrant et al. 2004). This increased prostate cancer risk may be due to AhR's activation of Cyp1A1 and related enzymes which can activate carcinogens. However, studies in TRAMP mouse models of prostate cancer have shown that blocking of AhR in these mice worsens the prostatic hyperplasia phenotype in these mice (Fritz, Lin et al. 2007). Treatment of TRAMP mice with the weak AhR ligand, 6-methyl-1, 3, 8-trichlorodibenzofuran, has also led to decreased carcinogenesis in this mouse model (Fritz, Lin et al. 2009). Levels of serum AhR ligands have also been associated with decreased incidence of BPH (Gupta, Ketchum et al. 2006). Some studies have suggested that involvement of AhR in the blocking of signaling through androgen receptor may possibly inhibit prostate growth (Morrow, Qin et al. 2004). We have been interested in the cross talk between AhR and estrogen signaling. Since ERβ but not ERα is the major estrogen receptor expressed in the prostate, we investigated the ventral prostates of AhR knockout mice.

The TGFβ super-family consists of a large number of extracellular proteins that signal by binding to their own cell-surface receptors (Barrack 1997). Binding to TGFβ receptors results in the phosphorylation of Smad proteins, which then enter the nucleus

and act as transcription factors for a wide variety of TGFβ target genes (Strelau, Bottner et al. 2000). ERβ can alter TGF β signaling by increasing inhibin (Fan, Gabbi et al. 2010) and the inhibin receptor TGFβ receptor 3 (unpublished data).

The ventral prostates of ER β knockout mice develop hyperplasia of the epithelium and increased expression of the anti-apoptotic factor Bcl2 (Imamov, Morani et al. 2004). Since TGF β can also be involved in these phenomena, we analyzed the prostates of ERβ-/- and AhR-/- mice for abnormalities in TGFβ signaling.

3.2 Results

3.2.1 Fibrosis of the AhR knockout mouse ventral prostate

Examination of the ventral prostate in AhR -/- mice demonstrated an abnormal stromal phenotype. There is usually a very small stromal compartment in the ventral prostates of wt mice. There was a substantial stromal compartment in prostates taken from 9.5, 10.5 and 11.5 month-old AhR -/- mice (figure 3). We also observed the growth of stroma into the ducts in areas of some AhR -/- mouse ventral prostates (figure 3). In the ventral prostates of approximately 50% of AhR -/- mice older than 12 months of age, there was epithelial hyperplasia and stromal overgrowth (figure 4). E-cadherin, a component of the adherens junctions which keeps epithelial cells organized, was still present in 13-month-old AhR -/- but at decreased levels (figure 4). With BrdU labeling of DNA, we could detect no increase over WT littermates in the number of proliferating epithelial cells in the ventral prostates of AhR-/- mice (9.5, 10.5 and 11.5-months of age) (figure 5).

In order to understand the mechanism behind the stromal overgrowth and occasional hyperplasia in the AhR -/- mouse ventral prostates we stained for several proteins important in the prostate. Expression of the Androgen receptor was high but not different from that of wild-type mouse prostates (figure 5). However, p-smad 2/3 in the nuclei of the ventral prostates of AhR -/- mice was higher than that of wild-type prostates, especially in areas of increased stromal over-growth (figure 5). Increased TGFβ signaling in the AhR -/- prostates suggests a possible mechanism for the stromal overgrowth in these animals (figure 5).

3.2.2 Alterations in the TGFβ pathway in ERβ knockout mouse ventral prostates

ERβ is known to regulate TGFβ signaling and ERβ is highly expressed in the ventral prostate of mice. We were interested to determine whether there are also alterations in the TGFβ pathway in ERβ knockout prostates such as we observed in AhR -/- prostates. We examined changes in the TGFβ pathway in ERβ knockout mice by staining for the downstream mediator of TGFβ signaling, p-smad 2/3. There were more epithelial cells in the ERβ knockout mice expressing p-smad 2/3 than in wild-type mice (figure 6).

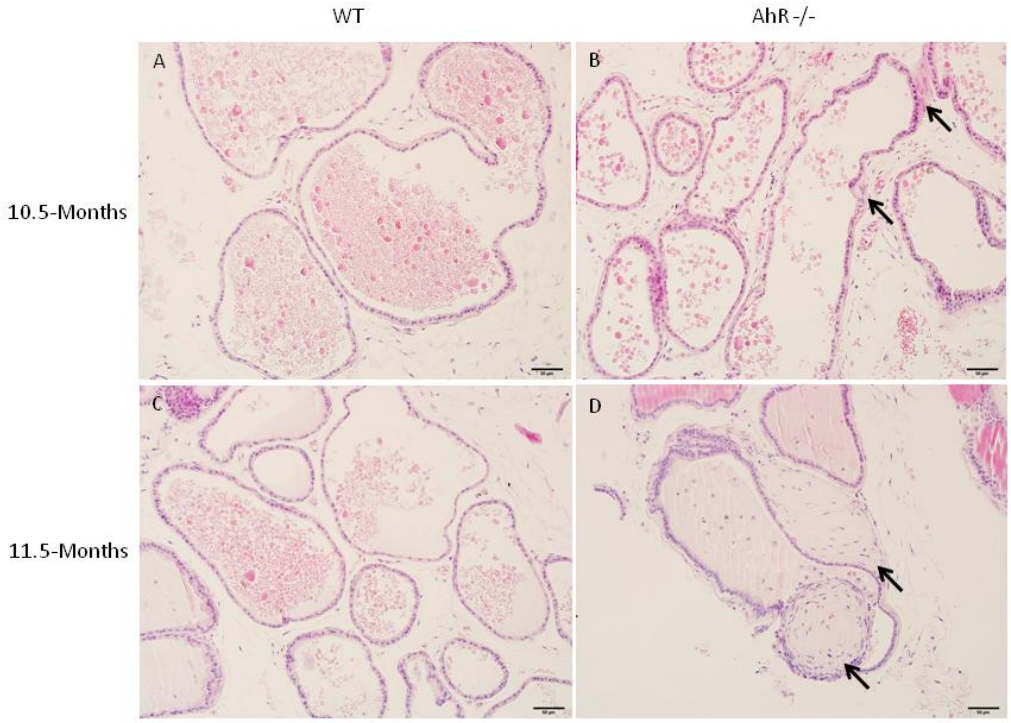


Figure 3. Hematoxylin and eosin staining of the ventral prostates in aging AhR -/- mice. 10.5-Month-old prostates of AhR -/- mice show slightly increased stromal thickness as indicated by arrowheads (B). 11.5-month-old AhR -/- mice develop large stromal growths (D) which invade the lumen of the prostatic ducts (arrowheads). Scale bars represent 50μm.

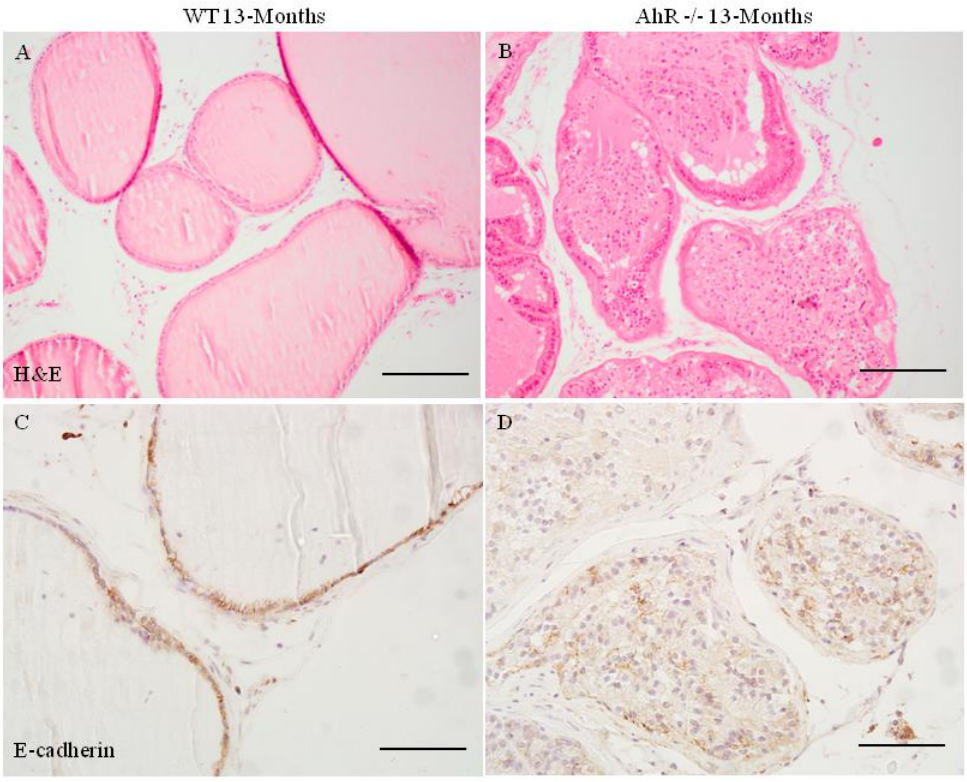


Figure 4. Hyperplasia of 13-month-old AhR -/- ventral prostates. Approximately 50% of the mice over 13-months of age had epithelial hyperplasia (B) in addition to the stromal fibrosis previously described. E-cadherin staining of AhR -/- ventral prostates (D) was present but not in all epithelial cells as in wild-type littermates (C). Scale bars in (A, B) represent 100 μm and 50 μm in (C, D).

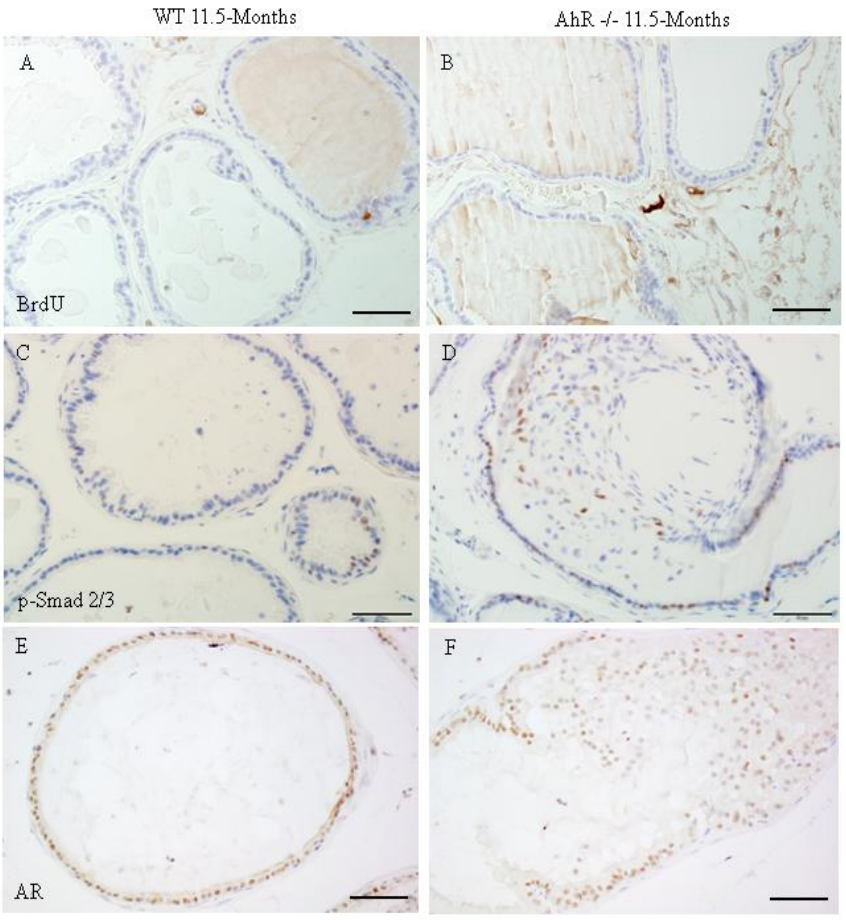


Figure 5. Immunohistochemical staining of ventral prostates of 11.5-month-old wild-type and AhR -/- mice. Staining for BrdU in the ventral prostates of mice injected with BrdU was not significantly different in epithelial cells between WT (A) and AhR -/- mice (B). Staining for p-Smad 2/3 was higher in areas of stromal over-growth in AhR -/- ventral prostates (D). AR was expressed throughout the prostates of both WT (E) and AhR -/- (F) animals. Scale bars represent 50μm.

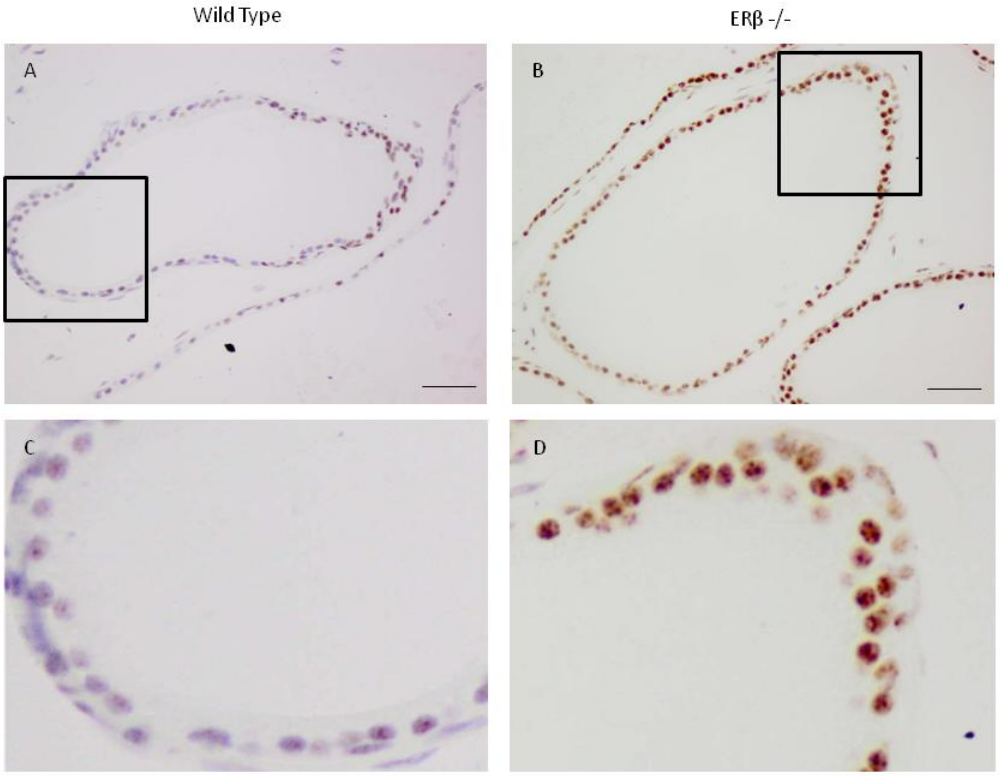


Figure 6. p-Smad 2/3 staining the in 1-year-old ERβ knockout mouse ventral prostate. There are a larger number of cells expressing p-smad 2/3 in ERβ knockout ventral prostates (B, D) than wild-type littermates (A, C). Scale bars represent 50 μm. C and D are increased magnifications of the boxed areas in A and B.

3.3 Discussion

Our results demonstrate that AhR knockout mice develop abnormalities in their ventral prostates as they age. These results complement previous studies that showed that some AhR ligands decreased prostatic growth in TRAMP mouse models as well as BPH occurrence in humans (Gupta, Schecter et al. 2006; Fritz, Lin et al. 2009). In the absence of AhR there is increased growth of the stromal layer in the ventral prostates of mice. As the mice grow older, some also show epithelial hyperplasia. Increased TGFβ signaling is known to increase fibrosis in different tissues as well as worsen outcome in certain cancers (Akhurst and Hata 2012). We found that TGFβ signaling was increased in AhR -/- prostates where fibrosis occurred, suggesting a possible cross-talk between AhR and TGFβ in the prostate.

In the ventral prostates of ERβ -/- mice there was an increase, over wild-type mice, in the number of p-smad 2/3-positive epithelial cells. Increased TGFβ signaling in this tissue may have led to some of the increased proliferation previously observed (Imamov, Morani et al. 2004) as well as induction of epithelial to mesenchymal transition.

AhR and ERβ both have functions in the prostate. The present study demonstrates a role for TGFβ in both AhR and ERβ signaling in this tissue. Future investigations may help elucidate the exact mechanism through which these receptors interact with TGFβ.

4. The Aryl Hydrocarbon Receptor and its Role in Uric Acid Bladder Stone Formation

4.1 Introduction

The aryl hydrocarbon receptor (AhR) has been extensively studied for its role in regulating xenobiotic-metabolizing enzymes and the curious observation that one of its most potent ligands is the environmental contaminant, 2, 3, 7, 8-tetra-chlorodibenzoparadioxin (TCDD) (Nebert, Dalton et al. 2004). Even though many of the enzymes regulated by AhR are involved in purine metabolism (table 1), no connections have been shown between AhR and human diseases related to hyperuricemia. Also, the AhR -/- mouse strains which have been generated and studied by several different labs have not yet described any phenotypes related to uric acid in these mice.

The aim of the study in chapter 3 was to investigate the AhR -/- mice generated in the laboratory of Fujii-Kuriyama to determine whether there is cross talk between AhR and ERβ and whether such interactions influenced any aspects of prostate physiology and pathology. In the process of removing ventral prostates we found urate stones in the urinary bladder. We investigated the source of these stones and found that they were due to abnormal cellular turnover in the urinary bladder itself. There was also extensive inflammation and development of bladder cancer in older mice.

4.2 Results

4.2.1 Uric acid stones and elevated urinary uric acid in the AhR -/- mice

All AhR-/- mice at 8 months of age had urinary bladder stones approximately 3-4 mm in diameter and their composition was almost 100% urate. These stones first appeared in some of the mice at 10 weeks of age and by the age of 6 months all of the mice had a stone occupying most of the bladder (figure 7). At three months of age, uric acid levels in the urine of these mice were approximately 10 fold higher than those of wild-type littermates (figure 8).

Interestingly, the serum levels of uric acid in the knockouts were not significantly different from the wild-type mice at 3 months (figure 8). There were also no urate stones or histological abnormalities in the kidneys or the joints of the AhR -/- mice, which are characteristics of elevated uric acid in humans.

Uric acid is the end point of purine metabolism in humans but in mice, unlike humans, there is an enzyme called uricase, which catalyzes the conversion of uric acid to allantoin. We examined several enzymes in the purine degradation pathway and found no significant difference in the RNA or protein levels in the liver for adenosine deaminase, uricase, hypoxanthine-guanine phosphoribosyltransferase or xanthine oxidase (Figure 9). This result is compatible with the lack of high urate in the circulation and suggested an abnormality in the bladder itself.

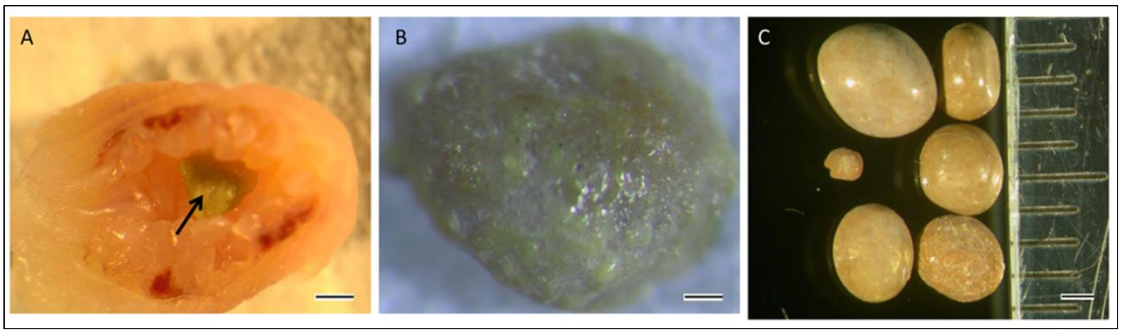


Figure 7. Urate stones in the urinary bladder of AhR knockout mice. Urinary bladder of a 10-week-old AhR knockout mouse (A) with a urate stone indicated by the arrow. These stones are rough and pitted in appearance (B). Urate stones at 6 months of age (C) with diameters of 3-4 mm. The scale bar in (A) represents 500 μm, in (B) it is 200 μm and in (C) it is 1 mm.

Figure 8. Urine solutes, urine volume and serum uric acid measurements of wild type and AhR knockout mice at 3 months of age. Urine creatinine (A), nitrate (D), volume (E), osmolality (F), potassium (G), chloride (H), calcium (I) and sodium (J) were not significantly different in the knockout mice when compared to their wild type littermates. Uric acid (B) was approximately 10 fold higher in the urine of knockout mice ($p < 0.001$). There was a small decrease in the total protein (C) in the AhR knockout mice ($p = 0.039$). Uric acid levels in the serum were not significantly changed at 3 months of age (K). Error bars in (K) represent standard deviation.

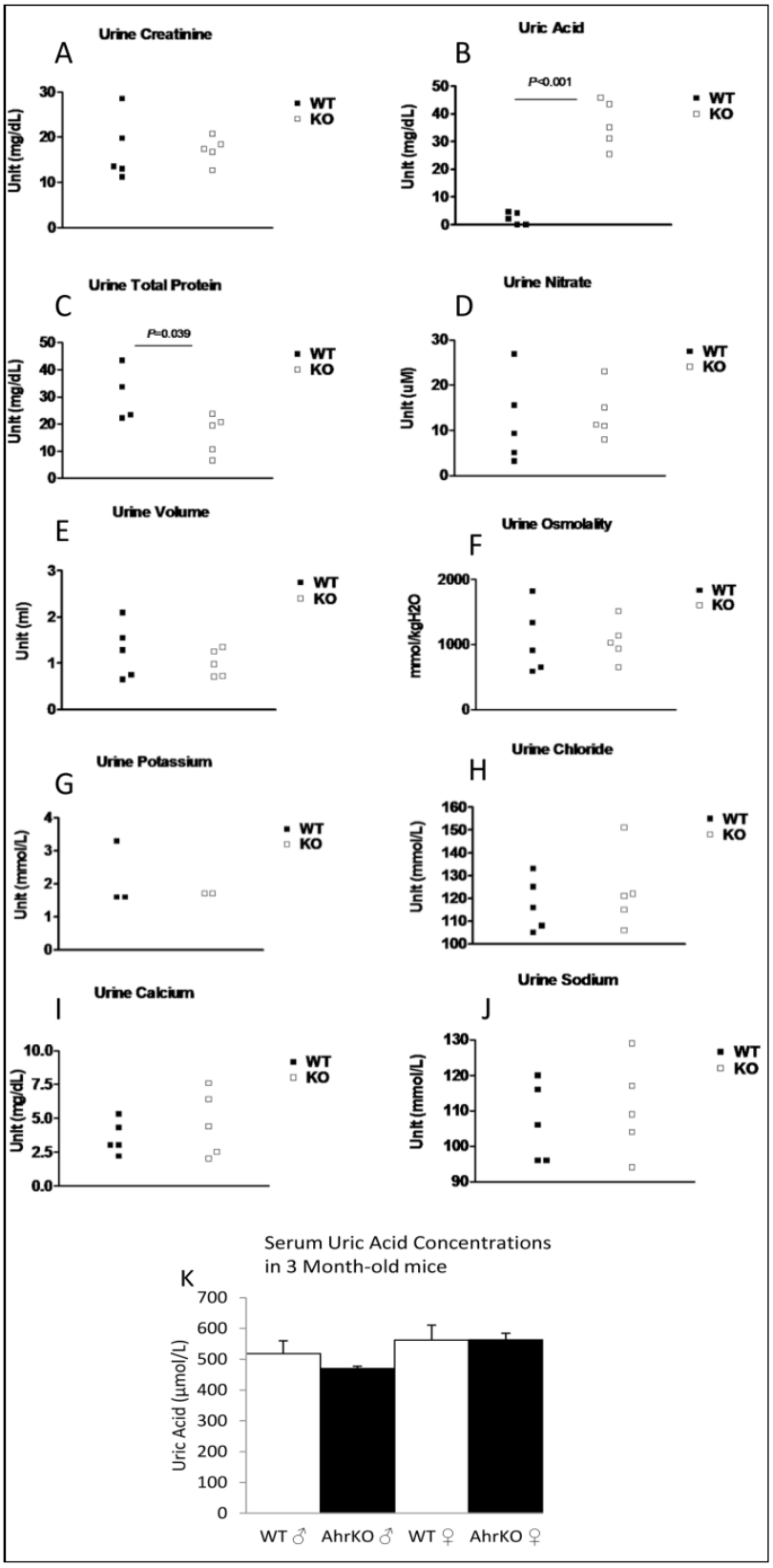
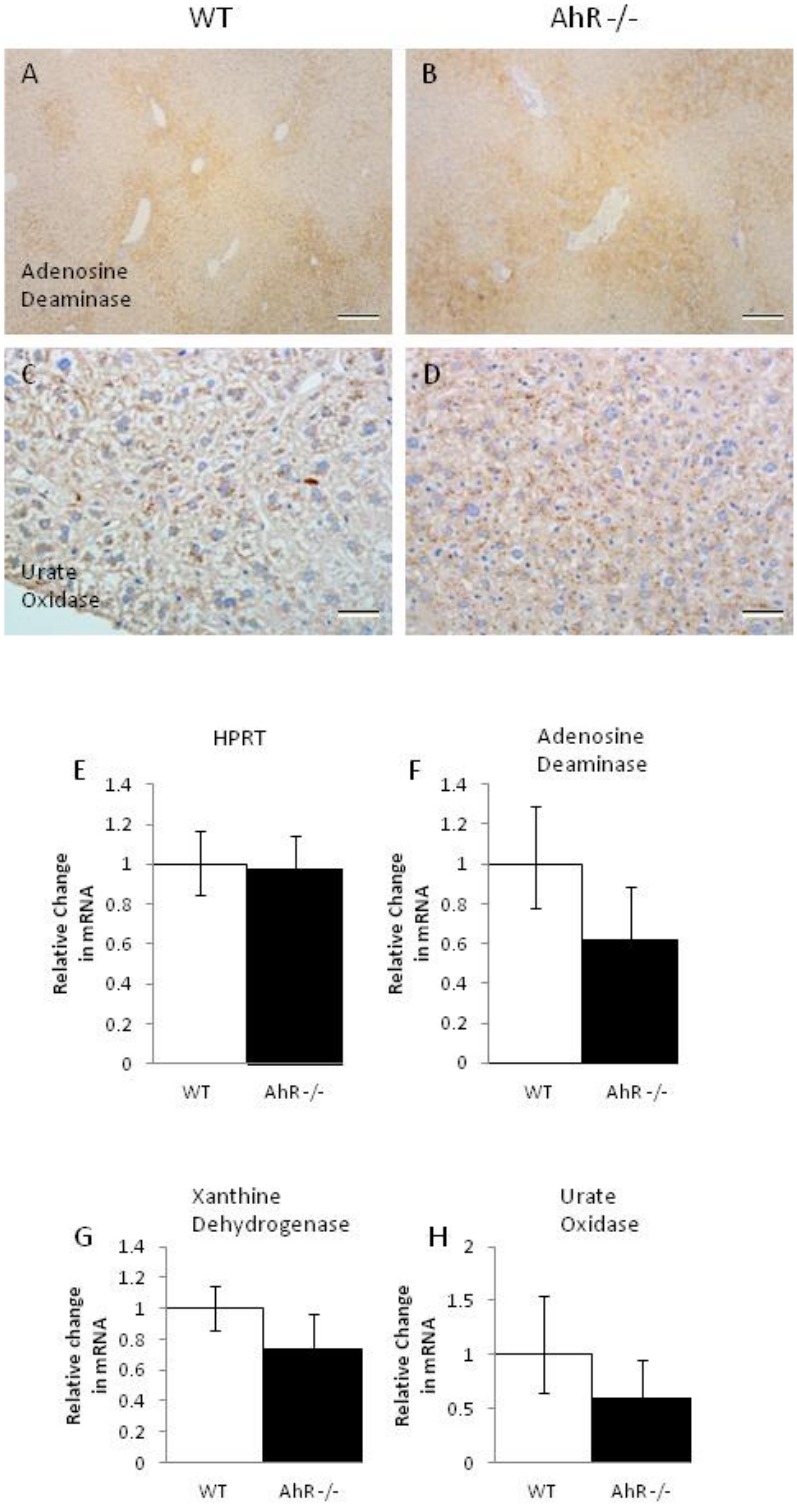

A
Urine Creatinine
Unit (mg/dL)
WT
KO
B
Uric Acid
P<0.001
Unit (mg/dL)
C
Urine Total Protein
P=0.039
Unit (mg/dL)
D
Urine Nitrate
Unit (uM)
E
Urine Volume
Unit (ml)
F
Urine Osmolality
mmol/kgH2O
G
Urine Potassium
Unit (mmol/L)
H
Urine Chloride
Unit (mmol/L)
I
Urine Calcium
Unit (mg/dL)
J
Urine Sodium
Unit (mmol/L)
K
Serum Uric Acid Concentrations
in 3 Month-old mice
Uric Acid (µmol/L)
WT ♂
AhrKO ♂
WT ♀
AhrKO ♀

Figure 9. Measurement of enzymes involved in the purine degradation pathway in AhR -/- mice. There was no noticeable change in the protein or RNA levels of several members of the purine degradation pathway in the livers of 6-month-old AhR -/- mice compared to those of wild-type littermates. There were six mice in each group. Immunohistochemistry shows similar staining patterns for adenosine deaminase (A, B) and urate oxidase (C, D) in the livers of 6-month-old wild-type and AhR -/- mice. The level of HRPT RNA in the liver was measured by real-time quantitative PCR. (E), adenosine deaminase (F) xanthine dehydrogenase (G) and urate oxidase (H) were not significantly different between the 6-month-old wild-type and AhR -/- littermates. For (A) and (B) scale bars represent 200 μm and for (C) and (D) scale bars are 20 μm. Error bars represent standard deviation.


WT
AhR -/-
A
B
Adenosine
Deaminase
C
D
Urate
Oxidase
HPRT
E
Relative Change in mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
AhR -/-
Adenosine
Deaminase
F
Relative Change in mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
AhR -/-
Xanthine
Dehydrogenase
G
Relative change in mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
AhR -/-
Urate
Oxidase
H
Relative Change in mRNA
2
1.5
1
0.5
0
WT
AhR -/-

4.2.2 Urine solute measurements and urate transporter levels

Changes in water transporters (aquaporins) in the kidney are known to cause the urine to become concentrated and may lead to the formation of stones. However, in the AhR-/- mice the urine osmolality was not different from their control littermates at 3 months (figure 8) or at 4, 5, and 6 months of age. There were also no changes in the total urine volume, nitrate, potassium, sodium, calcium, creatinine or chloride in these mice (figure 8). The pH was 6.5 in all knockout and wild-type mice. There was a small decrease in the total protein concentration in the urine (figure 8). The mRNA levels of several urate transporters were also unchanged in the kidney (figure 10).

4.2.3 Fibrosis in the AhR -/- mouse bladder submucosal layer.

In view of the lack of changes in other pathways that may have led to elevated uric acid in the urine, we examined the histology of the urinary bladders of mice at 10 weeks of age. There was increased fibrosis of the submucosal layer in AhR -/- mice when compared to their heterozygous littermates (figure 11). There was a thicker collagen layer in these bladders as well as very large, dilated blood vessels. This phenotype is even more severe at 6 months of age and in some of the mice epithelial cells had invaded the muscle and submucosal layers of the bladder (figure 12). It appeared possible that a high turnover of cells in the enlarged bladders may have caused an increase in nucleic acid degradation into uric acid. This is a situation similar to tumor lysis syndrome in leukemia when tumor cells are killed too rapidly.

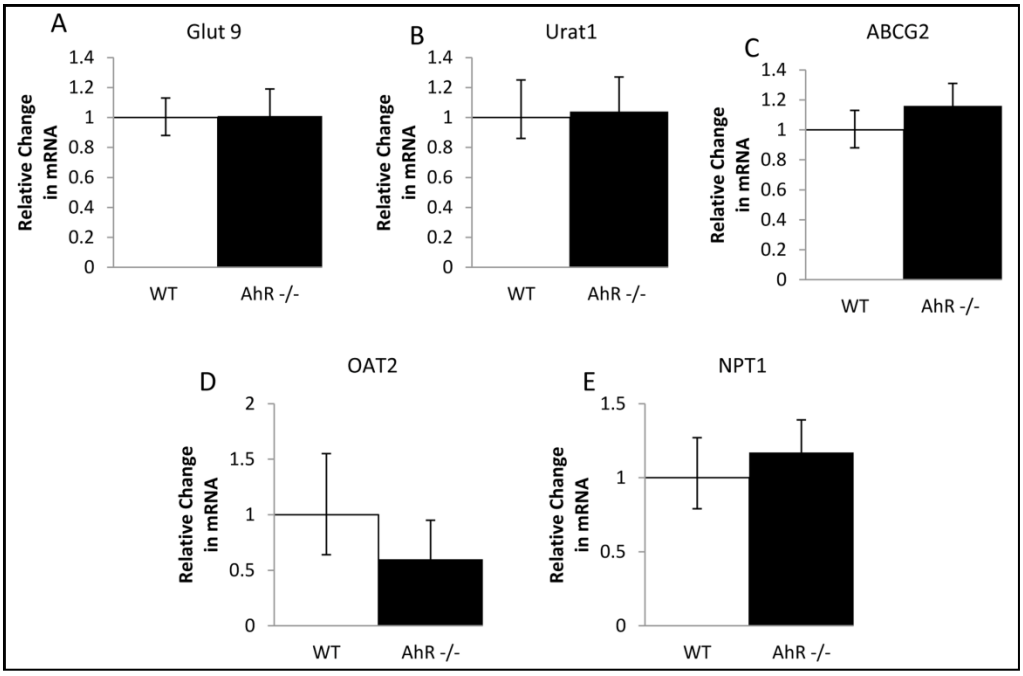


Figure 10. Measurements of urate transporters in AhR -/- kidneys. There was no significant difference in the mRNA levels of several different urate transporters in the kidneys of 6-month-old AhR -/- mice when compared to wild-type littermates. mRNA levels of 6 mice from each group were measured using real-time quantitative PCR. There was no significant change in Glut 9 (A), Urat1 (B), ABCG2 (C), OAT2 (D), or NPT1 (E). Error bars represent standard deviation.

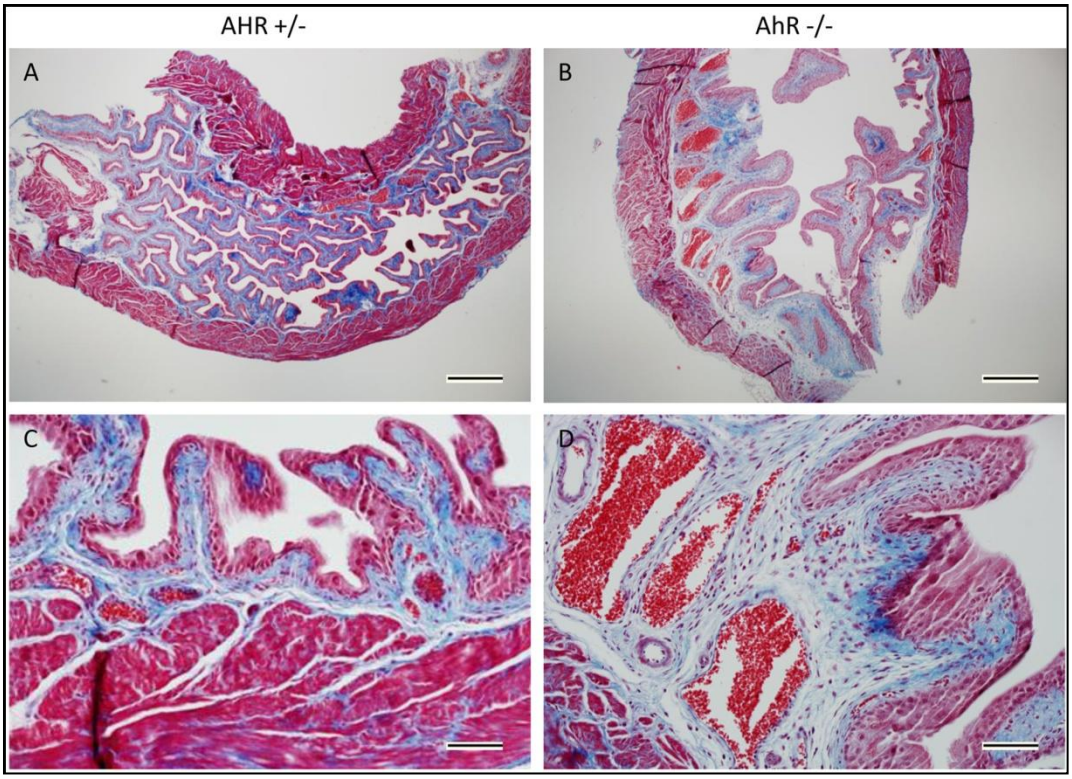


Figure 11. Fibrosis in the submuscosal layer of the 10-week-old AhR knockout bladders. Masson's trichrome stain of AhR +/- (A,C) and AhR -/- (B, D) bladders demonstrating fibrosis of the knockout bladder with an increased amount of collagen (blue) and enlarged blood vessels. Scale bars in (A) and (B) represent 500 μm and scale bars in (C) and (D) represent 20 μm.

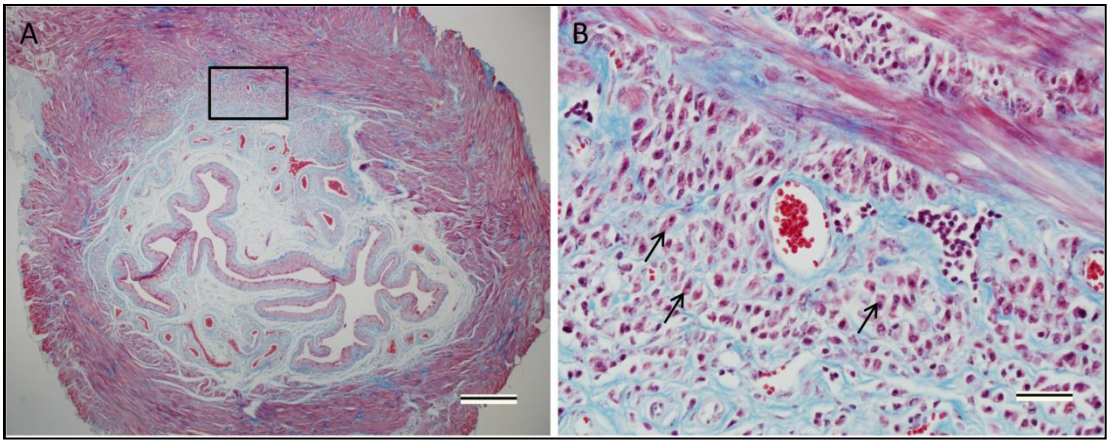


Figure 12. Invading epithelial cells are observed in the submucosal and muscle layers of the 6-month-old AhR -/- bladder. Some epithelial cells are marked with arrows (B). Masson's trichrome stain was used to stain collagen blue, cytoplasm red, and nuclei purple. The scale bar in (A) represents 500 μm and the scale bar in (B) represents 20 μm.

4.2.4 Numerous round structures in the cytoplasm and death of urothelium

In the urothelial cells in the bladder of AhR knockout mice there were numerous round structures concentrated towards the luminal surface which were not present in wild-type or heterozygous mice (figure 13). These structures stained positive for acidic substances, eosin. These structures also stained positive for uric acid, with methenamine silver, while the control mice stained negative (figure 13). We speculate that these structures are vesicles filled with uric acid. Therefore we treated some fresh 4-month-old AhR -/- bladders with uricase (an enzyme which converts uric acid to allantoin) to see if these structures would dissolve. Even though the hydrogen peroxide used in this reaction caused much of the epithelium to fall off, the remaining epithelium after the uricase treatment did not contain any round granules while the control did (figure 13). Since the uricase appeared to eliminate the presence of the epithelial inclusions, we conclude they contain uric acid.

To investigate the round vesicles in the urothelium of the AhR -/- mice further, we performed transmission electron microscopy (TEM). This technique allowed us to visualize the tissue at very high magnifications and resolutions in order to better understand how these structures form. From the images taken, we noticed that the vesicles appear dark in TEM which indicates that they contain an electron-dense material (figure 14). In light microscopy, the structures could only be seen near the luminal surface of the urothelium but the TEM shows that smaller vesicles also exist closer to the basal surface (figure 14). It appears that numerous small vesicles fuse together to form the larger vesicles which seem to be released into the urine.

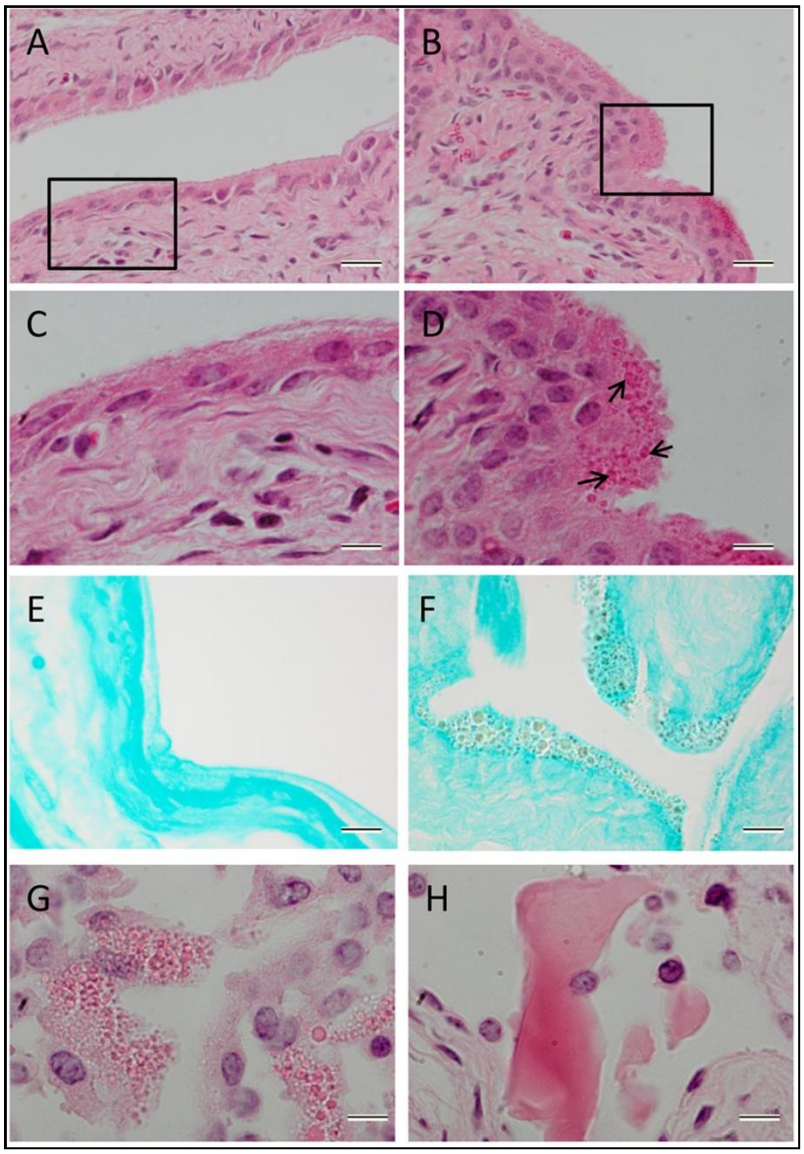
4.2.5 Immunohistochemical characteristics of the AhR -/- bladder.

Since increased cell death is a possible explanation for the elevated uric acid we measured apoptosis in the AhR -/- bladders with the Tunel assay. There were more Tunel–positive cells in the 4-month-old knockout bladders than in heterozygous littermates and there were several areas in the knockouts where cells had lost their cytoplasm and the nuclei were condensed (figure 15).

E-cadherin, an important constituent of the epithelial adherens junction, was lower in the AhR -/- bladders with almost complete absence in some disorganized areas of epithelium from 6-month-old mice (figure 16). There was a marked increase in the number of macrophages (positive staining for the macrophage marker F4/80) in the stroma of the AhR-/- mouse bladders (figure 16). Since macrophages can degrade purines and secrete uric acid, these cells may be the source of uric acid in the urine of the knockout mice.

As AhR -/- mice aged, the pathological changes in the urinary bladder became more severe. By the time the mice were of 10.5-months of age, the stromal and epithelial layers of the bladder were thicker than that seen in younger mice (figure 17). There were also changes in morphology of some of the urothelial cells in AhR -/- mice into an abnormal, elongated shape, characteristic of epithelial to mesenchymal transition (figure 17). There were many more BrdU-positive cells in both the epithelium and stroma of the urinary bladders of 10.5-month-old AhR -/- mice than wild-type mice. This indicates a higher rate of proliferation in the bladders of aging AhR -/- mice (figure 17).

Figure 13. Histological characteristics of the AhR -/- mouse urothelium. Numerous round particles were visible in the luminal side of the urothelium of a 10-week-old AhR knockout mouse, (B, D) while no particles existed in the heterozygous control (A, C). Cytoplasm and particles are stained pink with eosin and nuclei are stained purple with Mayer's hemotoxylin. These structures stain positive (brown-black color) for Grocott's Methenamine Silver stain which can be a marker for uric acid (F) while the control bladder remains negative (E). After 1 h treatment with uricase and hydrogen peroxide, these granules are not present in the epithelium of the 4-month-old AhR -/- bladder (H). The granules are still present after the control treatment of only hydrogen peroxide in PBS (G). Scale bars for (A), (B), (E) and (F) represent 20 μm, scale bars for (C), (D), (G), and (H) represent 10 μm.


A
B
C
D
E
F
G
H

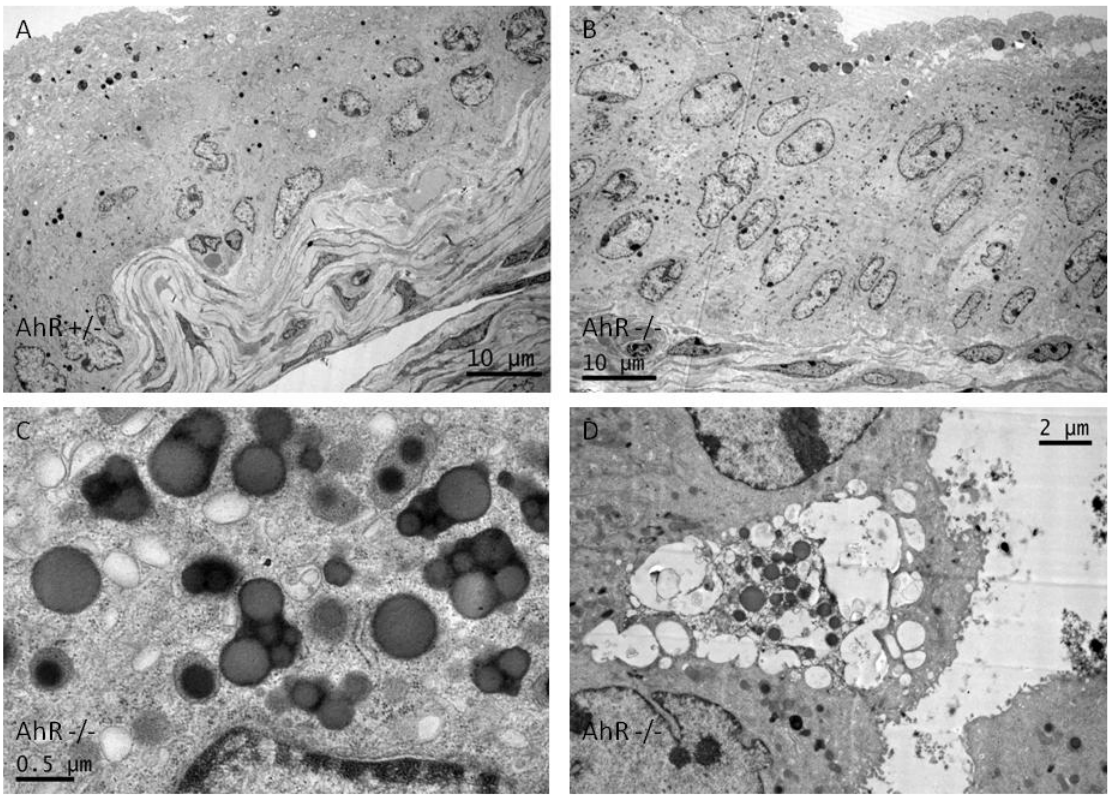


Figure 14. Transmission electron microscopy of AhR -/- urothelia from 4-month-old mice. Vesicles can be seen in the luminal and basal areas of the urothelium in AhR -/- mice (B). Nuclei are also larger in AhR -/- urothelium (B) compared to wild-type controls (A). Dark-colored, electron-dense small vesicles appear to fuse together to form larger vesicles (C). Vesicles which are about to be released from the urothelium into the urine (D).

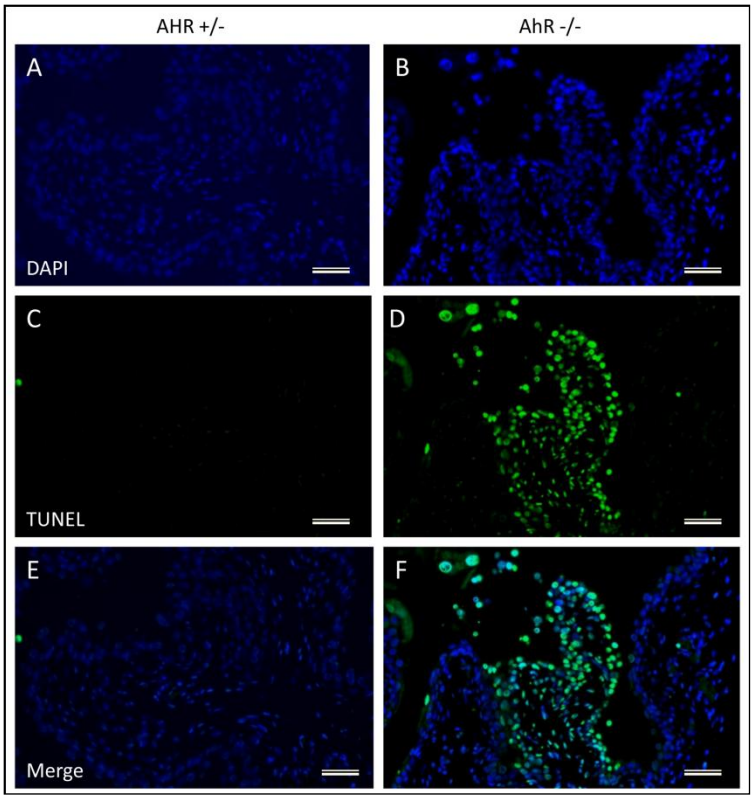


Figure 15. Cell death in the AhR -/- urothelium. Apoptotic cells are present in some areas of the 4-month-old AhR -/- bladder (B, D, F) while there are very few in the AhR +/- mouse (A, C, E). DAPI is used to stain the nuclei blue (A, B), FITC stains Tunel-positive cells green (C, D). All scale bars represent 20 μm

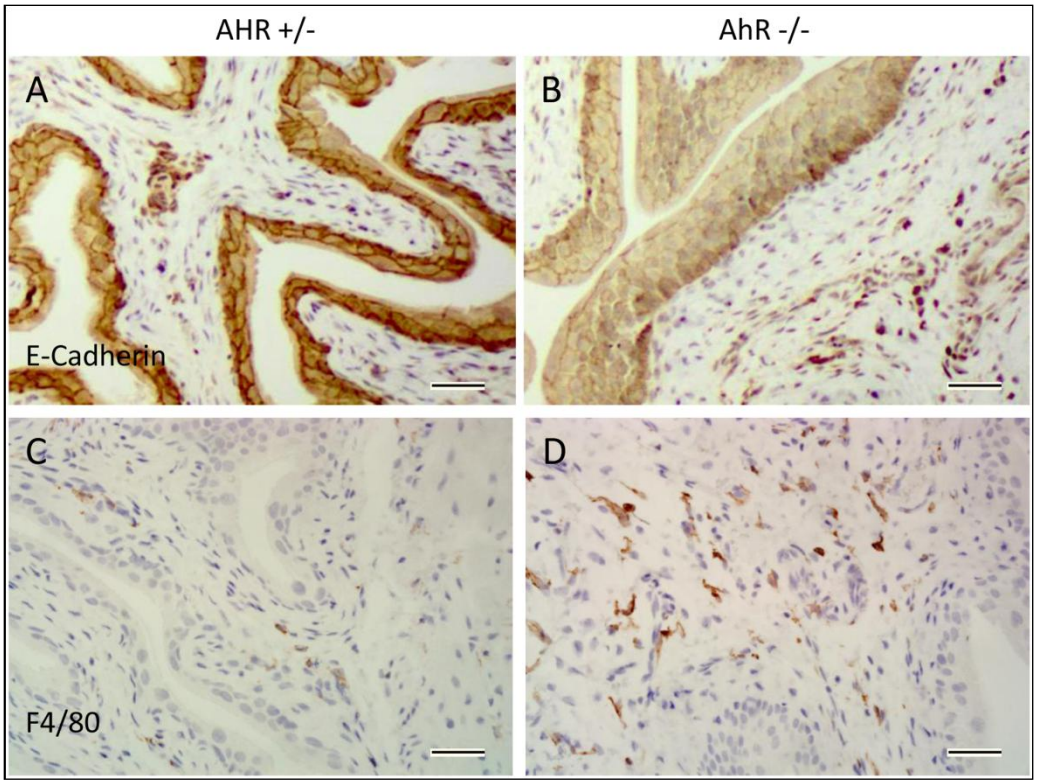
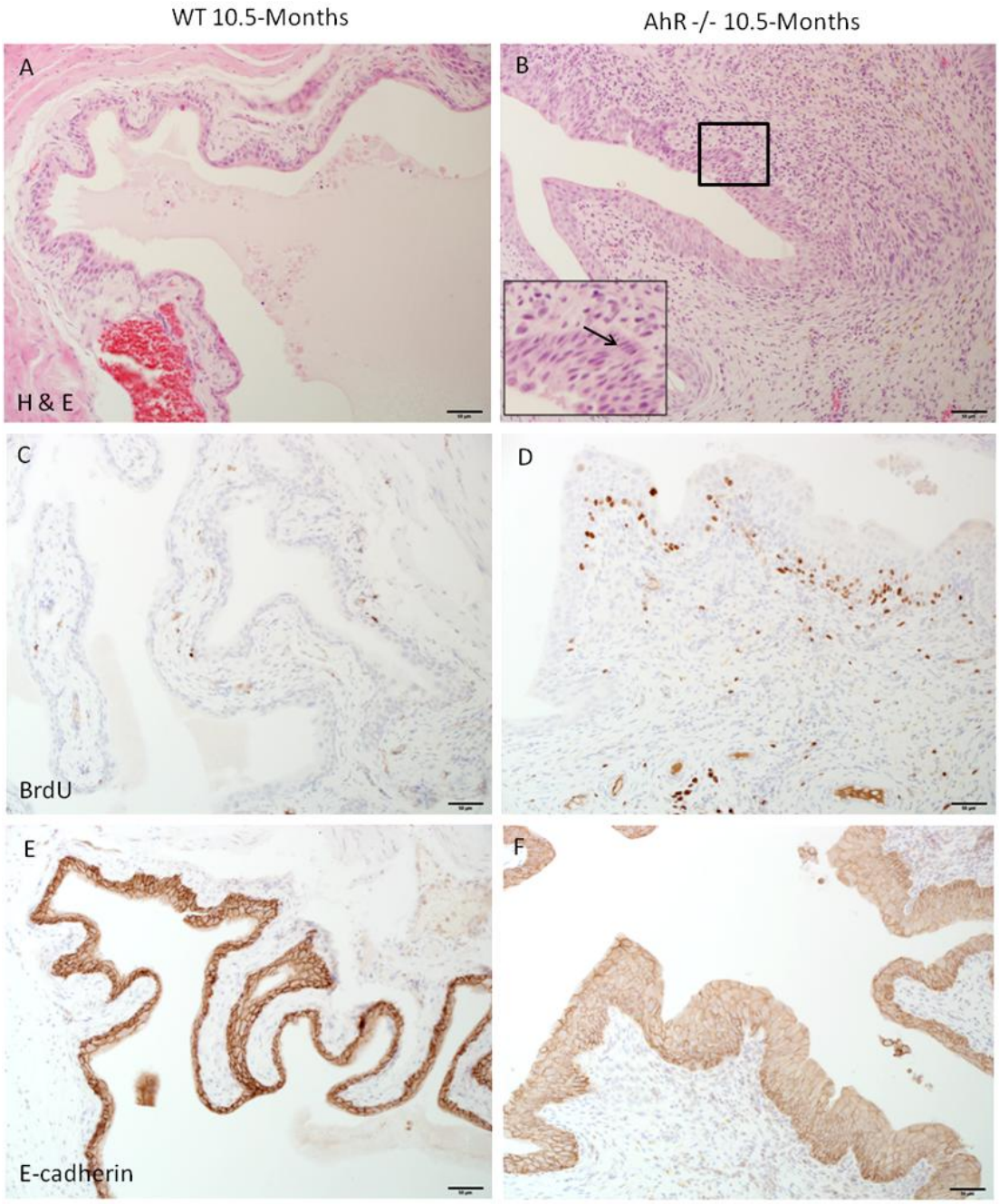


Figure 16. E-Cadherin and F4/80 staining in 10-week-old AhR -/- and +/- mice. There is loss of E-cadherin in some areas of the AhR -/- urothelium (B) while the heterozygous mice appear to have normal E-cadherin expression throughout the bladder (A). There were many more cells that stained positive for the macrophage marker, F4/80, in the stroma of the knockout bladders (D) when compared to the heterozygous controls (C). All scale bars represent 20 μm.

Figure 17. Increased proliferation in the bladders of 10.5-month-old AhR -/- bladders. Urinary bladders from AhR -/- mice (B) had very thick epithelial and stromal layers when compared to wild type animals (C) which were more severe than younger mice examined. The AhR -/- mice also had some abnormal, elongated epithelial cells which may have been undergoing EMT (B inset). Proliferation of the epithelium was much higher in AhR -/- bladders (D) than the wild-types (C) as measured by BrdU incorporation in the nuclei. E-caherin was still present in most of the AhR -/- urothelium (F) but was decreased when compared to wild-type urothelium (E). Scale bars represent 50 μm.


WT 10.5-Months
AhR -/- 10.5-Months
A
B
H & E
C
D
BrdU
E
F
E-cadherin

Immunohistochemical staining of E-cadherin was still present throughout the urothelium of AhR -/- mice, however its expression was markedly decreased in some those areas where the epithelial cells were becoming more fibroblastic in shape (figure 17).

4.3 Discussion

In this study we have described a novel phenotype of the aryl hydrocarbon receptor knockout mouse, i.e., uric acid stones in the urinary bladder. In most human diseases that involve elevated urinary uric acid – such as gout, and lesch-nayhan disease – the serum uric acid is also elevated (Stark, Reinhard et al. 2009; Jinnah, Ceballos-Picot et al. 2010). The AhR knockout mice in our study had elevated uric acid in the urine but normal levels of serum uric acid, setting this model apart from any human diseases. Unlike humans, mice have an enzyme, urate oxidase (uricase), which catalyzes the conversion of uric acid to allantoin. Because of the presence of uricase in the liver, mice excrete very low levels of uric acid in their urine and do not suffer from uric acid-related diseases. Uricase knockout mice have elevated serum uric acid, stones in the kidney, and a very high mortality rate (Wu, Wakamiya et al. 1994), none of which occurs in the AhR knockout mice. Because there is no uricase expressed in humans, loss of AhR in humans would be expected to lead to a much more severe disease with elevated levels of uric acid in the circulation and in joints. For this reason, defective AhR signaling should be considered as a risk factor for development of gout.

In the AhR knockout mice there were no detectable abnormalities in the purine degradation pathway during which uric acid is formed. There were normal protein and

RNA levels of uricase and other members of this pathway in the knockout mice (Figure 9). There were also no significant changes in the urine volume, osmolality or concentrations of any other solutes measured (figure 8) which rules out the involvement of water transporters concentrating the urine. No significant changes in the mRNA levels of several urate transporters in the kidney were found (Figure 10). These results led us to the conclusion that there was a problem with the urinary bladder itself in these mice.

AhR is known to regulate several enzymes involved in the metabolism of toxic substances including Cyp1A1, Cyp1B1, and glutathione-S-transferase (GST) (Rowlands and Gustafsson 1997). The cytochrome P450 1A1 and 1B1 enzymes are capable of converting polycyclic aromatic hydrocarbons into their most carcinogenic forms and AhR knockout mice are less susceptible to the cancer-causing effects of some of these substances (Matsumoto, Ide et al. 2007). Some of the GST enzymes, however, are known to detoxify many harmful substances which can end up in the bladder and a characteristic of the GSTM1-null genotype is bladder carcinogenesis (Chico and Listowsky 2005). Since levels of GST are much reduced in the AhR knockout mice, it is possible that more unmetabolized toxins are entering the bladder, leading to cytotoxicity and bladder cancer. The urothelial layer of the AhR -/- bladders contains more cells, have more disorganized areas with decreased expression of E-cadherin and in some cases there is invasion of the muscle layers by epithelial cells (figure 12).

Tumor lysis syndrome in humans is a situation when a large amount of cellular components from the dying cells in a tumor are released into the bloodstream, causing

hyperuricemia (Kennedy and Ajiboye 2010). There have been cases of tumor lysis which led to large amounts of uric acid in the bladder, causing stone formation (Chubb, Maloney et al. 2010). In the AhR-/- mice, the urate seemed to accumulate as granules on the luminal side of the urothelial cells, many of which appeared to be undergoing necrosis or apoptosis. Since the granules present in the urothelium stained positive for a uric acid marker and were degraded with uricase (figure 13), we believe uric acid is being secreted from the bladder into the urine by these structures. There is a possibility that the process ongoing in the AhR -/- bladder is similar to tumor lysis syndrome in that the large number of cells in the stroma and urothelium may be releasing their components into the bladder. If uric acid produced in the bladder, entered the bloodstream it would be detoxified by uricase in the liver. This would explain why urate levels in the serum are not elevated.

In human beings there is a rare disorder, keratinizing squamous metaplasia, in which the epithelial cell layer of the bladder grows and the stromal layer becomes thicker (Ahmad, Barnetson et al. 2008). This disorder can be caused by irritation of urinary tract by objects such as catheters, stones or infections but may also occur due to genetic factors (Ahmad, Barnetson et al. 2008). There have also been studies showing that foreign bodies introduced in animal bladders can cause inflammation and proliferation of the urothelium (Shirai, Ikawa et al. 1986). At present, we are unsure of the exact cause of the elevated uric acid in AhR knockout mouse bladders. We do not know to what extent the pathological changes are due to uric acid itself and what is the contribution of irritation caused by the presence of stones. The macrophages in the stromal and muscle layers of

the AhR -/- bladders may have been recruited to the bladder to clear away cells damaged by toxins in the urine. Macrophages engulf cell debris and can degrade DNA to produce and secrete uric acid (Chan 1979). It is possible that the large numbers of macrophages in these bladders are secreting uric acid which ends up in the urine. The presence of large stones in the bladder is a further irritant leading to more inflammation and a worsening of pathological changes. If cells in the urinary bladder are dying because of exposure to toxins, and the elevated uric acid is due to degradation of these cells, the process is ongoing in very young mice since bladder stones were detectable when mice were 10 weeks of age.

In conclusion, we have demonstrated that the AhR knockout mice developed in the Fuji-Kuriyama laboratory have markedly elevated uric acid in the urine and develop urate stones in their bladders. The bladders of the AhR knockout mice had an enlarged submucosal area, numerous round particles in the urothelium and increased macrophage infiltration. In some older mice there was invasion of the epithelium into the stromal and muscle compartment indicating the development of malignant changes. Thus the absence of AhR confers a predisposition for bladder toxicity without exposure of mice to any known carcinogen. Other AhR -/- mouse strains remain to be studied with regard to this bladder phenotype and it would be valuable to see if the phenotype is consistent across all strains. While we are not certain about the mechanism for this phenomenon, we have described a novel connection between AhR and uric acid production with possible associations to human diseases such as gout.

5. Chronic Myeloid Leukemia in the Aryl Hydrocarbon Receptor knockout mouse

5.1 Introduction

AhR was first recognized to have functions in the immune system when people exposed to TCDD suffered from immune suppression. Like AhR, ERβ is also highly expressed in the immune system and is an important regulator of different types of immune cells (Koehler, Helguero et al. 2005). ERβ knockout mice develop a severe myeloproliferative disorder similar to chronic myeloid leukemia (Shim, Wang et al. 2003). ERβ has anti-inflammatory functions in the microglia of the brain, Th17 T cells, macrophages and many other types of cells (Stygar, Masironi et al. 2007; Subramanian and Shaha 2009; Saijo, Collier et al. 2011). ERβ ligands may prove to be effective drugs for treatment of inflammation and autoimmune diseases in the future.

Our previous findings (chapter 4) demonstrated that AhR -/- mice from the lab of Dr. Fujii-Kuriyama develop urinary bladder stones comprised of uric acid. We concluded that the increased uric acid in the bladders of these mice most likely originated from the metabolism of purines released from the DNA of dying cells in the bladder. Tumor lysis syndrome occurs in humans when there is a rapid death of tumor in response to chemotherapy. In tumor lysis syndrome, the burden of uric acid released from the DNA exceeds the capacity of the body and uric acid is deposited in several tissues. (Firwana, Hasan et al. 2012). Because there is severe immune infiltration in several tissues of the AhR-/- mice, we investigated whether immune cells were contributing to the formation of uric acid bladder stones.

5.2 Results

5.2.1 The immune phenotype of the AhR knockout mouse

The lymph nodes of the 1-year-old AhR -/- mice were much enlarged (figure 18). Blood smears indicated an increased circulating white blood cell count which was on average 2.6-fold higher than in wild-type mice (figure 18). Microscopic evaluation of the blood smears also demonstrated that there were many more circulating granulocytes in the AhR -/- mice. Enlarged lymph nodes in mice and an increased circulating white blood cell count in mice that do not have an infection indicate an overactive immune system.

AhR knockout mice were found to have splenomegaly which worsens with age. The spleens of these mice are two times the size of wild-type littermates and the increased sizes were due to an increased number of immune cells (figure 19). There were also many more immune cells invading into the white pulp (the part of the spleen containing mostly red blood cells) of the AhR -/- spleens compared to the wild-type mice (figure 19). In the spleens of the AhR -/- mice the number of granulocytes (positive for chloroacetate esterase) and myeloid cells (myeloperoxidase-positive) was higher than in wt mice (figure 19). There were also numerous megakaryocytes in the spleen, found in the spleen, in the the AhR -/- mice but these were rare in wild-type mice (figure 19). These phenotypes were present in all mice tested from 6 months to 14 months of age.

There was a very marked immune invasion in the livers of the AhR -/- mice (figure 20) but this was also seen in the white adipose tissue, pancreas, lungs, urinary bladders, and colon. In the liver, this immune infiltration forms very large lesions and

staining with myeloperoxidase and chloroacetate esterase demonstrated that most of these cells were granulocytes (figure 20).

The hind limbs from AhR -/- and AhR +/+ mice were taken at 1 year of age, bones were decalcified and bone marrow was examined histologically. The AhR -/- mouse bone marrow differed from that of wild-type mice in that it had more less-differentiated cells, similar to what is seen in a blast crisis in chronic myeloid leukemia (figure 21). The majority of these cells were of myeloid lineage (positive for myeloperoxidase) permitting the diagnosis of chronic myeloid leukemia (figure 21).

Since we were interested in ERβ signaling and previous studies have shown ERβ knockout mice to have a similar immune phenotype as the AhR knockout mice described here, we examined expression of ERβ. Immunohistochemical staining of ERβ in the spleens, bone marrow and invading cells of the liver demonstrated that ERβ is expressed in the immune system of AhR knockout mice (figure 22). There was also no significant difference in the expression of ERβ in the immune systems of AhR -/- and wild-type mice (figure 22).

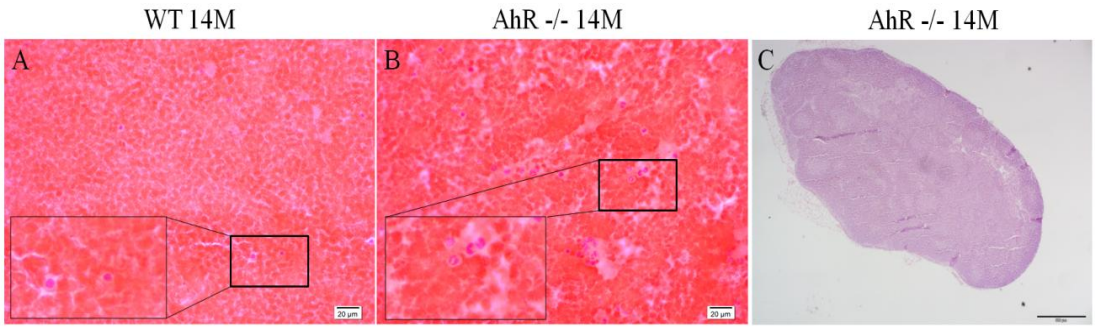


Figure 18. Blood smears and lymph nodes from 14-month-old AhR knockout mice. There is increased white blood cell count in AhR -/- mice (B) when compared to wild-type littermates (A). Lymph nodes were greatly enlarged in AhR -/- mice (C) and were not visible in wild-type mice. 5 mice in each group were examined, representative pictures are shown. Scale bars represent 20 μm.

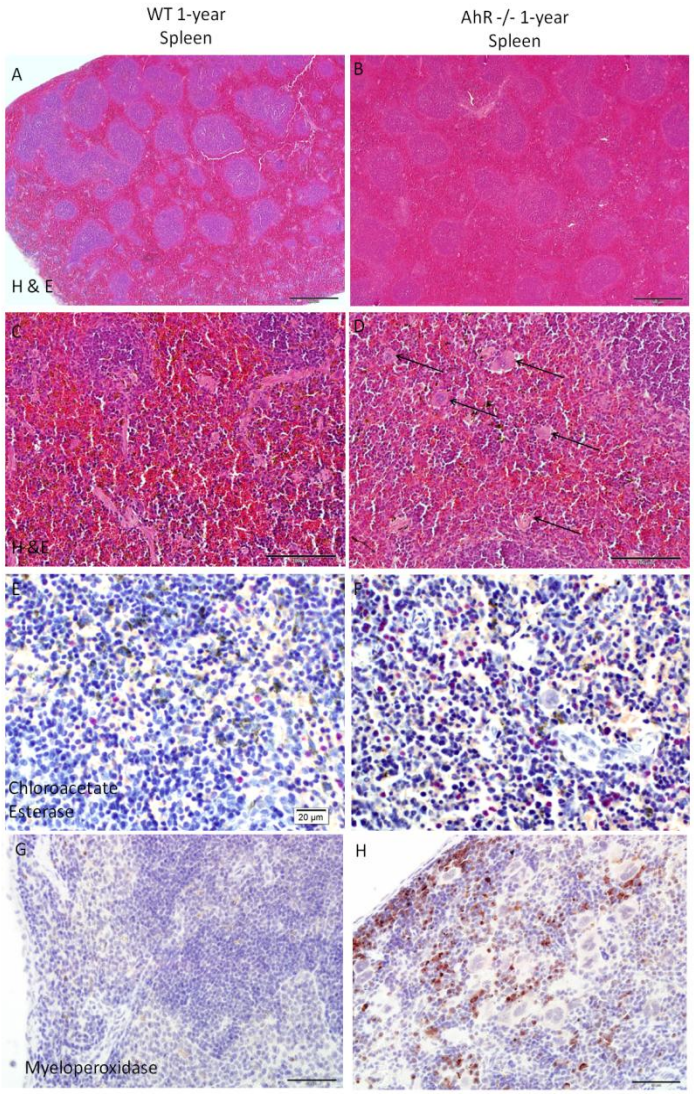


Figure 19. Abnormalities in the spleens of 1-year-old AhR -/- mice. AhR -/- mice suffer from splenomegaly and a loss of defined structure between the white and red pulp (B). There are many megakaryocytes invading the spleens of AhR -/- mice (D, arrowheads) while this type of cell is very rare in wild-type spleens (C). Both chloroacetate esterase (E, F) and myeloperoxidase (G, H) staining demonstrate there are many more granulocytes in the AhR -/- spleens (F, H) compared to wild-type animals (E, G).

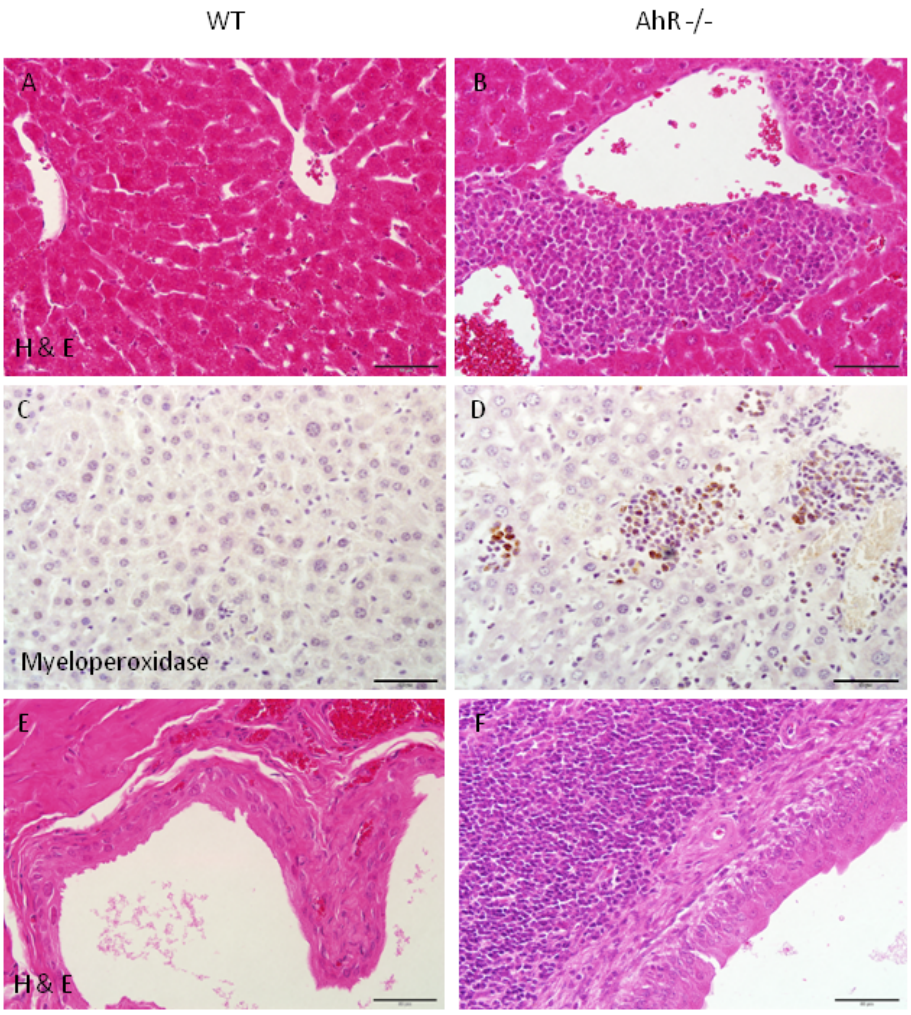


Figure 20. Immune invasion into the liver and bladders of the AhR -/- mice. There is massive immune invasion into the livers of AhR -/- mice which is very severe by 1-year of age (B). Myeloperoxidase staining demonstrates the majority of the invading cells are granulocytes (D). Immune invasion also occurs in other tissues such as the urinary bladder (F).

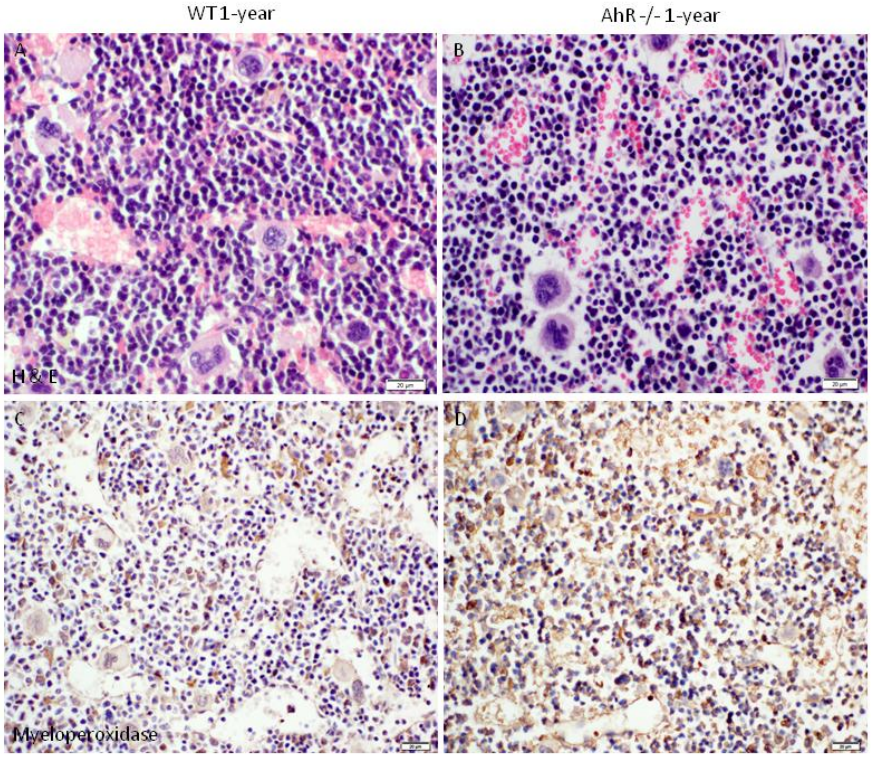


Figure 21. Bone marrow of 1-year-old AhR -/- mice. There are more immature cells in the AhR -/- bone marrow (B) and more cells stain for myeloperoxidase (D); these are characteristics of blast crisis in chronic myeloid leukemia.

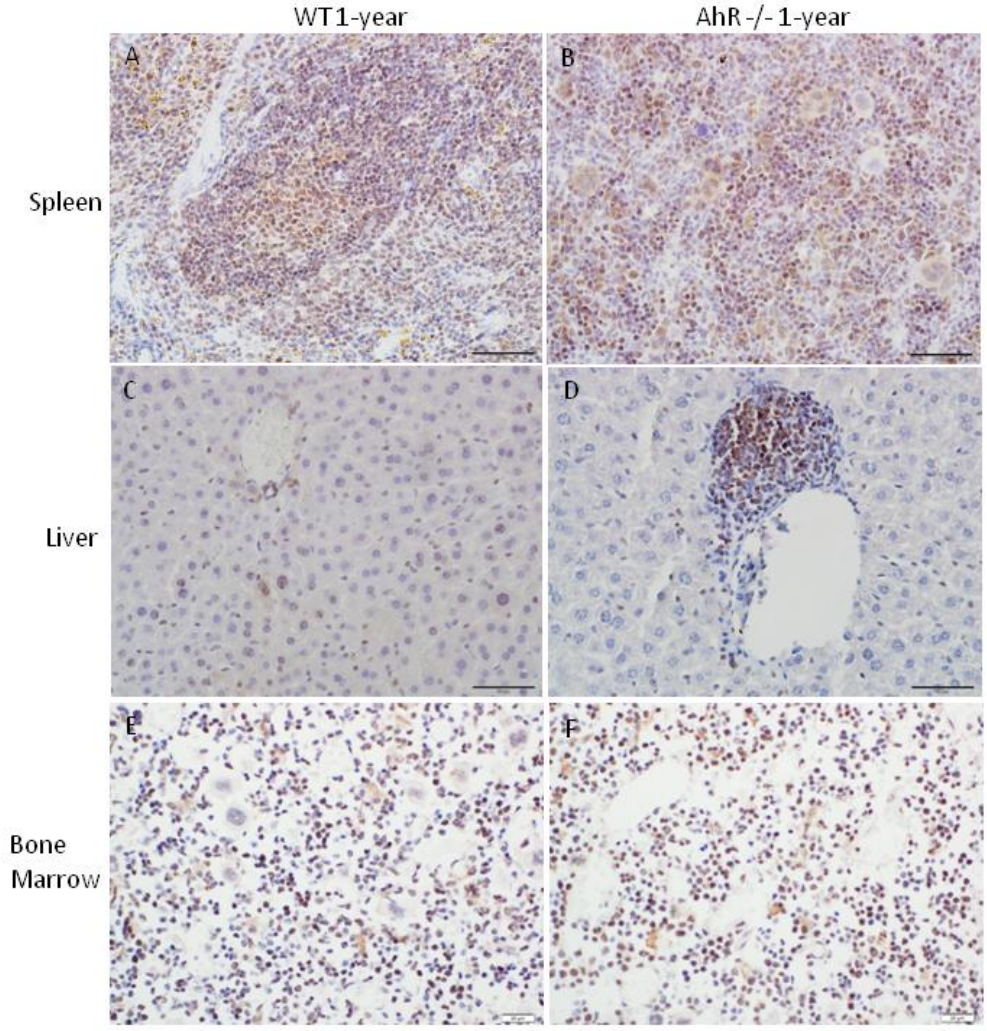


Figure 22. Immunohistochemical expression of ERβ in WT and AhR -/- 1-year-old mouse tissues. The immune cells of the spleen, liver and bone marrow of AhR -/- mice (B, D, F) express ERβ at similar levels as wild-type mice (A, C, E). Representative pictures are shown from 5 mice per group.

5.3 Discussion

These results help to elucidate the role of AhR in the immune system and also suggest a possible connection between AhR with chronic myeloid leukemia. We show here that AhR -/- mice develop an extremely severe immune phenotype after 1-year of age, with increased white blood cell count, splenomegaly, and immune invasion (especially myeloid cells) into many tissues. Our finding of an overactive immune system is compatible with a role for AhR in regulating Treg cells. It remains to be determined why one other strain of AhR knockout mice had decreased lymphocyte count in the spleen (Fernandez-Salguero, Pineau et al. 1995). There have been inconsistencies between the different AhR knockout strains over the years and the reasons for this may be due to differing knockout strategies or the variable environments the mice are raised in. It is also possible that deletion of some regions of the receptor in the different knockout mice may lead to a constitutively active receptor.

Previous studies have demonstrated that AhR is important in the activation of Treg cells (Gandhi, Kumar et al. 2010) and can influence the differentiation and proliferation of HSCs (Casado, Singh et al. 2011). It is reasonable to expect that if AhR is important in activating the inhibitory cells (Tregs) of the immune system, then a lack of AhR would result in an overactive immune system. It is also understandable that the progenitor cells of the AhR knockout mouse are altered due to the role of AhR in regulating HSC differentiation.

Chronic myeloid leukemia is characterized by increased numbers of circulating myeloid cells which may also cause spelomegaly and invade into other tissues (Jabbour and Kantarjian 2012). Later stages of CML are characterized by a build-up of more immature cells in the bone marrow, a phenomenon referred to a blast crisis (Radich 2011). Although CML is almost always caused by an ABL-BCR mutation, we demonstrate here that AhR knockout mice can develop a similar phenotype. It is possible that mutations in AhR in humans can also contribute to CML. A small number of studies have investigated AhR in relation to leukemia, however, they mostly focus on the pro-cancerous role of AhR ligands (Yu, Loehr et al. 2006; Xu, Li et al. 2007). Since some studies have shown a function for AhR in hematopoiesis (Singh, Wyman et al. 2009) and leukemia usually develops due to defects in this process, there is a strong possibility that AhR is involved in CML or other forms of leukemia.

We also noted in this study that the AhR knockout mice had a very large number of megakaryocytes in their spleens. This is an interesting finding because AhR has been shown to induce megakaryocyte polyploidization and AhR knockout mice were previously shown to have lower platelet counts (Lindsey and Papoutsakis 2011). It is possible that higher numbers of megakaryocytes are lodged in the spleens of AhR knockout mice and not able to function properly, explaining the lower platelet count.

The results shown here regarding the immune system of the AhR knockout mouse help to further explain the bladder phenotype described in chapter 4 (figure 23). The elevated uric acid in the previous study was determined to be caused by the breakdown of cells in the urinary bladder. Tumor lysis syndrome is most common in patients with

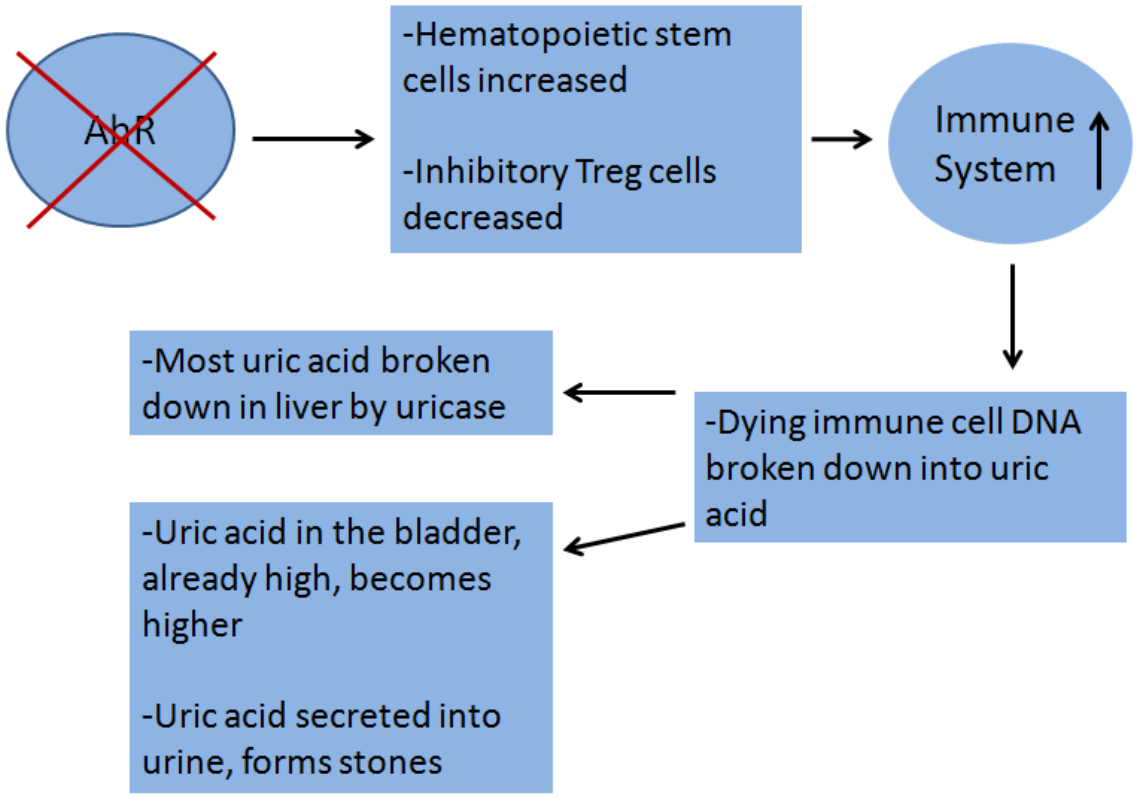


Figure 23. Proposed mechanism of the immune phenotype in AhR -/- mice and how it relates to the uric acid phenotype. AhR is important in activating Treg cells as well as inhibiting HSC proliferation and differentiation; therefore, deletion of AhR should increase HSCs and decrease Tregs. The immune system then becomes overactive (similar to CML) and the uric acid produced by the large number of dying immune cells contributes to the elevated uric acid in the bladder.

leukemia because a large breakdown of cells in the blood can lead to widespread hyperuricemia (Michallet, Tartas et al. 2005). Since the uric acid stones in the AhR knockout bladders grow larger as the mice age and the immune system phenotype becomes noticeable at later ages, it is possible that breakdown of these immune cells contributes to the elevated uric acid. Stones most likely begin to form by the death of cells in the bladder itself at younger ages and the leukemia at later ages causes the stones to grow further. Levels of uric acid in the blood do not increase in the AhR knockout mice, nor do stones form in parts of the body other than the bladder. The uric acid is confined to the bladder most likely because the enzyme uricase in the mouse liver is able to metabolize the uric acid that enters the bloodstream.

It is of particular interest that the immune phenotype of the AhR knockout mice is similar to that of the ERβ knockout mice (Shim, Wang et al. 2003). These mice also develop a myeloproliferative disorder similar to CML and, because of that, we investigated if ERβ was still present in the AhR knockout immune system. Our results showed that ERβ was present at normal levels in the AhR knockout mice (figure 22). Future studies are planned to test the effects of a specific ERβ ligand in AhR knockout mice. If these experiments are successful, AhR -/- mice would be relieved of their myeloproliferative disorder and ERβ could be a possible drug target for CML treatment.

In conclusion, we demonstrate an important function for AhR in the immune system. These studies also help to explain the urinary bladder phenotype in these mice, as well as suggest a possible role for AhR in CML. Future studies should further investigate

the mechanisms of AhR in the immune system, look for AhR mutations in CML patients and to test the effects of an ERβ ligand in the AhR knockout mice.

6. Hormone Replacement Therapy and its Effects on the Postmenopausal Breast

6.1 Introduction

Estrogen therapy is highly effective in relieving menopausal symptoms as well as for preventing postmenopausal osteoporosis, and reducing the incidence of colon and gastric cancer and improving life quality (Kronenberg and Fugh-Berman 2002; Nelson, Humphrey et al. 2002; Hickey, Davis et al. 2005; Wren 2009). The observation in the 1970s that increased endometrial cancer occurred in women taking estrogen alone led to the development of a standard hormonal therapy (HRT) with estrogen plus progestogen to women with intact uterus (Smith, Prentice et al. 1975; Kronenberg and Fugh-Berman 2002; Hickey, Davis et al. 2005). The risk of endometrial cancer was thus reduced by the combination of progestogen with estrogen treatment (Hickey, Davis et al. 2005; Yeh 2007). A major problem with HRT was confirmed in 2002, with the publication of the Women's Health Initiative (WHI) study (Rossouw, Anderson et al. 2002; Chlebowski, Hendrix et al. 2003; McTiernan, Martin et al. 2005) and Million Women Study (Beral 2003). These studies showed that the risk of breast cancer was increased in women taking estrogen-progestin therapy for more than 5 years when compared to women taking estrogen alone and non-users. These studies also failed to confirm a protective effect of HRT against arteriosclerosis. These reports resulted in a world-wide decrease in the use of HRT (Verkooijen, Bouchardy et al. 2009). Recently, the WHI study has been re-evaluated and HRT has again become a highly debated topic in the international menopause community (Gompel, Rozenberg et al. 2008; Pines 2008; Bluming and Tavris 2009).

There have been many studies on the contribution of progestogen to stimulation of breast proliferation and breast cancer development. However, a comparison of the findings reported from different studies is difficult because different types of estrogens and progestogens have been used in each study (Stahlberg, Pedersen et al. 2003; Sitruk-Ware and Plu-Bureau 2004; Campagnoli, Clavel-Chapelon et al. 2005; Biglia, Mariani et al. 2007; Yeh 2007; Skouby and Jespersen 2009). In the USA the most commonly used progestogen is medroxyprogesterone acetate (MPA), while in Europe the progestogen component of HRT is mainly represented by 19-nortestosterone (norethindrone) acetate derivatives (NETA) (Gadducci, Biglia et al. 2009). The difficulty of obtaining breast tissue from healthy postmenopausal women has impeded our understanding of the direct effect of progestogen-induced proliferation in normal breast. Studies using samples obtained by fine needle biopsies indicated that E2/NETA treatment could stimulate proliferation with an increase of the proliferation marker, Ki67 (Conner, Soderqvist et al. 2003; Conner, Christow et al. 2004). Usually, very few breast structures are obtained with fine needle biopsies of normal women. With middle needle biopsies under the guidance of ultrasound, we succeeded in obtaining breast tissue with normal structures from healthy postmenopausal women (Cheng, Wilczek et al. 2007). In the present study, we evaluated the effects of E2 and E2/NETA treatment on breast density, proliferation and expression of steroid hormone receptors in healthy postmenopausal women.

6.2 Results

6.2.1 Physiological effects of HRT on postmenopausal women

Of the 60 women, four dropped out due to side effects. The reasons were as follows: one taking estradiol alone (nausea) and three taking estradiol and norethindrone (hypertension, headache and nausea). Fifty-six women completed the full 12 weeks of study. The women in both groups were of similar age and years since menopause (YSM) (Table 3). Body weights were recorded for each of the women before and after treatment. No significant changes in weight or body mass index (BMI) were found in either group after treatment ($P>0.05$). There were no changes in blood pressure, kidney function, liver function (p-ASAT and p-ALAT), or thyroid function (S-TSH and S-T4) in either group. S-TSH was 2.2 ±0.9 and 2.3±1.0 before and after E2/NA treatment (mE/L, $P>0.05$), 1.9±0.8 and 2.1±1.0 before and after E2 treatment (mE/L, $P>0.05$), respectively. S-T4 was 15.9 ±2.5 and 15.3±1.7 before and after E2/NA treatment (pmol/L, $P>0.05$), 14.9±2.9 and 14.7±3.0 before and after E2 treatment (pmol/L, $P>0.05$), respectively.

In this study, 27 women showed varied degrees of symptoms of hot flashes and sweating during the day and night. The scores for hot flashes and sweating, which were calculated as frequency multiplied by intensity, were significantly reduced by both treatments (Table 4).

Blood samples from 56 women were analyzed for cholesterol, triglycerides, high-density-lipoprotein-cholesterol (HDL-C) or low-density-lipoprotein-cholesterol (LDL-C). No significant changes of lipids were induced by E2 ($P>0.05$). However, E2/NA induced

a significant decrease in cholesterol (5.7±0.9 vs 5.0±0.9, P<0.05), LDL-C (3.3±0.7 vs 2.9±0.8, P<0.05), and HDL-C (1.9±0.4 vs 1.6±0.4, P<0.05) (Table 4).

6.2.2 Breast density how it relates to HRT and collagen

Mammography was performed before and after treatment in all women and 114 samples were obtained. Normal breast structure could be identified in 93 samples as we previously described (Cheng, Wilczek et al. 2007) . In each section 60 to 5000 (average 630) epithelial cells in lobules or ducts could be found. In 21 samples there were no epithelial cells but only fat tissue or connective tissue.

When breast density was classified by mammography, none of the participants presented with complete breast density i.e, a score of 5. Of the 29 women who were treated with estradiol alone, only one women showed increased density from score 2 before treatment to score 3 after treatment. The changes in score were as follows: 1→1, 13 women; 2→2, 11 women; 3→3, 4 women; 2→3, 1 woman. Of the 27 women who were treated with estradiol and norethindrone acetate, 5 women showed increased breast density after treatment. The changes in their scores were as follows: 1→1, 11 women; 1→2, 2 women; 2→2, 8 women; 2→3, 2 women; 3→3, 1 woman; 3→4, 1 woman; 4→4, 2 women. There was no statistical difference in breast density changes between the two treatments (chi-square = 3.32, P=0.068).

Table 3. Basic patient information

	E2/NA	E_2
Case	27	29
Age	62.3 ± 5.7	61.4 ± 5.3
YSM	12.2 ± 5.7	11.6 ± 5.4
Height (cm)	163.5 ± 6.8	165.1 ± 7.2
Weight (Kg)	69.4 ± 14.3	68.6 ± 15.5
BMI	25.7 ± 4.0	25.1 ± 4.6

Data are mean ± SD. YSM: years since menopause. BMI, body mass index= weight (kg)/height $(m)^2$.

Table 4. Changes in climacteric symptoms

	No	Hot flashes		Sweating	
		Day	night	day	night
E2/NA					
Before	10	3.6 ± 0.8	3.6 ± 1.0	3.9 ± 1.4	2.2 ± 0.6
After	10	0.3 ± 0.2**	0.6 ± 0.3*	1.1 ± 0.7*	1.1 ± 0.5
E2					
Before	17	3.7 ± 1.1	4.3 ± 1.4	3.7 ± 1.0	3.6 ± 1.0
After	17	0.8 ± 0.4*	1.3 ± 0.5*	0.9 ± 0.3*	1.1 ± 0.4**

Data are mean ± SD. * P<0.05; ** P<0.01 (Compared with pretreatment by paired-sample t-test).

Table 5. Levels and changes of lipoprotein lipids (mmol/L)

	No	Cholesterol	Triglyceride	HDL-C	LDL-C
E2/NA					
Before	27	5.7 ± 0.9	1.3 ± 0.5	1.9 ± 0.4	3.3 ± 0.7
After	27	5.0 ± 0.9**	1.1 ± 0.4	1.6 ± 0.4*	2.9 ± 0.8*
E2					
Before	29	5.5 ± 1.0	1.2 ± 0.7	2.0 ± 0.7	2.9 ± 0.8
After	29	5.3 ± 1.1	1.3 ± 0.6	1.9 ± 0.4	2.8 ± 1.1

Data are mean ± SD. * $P<0.05$, ** $P<0.01$ (compared with before treatment by paired t-test).

All sections were stained for collagen with Masson's trichrome. Breasts that were evaluated by the mammographer and pathologists as dense all stained strongly for collagen (blue, 33 samples) while those which were evaluated as not dense (slight traces of blue, all remaining samples) had only sparse collagen fibrils (figure 24). The difference was very clear.

6.2.3 The effects of HRT on the expression of nuclear receptors in the breast

Expression of estrogen receptors (ERα, ERβ, and ERβcx) was measured in the biopsy samples by immunohistochemistry. ERα was detected in 82% of the biopsies and in these less than 10% of epithelial cell nuclei were ERα-positive (figure 25). ERβ was detected in most of the samples and more than 50% of the epithelial nuclei were ERβ-positive (figure 25). ERβcx was not detected in the nuclei of the epithelial cells but some stromal cells were positive. Percentages of $ER\alpha^{+}$ or $ER\beta^{+}$ cells were counted in each sample and compared before and after treatment. No significant changes in ERα or ERβ were found in either group after treatment as evaluated by comparison of the means (Table 6, figure 25, figure 26).

Progesterone receptor isoforms, PR-A and PR-B, were detected in 83% of the breast biopsies. In both groups, PR-A and PR-B expression in the samples taken after treatment were significantly up-regulated as compared with those in samples taken before treatment ($P<0.05$) (figure 26). The average percentage of positive cells of PR-A and PR-B was increased by 2-3 fold by E2 and E2/NA (Table 6, figure 25).

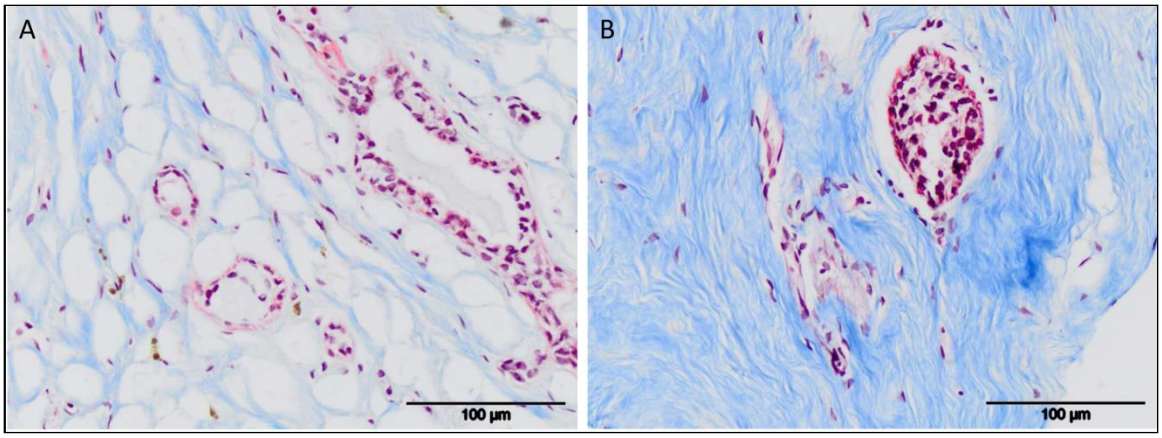


Figure 24. Breast density is correlated with collagen content in postmenopausal breasts. Representative pictures of Masson's trichrome staining for collagen (blue). In the breasts determined to be non-dense by mammography there are few collagen fibrils (A). In breasts evaluated by mammography to be dense, there was abundant staining for collagen (B). Both samples are from the untreated group. Scale bars represent 100 µm.

Aryl hydrocarbon receptor was present in the breast epithelium of almost all of the samples measured where it was localized in the cytoplasm and nuclei (figure 26). There was no change in the expression levels of AhR upon treatment with E2 or E2/NA.

Androgen receptor (AR) was detected in 79% of the samples. No significant changes in the number of AR-positive cells were found in either group after treatment (Table 6, figure 25).

6.2.4 Proliferation in the postmenopausal breast and its relation to HRT and HER2

Proliferation was evaluated by Ki67 expression. The percentage of proliferating cells in the samples was low, ranging between 0.5 to 2.5% and did not change upon either treatment regimen. What was most interesting was that the proliferating cells were located in patches of breasts that were otherwise totally silent. Furthermore, the presence of KI67-positive nuclei bore no relationship to any of the steroid receptors. However, human epithelial growth factor receptor 2 (HER2) was expressed in the ducts harboring proliferating epithelial cells. Out of 31 samples that were stained, resting ducts did not express HER2 (21 samples) but in ducts with Ki67-positive cells, (10 samples) there was clear plasma membrane staining of HER2 (figure 27).

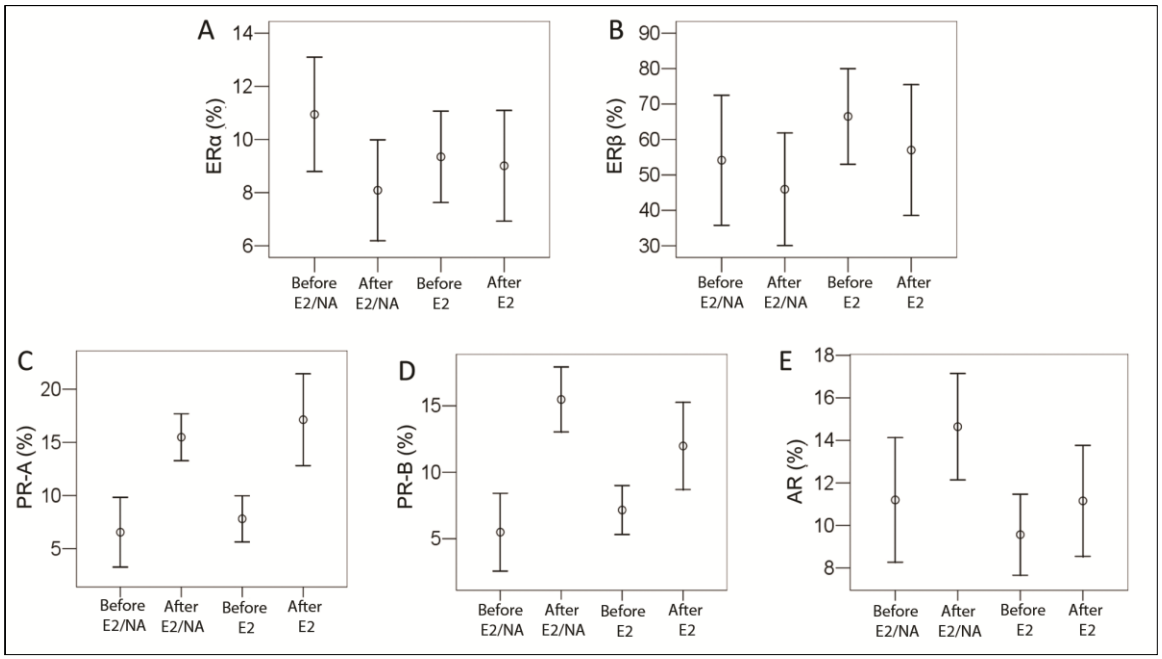


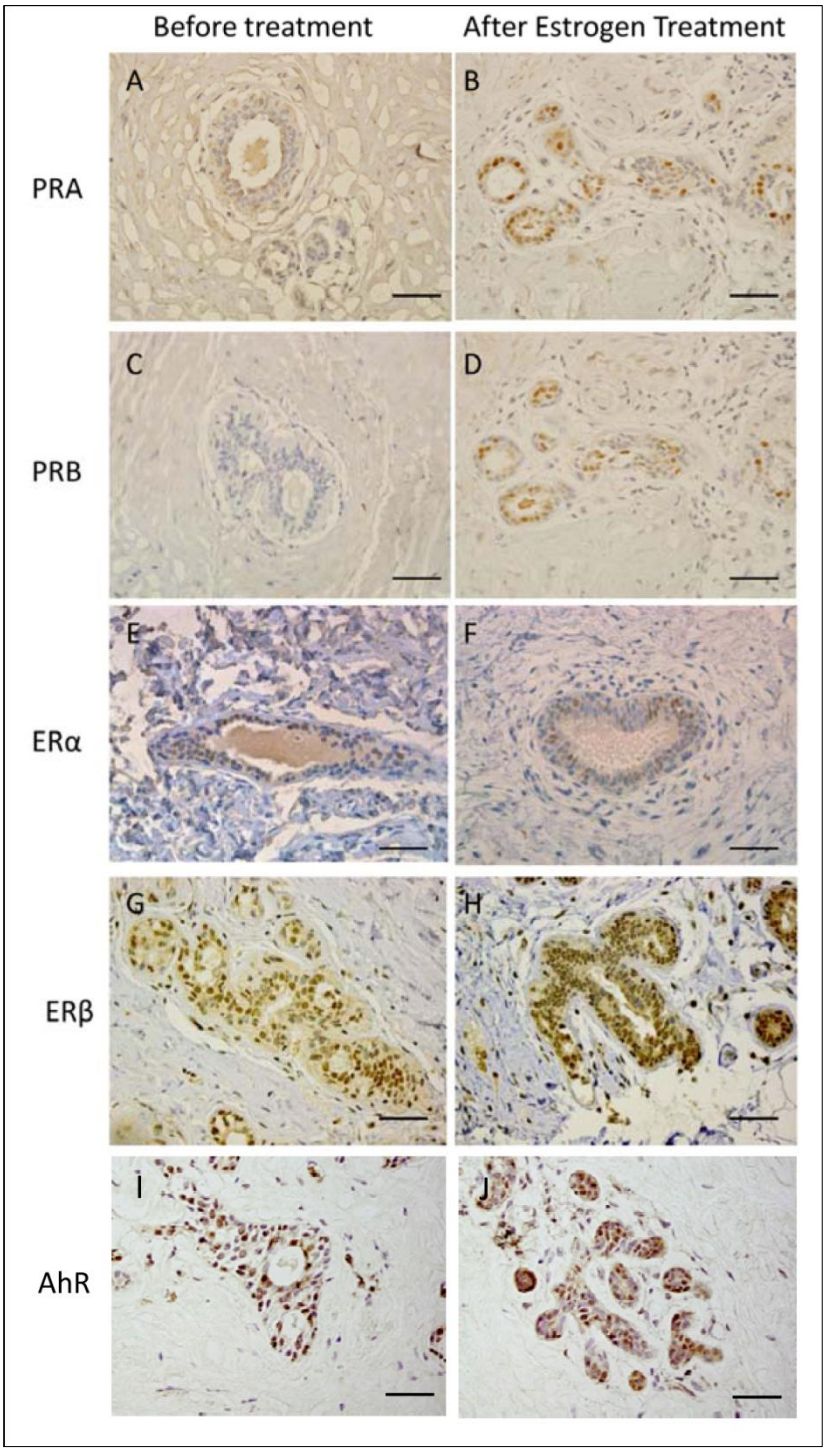
Figure 25. Comparison of the average percentages of cell nuclei positive for nuclear receptors in postmenopausal breasts treated with HRT. Increases in PR-A (C, $P<0.05$) and PR-B (D, $P<0.05$) are seen after treatment in both E2/NA and E2 groups while there was no significant change in the other receptors (A, B, E, $P>0.05$). E2/NA group before treatment, n=20; E2/NA group after treatment, n=20; E2 group before treatment, n=22; E2 group after treatment, n=22. The percentage of cells stained positive for each receptor was taken for each slide and results are shown as means ± SD for each group.

Table 6. Percentages of nuclear receptor-positive cells before and after HRT treatment.

	No	ERα	ERβ	PR-A	PR-B	AR
E2/NA						
Before	20	11 ± 10	54 ± 34	7 ± 15	6 ± 13	11 ± 13
After	20	8 ± 9	46 ± 31	16 ± 10*	16 ± 11*	15 ± 11
E2						
Before	22	9 ± 9	67 ± 21	8 ± 10	7 ± 9	10 ± 9
After	22	9 ± 10	57 ± 29	17 ± 20*	12 ± 14	11 ± 12

Data are mean ± SD. * P<0.05; ** P<0.01 (Compared with pretreatment by t-test).

Figure 26. The number of cells expressing progesterone receptors increases after HRT while estrogen receptors remain unchanged. The number of PRA and PRB-positive cells was significantly higher in breast samples after estrogen treatment (B, D) than before treatment (A, C). The majority of epithelial cells are ERα negative both before treatment (E) and after treatment (F). More than 50% of epithelial cells are ERβ-positive before (G) and after treatment (H). AhR was present in the nuclei and cytoplasm of most epithelial cells but was not changed upon estrogen treatment (I, J). Representative pictures are shown. Results are similar in all women from the estrogen only treatment and with women in the combined treatment group. Scale bars represent 20 μm.


Before treatment
After Estrogen Treatment
PRA
PRB
ERα
ERβ
AhR
A
B
C
D
E
F
G
H
I
J

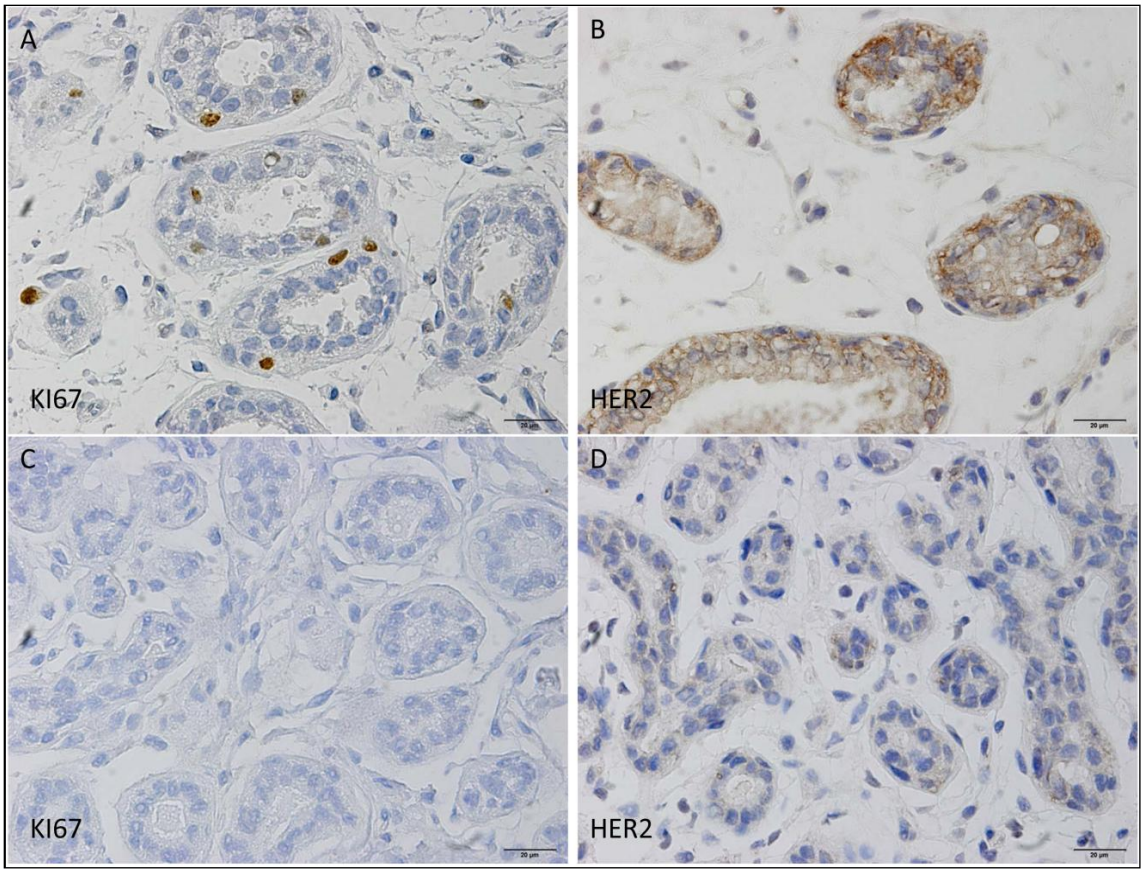


Figure 27. Expression of HER 2 in proliferating breast tissue. Ducts with proliferating epithelial cells (A) also expressed HER 2 (B). Non-proliferating breasts (C) were HER 2 negative (D). (A) and (B) are from the same patient and (C) and (D) are from the same patient. Scale bars represent 20 μm.

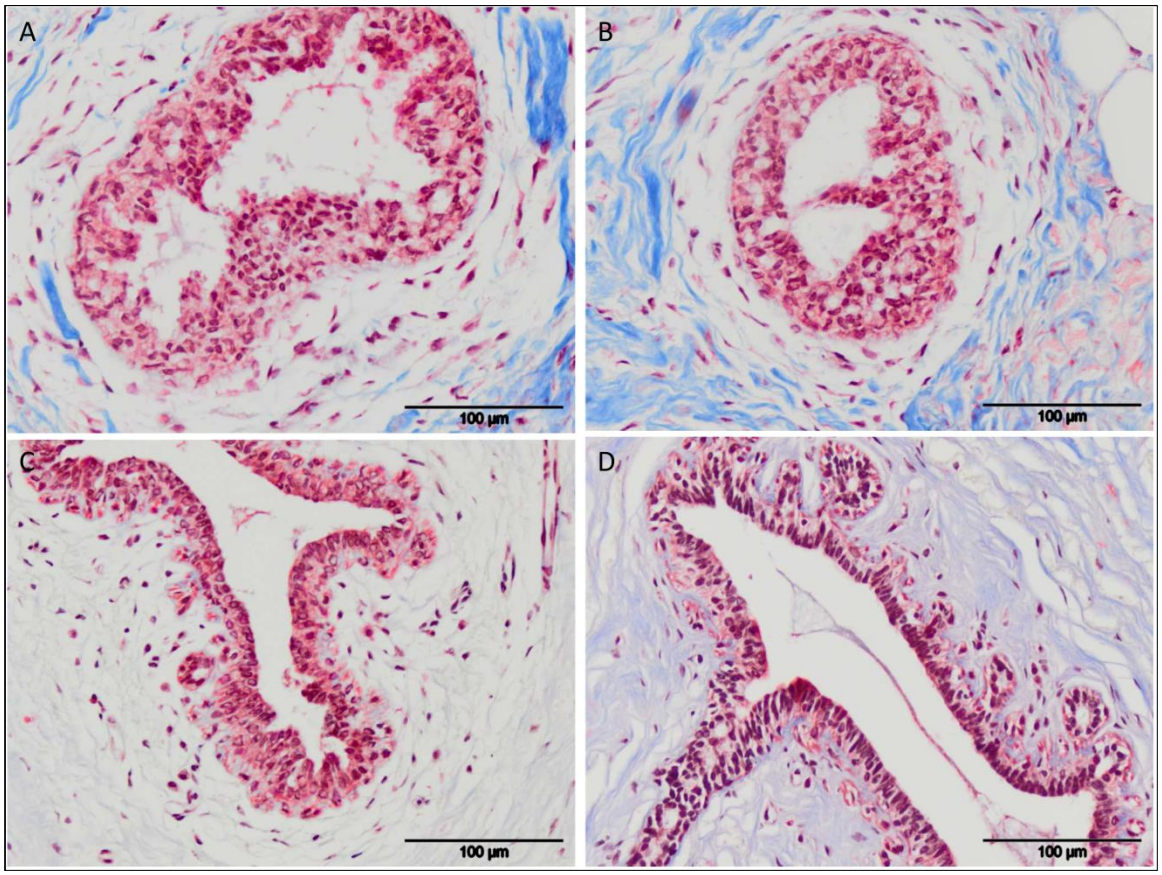


Figure 28. Histological abnormalities in some of the postmenopausal breast samples. Most of the breast samples with areas of high proliferation were determined to have usual ductal hyperplasia (UDH) (A, B) while in one sample there was atypical lobular hyperplasia (ALH) (C). In one sample there was columnar cell changes (D). Samples were stained with Masson's trichrome, scale bars represent 100 µm.

The breast samples which contained an abnormal number of proliferating cells were evaluated by two pathologists, Dr. Roger Price and Dr. Alejandro Contreras, both from Baylor College of Medicine. In almost all of the proliferating samples there was usual ductal hyperplasia, a benign growth of epithelial cells within the duct. Only one sample had atypical lobular hyperplasia, a benign proliferation of epithelial cells with some atypical cellular characteristics. In one sample there was columnar cell change which is a change in morphology of epithelial cells to a more elongated shape. This change is related to an increasing the risk of malignancy (figure 28).

6.3 Discussion

The main purpose of this study was to investigate the effect of short term exposure to estrogen and progestin on proliferation and hormone receptor expression in breast of postmenopausal women. The secondary aim was to evaluate the effects on lipids and climacteric symptoms. The drugs used in this study, E2/NA and E2, are the most prescribed HRT treatments in northern Europe for postmenopausal women.

To evaluate the effects of HRT, we have successfully used middle needle biopsy of breast tissue by employing ultrasound to identify glandular tissue. When we began this study we took fine needle biopsies from 30 patients. All we got was some fat and some red blood cells. With the same clinician and the same histochemist, we switched to middle needle biopsies and got very nice samples with epithelial structures. In a previous study, lobular or ductal structures could be shown in 52% (53/102) of samples with a single puncture of breast biopsy (Cheng, Wilczek et al. 2007). In the present study, we

performed two punctures for each biopsy. Epithelial cells and normal breast structures could be identified in 81% (93/114) of the biopsies in which there were 40 to 5000 (average 630) epithelial cells in each section.

Ki67 is a widely used proliferation marker in breast (Yerushalmi, Woods et al.). The maximal proliferation of the breast epithelium of sexually mature and normally cyclic women occurs during the luteal phase but not during the follicular phase, indicating that progesterone stimulates breast proliferation (L'Hermite, Simoncini et al. 2008). In the normal breasts of postmenopausal women the number of proliferating cells was quite low, ranging between 0.7-2.5% in the biopsy samples from women with or without treatment. We found no change in the number of proliferating epithelial cells following the HRT.

In most of the samples there were very few proliferating cells, however, the distribution of proliferating cells was not homogeneous and in some samples, there were nests of proliferating epithelial cells. Some of these proliferating nests were found in untreated women. The regions of the breast samples that were proliferating were diagnosed by the pathologist as regions of benign hyperplasia. We found that HER2 was expressed in some of the proliferating breasts (figure 27). HER2 is a member of the epidermal growth factor receptor (EGFR) family (Ross, Slodkowska et al. 2009). HER2 gene amplification and protein expression is correlated with increased cell proliferation, metastasis and decreased survival rates for breast cancer patients (Slamon, Clark et al. 1987; Ross, Slodkowska et al. 2009). Our data reveals that HER 2 may be expressed in

some normal breasts. The significance of the presence of this receptor and its relationship to breast proliferation requires further examination.

In this study we found that ERα was expressed in less than 11% of breast epithelial cells in postmenopausal women while ERβ was expressed in more than 50% of the epithelial cells. ERβcx was not detected in the breast epithelium. ERβcx is a splice variant of ERβ which does not bind estradiol but can inhibit the effects of ERα. It is not expressed in normal tissue but has been found in some cancers (Omoto, Kobayashi et al. 2002). In the stromal cells, there was nuclear staining of ERβ and ERβcx. No marked changes in ERα, ERβ, or ERβcx expression were observed after the 3-month treatment with E2 or E2/NA. However, we found significant increases in progesterone receptors after both treatments. The average percentage of cells positive for PR-A and PR-B was increased by 2- 3 fold after either hormone treatment. The up-regulation of progesterone receptors might be related to the activation of ERα. In a previous study using the same method, we did not observe obvious changes in progesterone receptors in the breasts of women who were treated with isoflavones (Cheng, Wilczek et al. 2007).

AR is another member of the nuclear receptor super-family. In a recent study, Hofling et al. reported that AR expression was markedly increased by tibolone and suppressed by combined conjugated equine estrogens (CEE)/MPA in macaques (Hofling, Ma et al. 2009). It is also known that norethindrone acetate can bind to AR (Skouby and Jespersen 2009). In the present study, however, we did not find any changes in AR after the 3-month treatment with estradiol or estradiol plus norethindrone acetate.

In summary, this small short-term prospective study showed that the administration of estradiol (E2) and estradiol plus norethindrone acetate (E2/NA) daily to postmenopausal women, effectively relieved hot flashes, and up-regulated expression of progesterone receptors (PR-A and PR-B) in breast epithelium in postmenopausal women, while estrogen receptors, androgen receptor, proliferation, and breast density were not affected by either of the HRT.

7. General Summary and Conclusions

AhR and ERβ are two transcription factors with key roles in human health and disease. The initial aim of the studies was to establish whether there is an interaction between ERβ and AhR as there is between AhR and ERα. The optimal tissue for such a study is the prostate which expresses both AhR and ERβ. In the very first attempts to investigate this crosstalk, we collected prostates from wild-type and AhR -/- mice. At the end of the tissue collection the petri dish with the AhR-/- prostates was filled with small round green-yellow stones. It turned out that in order to remove the ventral prostate, the urinary bladder had to be lifted with a pair of forceps and clipped off. The urinary bladder of the AhR -/- mice was filled with stones. The presence of stones packed like in peas in a pod in the urinary bladder was far too fascinating to pass up so we allowed ourselves to be distracted from our initial goal in order to understand the meaning of this mystery.

The studies in this dissertation have revealed roles for AhR signaling in the immune system and prostate which could not have been found if we had not followed our instincts and investigated the bladder stones. The role of HRT in the postmenopausal breast revealed some interesting data about hormones, breast proliferation and breast density but showed no obvious connection with AhR or ERβ.

For decades, AhR has been known to be a receptor for environmental contaminants such as the HAHs and PAHs. The roles AhR plays in mediating the hazardous effects of these compounds have been studied extensively, however, the

normal physiological functions of AhR are much less understood. The development of AhR knockout mice by three different labs has aided in the understanding of AhR biology but the inconsistent phenotypes between different strains of these mice have led to confusion in the field. We investigated the phenotype of the AhR knockout mice developed by the lab of Dr. Fujii-Kuriyama with special interest in the connection between AhR and estrogen signaling. We made the discovery that the AhR knockout mice develop stones comprised of uric acid in their urinary bladders; a phenotype which very rarely occurs in mice.

In pursuing the source of the uric acid bladder stones we were led not to a role of AhR in purine metabolism but to a role for AhR in preventing cytoxicity in the bladder and in control of the immune system.

There are several human diseases related to elevated levels of uric acid including urinary bladder/ kidney stones, gout and Lesch-Nyhan disease. These diseases may be understood more clearly due to these studies and AhR could be investigated further as a possible target for treatment in uric acid-related conditions.

The roles of AhR in regulating the immune system have been studied by many different investigators but much remains to be understood. The present studies demonstrate a possible role for AhR in chronic myeloid leukemia. CML in humans usually is caused by a mutation in a tyrosine kinase; however, these studies demonstrate a possible role of AhR in this disease.

Prostatic diseases, including prostate cancer and BPH, affect a large number of men in the United States and are a growing health concern in an aging population. The present studies demonstrate a role for AhR in prostatic growth in older mice; primarily in the stroma of the ventral prostate. Since AhR may have an anti-proliferative role in the prostate, it may be an important candidate for future research in prostate cancer and BPH.

This was also one of very few studies where breast biopsies were taken from normal women. We demonstrated that 3 months of hormone replacement therapy had no significant effects on proliferation of the breast or induced changes in any markers studied besides progesterone receptors. We did demonstrate, however, that some normal breasts with non-malignant hyperplasia expressed HER2 and we suggest that HER2 may cause pre-cancerous proliferation in breasts. We also found that breast density is not affected by HRT but is in fact reflective of breast fibrosis which can be visualized by histochemical stain for collagen. Since dense breast is a risk factor in breast cancer, future studies explaining the mechanism of increased breast fibrosis could aid in the understanding of breast cancer. Since this study only used a relatively short-term HRT, future studies using samples from patients treated for a longer period of time may demonstrate more effects of HRT on the breast.

In conclusion, these studies help elucidate roles for ligand-activated transcription factors, primarily AhR and ERβ, in several human diseases. AhR has long been considered to primarily mediate harmful effects of toxic compounds and studies such as these help add to the growing understanding of AhR signaling. While much remains to be

understood in regard to signaling through these receptors, the studies in this thesis provide a foundation upon which to build future investigations.

8. References

Abbouni, B., H. M. Elhariry, et al. (2004). "Overproduction of NAD+ and 5'-inosine monophosphate in the presence of 10 microM Mn2+ by a mutant of Corynebacterium ammoniagenes with thermosensitive nucleotide reduction (nrd(ts)) after temperature shift." Arch Microbiol **182**(2-3): 119-125.

Abel, J. and T. Haarmann-Stemmann (2010). "An introduction to the molecular basics of aryl hydrocarbon receptor biology." Biol Chem **391**(11): 1235-1248.

Ahmad, I., R. J. Barnetson, et al. (2008). "Keratinizing squamous metaplasia of the bladder: a review." Urol Int **81**(3): 247-251.

Akhtar, F. Z., D. H. Garabrant, et al. (2004). "Cancer in US Air Force veterans of the Vietnam War." J Occup Environ Med **46**(2): 123-136.

Akhurst, R. J. and A. Hata (2012). "Targeting the TGFbeta signalling pathway in disease." Nat Rev Drug Discov **11**(10): 790-811.

Andriole, G., N. Bruchovsky, et al. (2004). "Dihydrotestosterone and the prostate: the scientific rationale for 5alpha-reductase inhibitors in the treatment of benign prostatic hyperplasia." J Urol **172**(4 Pt 1): 1399-1403.

Baba, T., J. Mimura, et al. (2001). "Structure and expression of the Ah receptor repressor gene." J Biol Chem **276**(35): 33101-33110.

Baba, T., Y. Shima, et al. (2008). "Disruption of aryl hydrocarbon receptor (AhR) induces regression of the seminal vesicle in aged male mice." Sex Dev **2**(1): 1-11.

Baccarelli, A., A. C. Pesatori, et al. (2004). "Aryl-hydrocarbon receptor-dependent pathway and toxic effects of TCDD in humans: a population-based study in Seveso, Italy." Toxicol Lett **149**(1-3): 287-293.

Bardin, A., N. Boulle, et al. (2004). "Loss of ERbeta expression as a common step in estrogen-dependent tumor progression." Endocr Relat Cancer **11**(3): 537-551.

Barkhem, T., B. Carlsson, et al. (1998). "Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists." Mol Pharmacol **54**(1): 105-112.

Barrack, E. R. (1997). "TGF beta in prostate cancer: a growth inhibitor that can enhance tumorigenicity." Prostate **31**(1): 61-70.

Beischlag, T. V., J. Luis Morales, et al. (2008). "The aryl hydrocarbon receptor complex and the control of gene expression." Crit Rev Eukaryot Gene Expr **18**(3): 207-250.

Beral, V. (2003). "Breast cancer and hormone-replacement therapy in the Million Women Study." Lancet **362**(9382): 419-427.

Bergander, L., N. Wahlstrom, et al. (2003). "Characterization of in vitro metabolites of the aryl hydrocarbon receptor ligand 6-formylindolo[3,2-b]carbazole by liquid chromatography-mass spectrometry and NMR." Drug Metab Dispos **31**(2): 233-241.

Betterle, C. and L. Morlin (2011). "Autoimmune Addison's disease." Endocr Dev **20**: 161-172.

Biglia, N., L. Mariani, et al. (2007). "Increased incidence of lobular breast cancer in women treated with hormone replacement therapy: implications for diagnosis, surgical and medical treatment." Endocr Relat Cancer **14**(3): 549-567.

Bluming, A. Z. and C. Tavris (2009). "Hormone replacement therapy: real concerns and false alarms." Cancer J **15**(2): 93-104.

Boffetta, P., K. A. Mundt, et al. (2011). "TCDD and cancer: a critical review of epidemiologic studies." Crit Rev Toxicol **41**(7): 622-636.

Brent, G. A. (2012). "Mechanisms of thyroid hormone action." J Clin Invest **122**(9): 3035-3043.

Brzozowski, A. M., A. C. Pike, et al. (1997). "Molecular basis of agonism and antagonism in the oestrogen receptor." Nature **389**(6652): 753-758.

Burbach, K. M., A. Poland, et al. (1992). "Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor." Proc Natl Acad Sci U S A **89**(17): 8185-8189.

Burger, H. G., E. C. Dudley, et al. (2002). "Hormonal changes in the menopause transition." Recent Prog Horm Res **57**: 257-275.

Burns, C. M. and R. L. Wortmann (2011). "Gout therapeutics: new drugs for an old disease." Lancet **377**(9760): 165-177.

Campagnoli, C., F. Clavel-Chapelon, et al. (2005). "Progestins and progesterone in hormone replacement therapy and the risk of breast cancer." J Steroid Biochem Mol Biol **96**(2): 95-108.

Casado, F. L., K. P. Singh, et al. (2011). "Aryl hydrocarbon receptor activation in hematopoietic stem/progenitor cells alters cell function and pathway-specific gene modulation reflecting changes in cellular trafficking and migration." Mol Pharmacol **80**(4): 673-682.

Chan, T. S. (1979). "Purine excretion by mouse peritoneal macrophages lacking adenosine deaminase activity." Proc Natl Acad Sci U S A **76**(2): 925-929.

Cheng, G., B. Wilczek, et al. (2007). "Isoflavone treatment for acute menopausal symptoms." Menopause **14**(3 Pt 1): 468-473.

Chico, D. E. and I. Listowsky (2005). "Diverse expression profiles of glutathione-S-transferase subunits in mammalian urinary bladders." Arch Biochem Biophys **435**(1): 56-64.

Chlebowski, R. T., S. L. Hendrix, et al. (2003). "Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial." Jama **289**(24): 3243-3253.

Chubb, E. A., D. Maloney, et al. (2010). "Tumour lysis syndrome: an unusual presentation." Anaesthesia **65**(10): 1031-1033.

Collins, A. and B. M. Landgren (1997). "Psychosocial factors associated with the use of hormonal replacement therapy in a longitudinal follow-up of Swedish women." Maturitas **28**(1): 1-9.

Conner, P., A. Christow, et al. (2004). "A comparative study of breast cell proliferation during hormone replacement therapy: effects of tibolon and continuous combined estrogen-progestogen treatment." Climacteric **7**(1): 50-58.

Conner, P., G. Soderqvist, et al. (2003). "Breast cell proliferation in postmenopausal women during HRT evaluated through fine needle aspiration cytology." Breast Cancer Res Treat **78**(2): 159-165.

Conney, A. H. (1982). "Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture." Cancer Res **42**(12): 4875-4917.

Davarinos, N. A. and R. S. Pollenz (1999). "Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export." J Biol Chem **274**(40): 28708-28715.

Detmar, J., T. Rabaglino, et al. (2006). "Embryonic loss due to exposure to polycyclic aromatic hydrocarbons is mediated by Bax." Apoptosis **11**(8): 1413-1425.

Dey, P., P. Jonsson, et al. (2012). "Estrogen receptors beta1 and beta2 have opposing roles in regulating proliferation and bone metastasis genes in the prostate cancer cell line PC3." Mol Endocrinol **26**(12): 1991-2003.

Ebert, B., A. Seidel, et al. (2005). "Identification of BCRP as transporter of benzo[a]pyrene conjugates metabolically formed in Caco-2 cells and its induction by Ah-receptor agonists." Carcinogenesis **26**(10): 1754-1763.

Eggebeen, A. T. (2007). "Gout: an update." Am Fam Physician **76**(6): 801-808.

Ellem, S. J. and G. P. Risbridger (2009). "The dual, opposing roles of estrogen in the prostate." Ann N Y Acad Sci **1155**: 174-186.

Enmark, E., M. Pelto-Huikko, et al. (1997). "Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern." J Clin Endocrinol Metab **82**(12): 4258-4265.

Eskenazi, B., P. Mocarelli, et al. (2003). "Maternal serum dioxin levels and birth outcomes in women of Seveso, Italy." Environ Health Perspect **111**(7): 947-953.

Esser, C. (1994). "Dioxins and the immune system: mechanisms of interference. A meeting report." Int Arch Allergy Immunol **104**(2): 126-130.

Esslimani-Sahla, M., A. Kramar, et al. (2005). "Increased estrogen receptor betacx expression during mammary carcinogenesis." Clin Cancer Res **11**(9): 3170-3174.

Faderl, S., H. M. Kantarjian, et al. (1999). "Chronic myelogenous leukemia: update on biology and treatment." Oncology (Williston Park) **13**(2): 169-180; discussion 181, 184.

Faderl, S., M. Talpaz, et al. (1999). "Chronic myelogenous leukemia: biology and therapy." Ann Intern Med **131**(3): 207-219.

Faderl, S., M. Talpaz, et al. (1999). "The biology of chronic myeloid leukemia." N Engl J Med **341**(3): 164-172.

Fan, X., C. Gabbi, et al. (2010). "Gonadotropin-positive pituitary tumors accompanied by ovarian tumors in aging female ERbeta-/- mice." Proc Natl Acad Sci U S A **107**(14): 6453-6458.

Fernandez-Salguero, P., T. Pineau, et al. (1995). "Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor." Science **268**(5211): 722-726.

Firwana, B. M., R. Hasan, et al. (2012). "Tumor lysis syndrome: a systematic review of case series and case reports." Postgrad Med **124**(2): 92-101.

Follettie, M. T., M. Pinard, et al. (2006). "Organ messenger ribonucleic acid and plasma proteome changes in the adjuvant-induced arthritis model: responses to disease

induction and therapy with the estrogen receptor-beta selective agonist ERB-041." Endocrinology **147**(2): 714-723.

Forster, C., S. Kietz, et al. (2004). "Characterization of the ERbeta-/-mouse heart." Proc Natl Acad Sci U S A **101**(39): 14234-14239.

Forster, C., S. Makela, et al. (2002). "Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium." Proc Natl Acad Sci U S A **99**(24): 15578-15583.

Fradet, Y. (2012). "Radical prostatectomy is the most cost-effective primary treatment modality for men diagnosed with high-risk prostate cancer." Can Urol Assoc J **6**(5): 396-398.

Freedman, M. A. (2002). "Quality of life and menopause: the role of estrogen." J Womens Health (Larchmt) **11**(8): 703-718.

Freeman, E. W., M. D. Sammel, et al. (2007). "Symptoms associated with menopausal transition and reproductive hormones in midlife women." Obstet Gynecol **110**(2): 230-240.

Fritz, W. A., T. M. Lin, et al. (2007). "The aryl hydrocarbon receptor inhibits prostate carcinogenesis in TRAMP mice." Carcinogenesis **28**(2): 497-505.

Fritz, W. A., T. M. Lin, et al. (2009). "The selective aryl hydrocarbon receptor modulator 6-methyl-1,3,8-trichlorodibenzofuran inhibits prostate tumor metastasis in TRAMP mice." Biochem Pharmacol **77**(7): 1151-1160.

Gadducci, A., N. Biglia, et al. (2009). "Progestagen component in combined hormone replacement therapy in postmenopausal women and breast cancer risk: a debated clinical issue." Gynecol Endocrinol **25**(12): 807-815.

Gandhi, R., D. Kumar, et al. (2010). "Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells." Nat Immunol **11**(9): 846-853.

Garay, R. P., M. R. El-Gewely, et al. (2012). "Therapeutic perspectives on uricases for gout." Joint Bone Spine **79**(3): 237-242.

Gompel, A., S. Rozenberg, et al. (2008). "The EMAS 2008 update on clinical recommendations on postmenopausal hormone replacement therapy." Maturitas **61**(3): 227-232.

Gu, Y. Z., J. B. Hogenesch, et al. (2000). "The PAS superfamily: sensors of environmental and developmental signals." Annu Rev Pharmacol Toxicol **40**: 519-561.

Gupta, A., N. Ketchum, et al. (2006). "Serum dioxin, testosterone, and subsequent risk of benign prostatic hyperplasia: a prospective cohort study of Air Force veterans." Environ Health Perspect **114**(11): 1649-1654.

Gupta, A., A. Schecter, et al. (2006). "Dioxin exposure and benign prostatic hyperplasia." J Occup Environ Med **48**(7): 708-714.

Hahn, M. E., L. L. Allan, et al. (2009). "Regulation of constitutive and inducible AHR signaling: complex interactions involving the AHR repressor." Biochem Pharmacol **77**(4): 485-497.

Hamilton, R. J. and S. J. Freedland (2011). "5-alpha reductase inhibitors and prostate cancer prevention: where do we turn now?" BMC Med **9**: 105.

Hartman, J., A. Strom, et al. (2012). "Current concepts and significance of estrogen receptor beta in prostate cancer." Steroids **77**(12): 1262-1266.

Hasegawa, E. M., R. Fuller, et al. (2012). "Increased prevalence of simple renal cysts in patients with gout." Rheumatol Int.

Heath-Pagliuso, S., W. J. Rogers, et al. (1998). "Activation of the Ah receptor by tryptophan and tryptophan metabolites." Biochemistry **37**(33): 11508-11515.

Heisterkamp, N., J. R. Stephenson, et al. (1983). "Localization of the c-ab1 oncogene adjacent to a translocation break point in chronic myelocytic leukaemia." Nature **306**(5940): 239-242.

Heldring, N., A. Pike, et al. (2007). "Estrogen receptors: how do they signal and what are their targets." Physiol Rev **87**(3): 905-931.

Hickey, M., S. R. Davis, et al. (2005). "Treatment of menopausal symptoms: what shall we do now?" Lancet **366**(9483): 409-421.

Hofling, M., L. Ma, et al. (2009). "Expression of the androgen receptor and syndecan-1 in breast tissue during different hormonal treatments in cynomolgus monkeys." Climacteric **12**(1): 72-79.

Horvath, L. G., S. M. Henshall, et al. (2001). "Frequent loss of estrogen receptor-beta expression in prostate cancer." Cancer Res **61**(14): 5331-5335.

Huang, P., V. Chandra, et al. (2010). "Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics." Annu Rev Physiol **72**: 247-272.

Hughes, Z. A., F. Liu, et al. (2008). "WAY-200070, a selective agonist of estrogen receptor beta as a potential novel anxiolytic/antidepressant agent." Neuropharmacology **54**(7): 1136-1142.

Imamov, O., A. Morani, et al. (2004). "Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate." Proc Natl Acad Sci U S A **101**(25): 9375-9380.

Jabbour, E. and H. Kantarjian (2012). "Chronic myeloid leukemia: 2012 update on diagnosis, monitoring, and management." Am J Hematol **87**(11): 1037-1045.

Jinnah, H. A., I. Ceballos-Picot, et al. (2010). "Attenuated variants of Lesch-Nyhan disease." Brain **133**(Pt 3): 671-689.

Joel, P. B., A. M. Traish, et al. (1998). "Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase." J Biol Chem **273**(21): 13317-13323.

Johnson, J. L. and D. O. Toft (1994). "A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23." J Biol Chem **269**(40): 24989-24993.

Katzenellenbogen, B. S., I. Choi, et al. (2000). "Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology." J Steroid Biochem Mol Biol **74**(5): 279-285.

Kawajiri, K., Y. Kobayashi, et al. (2009). "Aryl hydrocarbon receptor suppresses intestinal carcinogenesis in ApcMin/+ mice with natural ligands." Proc Natl Acad Sci U S A **106**(32): 13481-13486.

Kennedy, L. D. and V. O. Ajiboye (2010). "Rasburicase for the prevention and treatment of hyperuricemia in tumor lysis syndrome." J Oncol Pharm Pract **16**(3): 205-213.

Kerkvliet, N. I. and J. A. Brauner (1990). "Flow cytometric analysis of lymphocyte subpopulations in the spleen and thymus of mice exposed to an acute immunosuppressive dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)." Environ Res **52**(2): 146-154.

Kewley, R. J., M. L. Whitelaw, et al. (2004). "The mammalian basic helix-loop-helix/PAS family of transcriptional regulators." Int J Biochem Cell Biol **36**(2): 189-204.

Koehler, K. F., L. A. Helguero, et al. (2005). "Reflections on the discovery and significance of estrogen receptor beta." Endocr Rev **26**(3): 465-478.

Kolluri, S. K., C. Weiss, et al. (1999). "p27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells." Genes Dev **13**(13): 1742-1753.

Krege, J. H., J. B. Hodgin, et al. (1998). "Generation and reproductive phenotypes of mice lacking estrogen receptor beta." Proc Natl Acad Sci U S A **95**(26): 15677-15682.

Kronenberg, F. and A. Fugh-Berman (2002). "Complementary and alternative medicine for menopausal symptoms: a review of randomized, controlled trials." Ann Intern Med **137**(10): 805-813.

Kuiper, G. G., B. Carlsson, et al. (1997). "Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta." Endocrinology **138**(3): 863-870.

Kuiper, G. G., E. Enmark, et al. (1996). "Cloning of a novel receptor expressed in rat prostate and ovary." Proc Natl Acad Sci U S A **93**(12): 5925-5930.

Kushner, P. J., D. A. Agard, et al. (2000). "Estrogen receptor pathways to AP-1." J Steroid Biochem Mol Biol **74**(5): 311-317.

L'Hermite, M., T. Simoncini, et al. (2008). "Could transdermal estradiol + progesterone be a safer postmenopausal HRT? A review." Maturitas **60**(3-4): 185-201.

Lazennec, G., D. Bresson, et al. (2001). "ER beta inhibits proliferation and invasion of breast cancer cells." Endocrinology **142**(9): 4120-4130.

Lepor, H. (2011). "Medical treatment of benign prostatic hyperplasia." Rev Urol **13**(1): 20-33.

Leung, Y. K., P. Mak, et al. (2006). "Estrogen receptor (ER)-beta isoforms: a key to understanding ER-beta signaling." Proc Natl Acad Sci U S A **103**(35): 13162-13167.

Lin, P. H., C. H. Lin, et al. (2007). "2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces oxidative stress, DNA strand breaks, and poly(ADP-ribose) polymerase-1 activation in human breast carcinoma cell lines." Toxicol Lett **172**(3): 146-158.

Lindsey, S. and E. T. Papoutsakis (2011). "The aryl hydrocarbon receptor (AHR) transcription factor regulates megakaryocytic polyploidization." Br J Haematol **152**(4): 469-484.

Lioy, P. J. and A. Greenberg (1990). "Factors associated with human exposures to polycyclic aromatic hydrocarbons." Toxicol Ind Health **6**(2): 209-223.

Loriot, Y., A. Zoubeidi, et al. (2012). "Targeted therapies in metastatic castration-resistant prostate cancer: beyond the androgen receptor." Urol Clin North Am **39**(4): 517-531.

Lund, T. D., L. R. Hinds, et al. (2006). "The androgen 5alpha-dihydrotestosterone and its metabolite 5alpha-androstan-3beta, 17beta-diol inhibit the hypothalamo-pituitary-adrenal response to stress by acting through estrogen receptor beta-expressing neurons in the hypothalamus." J Neurosci **26**(5): 1448-1456.

Maehle, B. O., K. Collett, et al. (2009). "Estrogen receptor beta--an independent prognostic marker in estrogen receptor alpha and progesterone receptor-positive breast cancer?" APMIS **117**(9): 644-650.

Marshall, N. B. and N. I. Kerkvliet (2010). "Dioxin and immune regulation: emerging role of aryl hydrocarbon receptor in the generation of regulatory T cells." Ann N Y Acad Sci **1183**: 25-37.

Maru, Y. (2012). "Molecular biology of chronic myeloid leukemia." Cancer Sci **103**(9): 1601-1610.

Matsumoto, Y., F. Ide, et al. (2007). "Aryl hydrocarbon receptor plays a significant role in mediating airborne particulate-induced carcinogenesis in mice." Environ Sci Technol **41**(10): 3775-3780.

McPherson, S. J., S. Hussain, et al. (2010). "Estrogen receptor-beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen independent and TNFalpha mediated." Proc Natl Acad Sci U S A **107**(7): 3123-3128.

McTiernan, A., C. F. Martin, et al. (2005). "Estrogen-plus-progestin use and mammographic density in postmenopausal women: women's health initiative randomized trial." J Natl Cancer Inst **97**(18): 1366-1376.

Menasce, L. P., G. R. White, et al. (1993). "Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique." Genomics **17**(1): 263-265.

Meyer, B. K., J. R. Petrulis, et al. (2000). "Aryl hydrocarbon (Ah) receptor levels are selectively modulated by hsp90-associated immunophilin homolog XAP2." Cell Stress Chaperones **5**(3): 243-254.

Michallet, A. S., S. Tartas, et al. (2005). "Optimizing management of tumor lysis syndrome in adults with hematologic malignancies." Support Cancer Ther **2**(3): 159-166.

Mohammadi-Bardbori, A., J. Bengtsson, et al. (2012). "Quercetin, resveratrol, and curcumin are indirect activators of the aryl hydrocarbon receptor (AHR)." Chem Res Toxicol **25**(9): 1878-1884.

Moos, A. B. and N. I. Kerkvliet (1995). "Inhibition of tumor necrosis factor activity fails to restore 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced suppression of the antibody response to sheep red blood cells." Toxicol Lett **81**(2-3): 175-181.

Moriwaki, Y., T. Yamamoto, et al. (1999). "Enzymes involved in purine metabolism--a review of histochemical localization and functional implications." Histol Histopathol **14**(4): 1321-1340.

Morrow, D., C. Qin, et al. (2004). "Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation." J Steroid Biochem Mol Biol **88**(1): 27-36.

Moutsatsou, P. (2007). "The spectrum of phytoestrogens in nature: our knowledge is expanding." Hormones (Athens) **6**(3): 173-193.

Muralidhara, F. Matsumura, et al. (1994). "2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-induced reduction of adenosine deaminase activity in vivo and in vitro." J Biochem Toxicol **9**(5): 249-259.

Murre, C., G. Bain, et al. (1994). "Structure and function of helix-loop-helix proteins." Biochim Biophys Acta **1218**(2): 129-135.

Nair, S. C., E. J. Toran, et al. (1996). "A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1, and the aryl hydrocarbon receptor." Cell Stress Chaperones **1**(4): 237-250.

Nebert, D. W., T. P. Dalton, et al. (2004). "Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer." J Biol Chem **279**(23): 23847-23850.

Nelson, H. D. (2008). "Menopause." Lancet **371**(9614): 760-770.

Nelson, H. D., L. L. Humphrey, et al. (2002). "Postmenopausal hormone replacement therapy: scientific review." Jama **288**(7): 872-881.

Ngo, A. D., R. Taylor, et al. (2006). "Association between Agent Orange and birth defects: systematic review and meta-analysis." Int J Epidemiol **35**(5): 1220-1230.

Nilsson, S. and J. A. Gustafsson (2011). "Estrogen receptors: therapies targeted to receptor subtypes." Clin Pharmacol Ther **89**(1): 44-55.

Nilsson, S., S. Makela, et al. (2001). "Mechanisms of estrogen action." Physiol Rev **81**(4): 1535-1565.

Nomura, M., L. Durbak, et al. (2002). "Genotype/age interactions on aggressive behavior in gonadally intact estrogen receptor beta knockout (betaERKO) male mice." Horm Behav **41**(3): 288-296.

Nyhan, W. L., J. P. O'Neill, et al. (1993). Lesch-Nyhan Syndrome. GeneReviews. R. A. Pagon, T. D. Bird, C. R. Dolan, K. Stephens and M. P. Adam. Seattle (WA).

Oesch-Bartlomowicz, B., A. Huelster, et al. (2005). "Aryl hydrocarbon receptor activation by cAMP vs. dioxin: divergent signaling pathways." Proc Natl Acad Sci U S A **102**(26): 9218-9223.

Ogawa, S., S. Inoue, et al. (1998). "The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro." Biochem Biophys Res Commun **243**(1): 122-126.

Ogawa, S., D. B. Lubahn, et al. (1997). "Behavioral effects of estrogen receptor gene disruption in male mice." Proc Natl Acad Sci U S A **94**(4): 1476-1481.

Ohtake, F., A. Baba, et al. (2008). "Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings." Biochem Biophys Res Commun **370**(4): 541-546.

Ohtake, F., Y. Fujii-Kuriyama, et al. (2009). "AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions." Biochem Pharmacol **77**(4): 474-484.

Ohtake, F., Y. Fujii-Kuriyama, et al. (2011). "Cross-talk of dioxin and estrogen receptor signals through the ubiquitin system." J Steroid Biochem Mol Biol **127**(1-2): 102-107.

Omoto, Y., S. Kobayashi, et al. (2002). "Evaluation of oestrogen receptor beta wild-type and variant protein expression, and relationship with clinicopathological factors in breast cancers." Eur J Cancer **38**(3): 380-386.

Opitz, C. A., U. M. Litzenburger, et al. (2011). "An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor." Nature **478**(7368): 197-203.

Overington, J. P., B. Al-Lazikani, et al. (2006). "How many drug targets are there?" Nat Rev Drug Discov **5**(12): 993-996.

Pace, P., J. Taylor, et al. (1997). "Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha." J Biol Chem **272**(41): 25832-25838.

Pak, T. R., W. C. Chung, et al. (2005). "The androgen metabolite, 5alpha-androstane-3beta, 17beta-diol, is a potent modulator of estrogen receptor-beta1-mediated gene transcription in neuronal cells." Endocrinology **146**(1): 147-155.

Philippou, P., K. Moraitis, et al. (2012). "The management of bladder lithiasis in the modern era of endourology." Urology **79**(5): 980-986.

Pike, A. C., A. M. Brzozowski, et al. (2001). "Structural insights into the mode of action of a pure antiestrogen." Structure **9**(2): 145-153.

Pines, A. (2008). "Re-think HRT: behind the scene of perceptions." Climacteric **11**(6): 443-446.

Quintana, F. J., A. S. Basso, et al. (2008). "Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor." Nature **453**(7191): 65-71.

Radich, J. P. (2011). "The biology of chronic myelogenous leukemia progression: who, what, where, and why?" Hematol Oncol Clin North Am **25**(5): 967-980, v.

Ramadoss, P. and G. H. Perdew (2005). "The transactivation domain of the Ah receptor is a key determinant of cellular localization and ligand-independent nucleocytoplasmic shuttling properties." Biochemistry **44**(33): 11148-11159.

Reddy, E. P. and A. K. Aggarwal (2012). "The ins and outs of bcr-abl inhibition." Genes Cancer **3**(5-6): 447-454.

Reyes, H., S. Reisz-Porszasz, et al. (1992). "Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor." Science **256**(5060): 1193-1195.

Ribeiro, R. C. and C. H. Pui (2003). "Recombinant urate oxidase for prevention of hyperuricemia and tumor lysis syndrome in lymphoid malignancies." Clin Lymphoma **3**(4): 225-232.

Roehrborn, C. G. (2008). "Pathology of benign prostatic hyperplasia." Int J Impot Res **20 Suppl 3**: S11-18.

Roger, P., M. E. Sahla, et al. (2001). "Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors." Cancer Res **61**(6): 2537-2541.

Ross, J. S., E. A. Slodkowska, et al. (2009). "The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine." Oncologist **14**(4): 320-368.

Rossouw, J. E., G. L. Anderson, et al. (2002). "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial." Jama **288**(3): 321-333.

Rowlands, J. C. and J. A. Gustafsson (1997). "Aryl hydrocarbon receptor-mediated signal transduction." Crit Rev Toxicol **27**(2): 109-134.

Safe, S. (2001). "Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions." Vitam Horm **62**: 231-252.

Saijo, K., J. G. Collier, et al. (2011). "An ADIOL-ERbeta-CtBP transrepression pathway negatively regulates microglia-mediated inflammation." Cell **145**(4): 584-595.

Saji, S., E. V. Jensen, et al. (2000). "Estrogen receptors alpha and beta in the rodent mammary gland." Proc Natl Acad Sci U S A **97**(1): 337-342.

Santen, R. J., D. C. Allred, et al. (2010). "Postmenopausal hormone therapy: an endocrine society scientific statement." Journal of Clinical Endocrinology & Metabolism **95**(7): s1-s66.

Saurat, J. H., G. Kaya, et al. (2012). "The cutaneous lesions of dioxin exposure: lessons from the poisoning of Victor Yushchenko." Toxicol Sci **125**(1): 310-317.

Schecter, A., L. C. Dai, et al. (1995). "Agent Orange and the Vietnamese: the persistence of elevated dioxin levels in human tissues." Am J Public Health **85**(4): 516-522.

Schlesinger, N. (2004). "Management of acute and chronic gouty arthritis: present state-of-the-art." Drugs **64**(21): 2399-2416.

Schmidt, J. V., G. H. Su, et al. (1996). "Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development." Proc Natl Acad Sci U S A **93**(13): 6731-6736.

Shanle, E. K. and W. Xu (2010). "Selectively targeting estrogen receptors for cancer treatment." Adv Drug Deliv Rev **62**(13): 1265-1276.

Shim, G. J., L. Wang, et al. (2003). "Disruption of the estrogen receptor beta gene in mice causes myeloproliferative disease resembling chronic myeloid leukemia with lymphoid blast crisis." Proc Natl Acad Sci U S A **100**(11): 6694-6699.

Shimada, T. and Y. Fujii-Kuriyama (2004). "Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1." Cancer Sci **95**(1): 1-6.

Shimizu, Y., Y. Nakatsuru, et al. (2000). "Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor." Proc Natl Acad Sci U S A **97**(2): 779-782.

Shin, K. J., S. S. Bae, et al. (2000). "2,2',4,6,6'-pentachlorobiphenyl induces apoptosis in human monocytic cells." Toxicol Appl Pharmacol **169**(1): 1-7.

Shirai, T., E. Ikawa, et al. (1986). "Uracil-induced urolithiasis and the development of reversible papillomatosis in the urinary bladder of F344 rats." Cancer Res **46**(4 Pt 2): 2062-2067.

Siegel, R., C. DeSantis, et al. (2012). "Cancer treatment and survivorship statistics, 2012." CA Cancer J Clin **62**(4): 220-241.

Siegel, R., D. Naishadham, et al. (2012). "Cancer statistics, 2012." CA Cancer J Clin **62**(1): 10-29.

Simonoska, R., A. E. Stenberg, et al. (2009). "Inner ear pathology and loss of hearing in estrogen receptor-beta deficient mice." J Endocrinol **201**(3): 397-406.

Sinal, C. J. and J. R. Bend (1997). "Aryl hydrocarbon receptor-dependent induction of cyp1a1 by bilirubin in mouse hepatoma hepa 1c1c7 cells." Mol Pharmacol **52**(4): 590-599.

Singh, K. P., R. W. Garrett, et al. (2011). "Aryl hydrocarbon receptor-null allele mice have hematopoietic stem/progenitor cells with abnormal characteristics and functions." Stem Cells Dev **20**(5): 769-784.

Singh, K. P., A. Wyman, et al. (2009). "Treatment of mice with the Ah receptor agonist and human carcinogen dioxin results in altered numbers and function of hematopoietic stem cells." Carcinogenesis **30**(1): 11-19.

Sirelkhatim, A., D. Sejnova, et al. (2008). "Our experience with tumor lysis syndrome treatment." Bratisl Lek Listy **109**(12): 560-563.

Sitruk-Ware, R. and G. Plu-Bureau (2004). "Exogenous progestagens and the human breast." Maturitas **49**(1): 58-66.

Skouby, S. O. and J. Jespersen (2009). "Progestins in HRT: sufferance or desire?" Maturitas **62**(4): 371-375.

Slamon, D. J., G. M. Clark, et al. (1987). "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene." Science **235**(4785): 177-182.

Smith, D. C., R. Prentice, et al. (1975). "Association of exogenous estrogen and endometrial carcinoma." N Engl J Med **293**(23): 1164-1167.

Song, R. X. and R. J. Santen (2006). "Membrane initiated estrogen signaling in breast cancer." Biol Reprod **75**(1): 9-16.

Sotoca, A. M., H. van den Berg, et al. (2008). "Influence of cellular ERalpha/ERbeta ratio on the ERalpha-agonist induced proliferation of human T47D breast cancer cells." Toxicol Sci **105**(2): 303-311.

Stahlberg, C., A. T. Pedersen, et al. (2003). "Hormone replacement therapy and risk of breast cancer: the role of progestins." Acta Obstet Gynecol Scand **82**(4): 335-344.

Stark, K., W. Reinhard, et al. (2009). "Common polymorphisms influencing serum uric acid levels contribute to susceptibility to gout, but not to coronary artery disease." PLoS One **4**(11): e7729.

Stein, R., A. Schroder, et al. (2012). "Bladder augmentation and urinary diversion in patients with neurogenic bladder: surgical considerations." J Pediatr Urol **8**(2): 153-161.

Strelau, J., M. Bottner, et al. (2000). "GDF-15/MIC-1 a novel member of the TGF-beta superfamily." J Neural Transm Suppl(60): 273-276.

Stygar, D., B. Masironi, et al. (2007). "Studies on estrogen receptor (ER) alpha and beta responses on gene regulation in peripheral blood leukocytes in vivo using selective ER agonists." J Endocrinol **194**(1): 101-119.

Subramanian, M. and C. Shaha (2009). "Oestrogen modulates human macrophage apoptosis via differential signalling through oestrogen receptor-alpha and beta." J Cell Mol Med **13**(8B): 2317-2329.

Sugihara, K., S. Kitamura, et al. (2001). "Aryl hydrocarbon receptor (AhR)-mediated induction of xanthine oxidase/xanthine dehydrogenase activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin." Biochem Biophys Res Commun **281**(5): 1093-1099.

Sugiyama, N., S. Andersson, et al. (2009). "Spatiotemporal dynamics of the expression of estrogen receptors in the postnatal mouse brain." Mol Psychiatry **14**(2): 223-232, 117.

Sugiyama, N., R. P. Barros, et al. (2010). "ERbeta: recent understanding of estrogen signaling." Trends Endocrinol Metab **21**(9): 545-552.

Talpaz, M., N. P. Shah, et al. (2006). "Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias." N Engl J Med **354**(24): 2531-2541.

Tamburrino, L., F. Salvianti, et al. (2012). "Androgen receptor (AR) expression in prostate cancer and progression of the tumor: Lessons from cell lines, animal models and human specimens." Steroids **77**(10): 996-1001.

Taylor, S. E., P. L. Martin-Hirsch, et al. (2010). "Oestrogen receptor splice variants in the pathogenesis of disease." Cancer Lett **288**(2): 133-148.

Tiwari-Woodruff, S. and R. R. Voskuhl (2009). "Neuroprotective and anti-inflammatory effects of estrogen receptor ligand treatment in mice." J Neurol Sci **286**(1-2): 81-85.

Torres, R. J. and J. G. Puig (2007). "Hypoxanthine-guanine phosophoribosyltransferase (HPRT) deficiency: Lesch-Nyhan syndrome." Orphanet J Rare Dis **2**: 48.

van Grevenynghe, J., S. Rion, et al. (2003). "Polycyclic aromatic hydrocarbons inhibit differentiation of human monocytes into macrophages." J Immunol **170**(5): 2374-2381.

Vanden Heuvel, J. P. and G. Lucier (1993). "Environmental toxicology of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans." Environ Health Perspect **100**: 189-200.

Verkooijen, H. M., C. Bouchardy, et al. (2009). "The incidence of breast cancer and changes in the use of hormone replacement therapy: a review of the evidence." Maturitas **64**(2): 80-85.

Wang, L., S. Andersson, et al. (2003). "Estrogen receptor (ER)beta knockout mice reveal a role for ERbeta in migration of cortical neurons in the developing brain." Proc Natl Acad Sci U S A **100**(2): 703-708.

Warner, M. and J. A. Gustafsson (2006). "Nongenomic effects of estrogen: why all the uncertainty?" Steroids **71**(2): 91-95.

Warner, M., P. Mocarelli, et al. (2011). "Dioxin exposure and cancer risk in the Seveso Women's Health Study." Environ Health Perspect **119**(12): 1700-1705.

Warner, M., S. Nilsson, et al. (1999). "The estrogen receptor family." Curr Opin Obstet Gynecol **11**(3): 249-254.

Weihua, Z., S. Saji, et al. (2000). "Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus." Proc Natl Acad Sci U S A **97**(11): 5936-5941.

Whitelaw, M. L., J. McGuire, et al. (1995). "Heat shock protein hsp90 regulates dioxin receptor function in vivo." Proc Natl Acad Sci U S A **92**(10): 4437-4441.

Wren, B. G. (2009). "The benefits of oestrogen following menopause: why hormone replacement therapy should be offered to postmenopausal women." Med J Aust **190**(6): 321-325.

Wu, X., M. Wakamiya, et al. (1994). "Hyperuricemia and urate nephropathy in urate oxidase-deficient mice." Proc Natl Acad Sci U S A **91**(2): 742-746.

Xu, M., D. Li, et al. (2007). "Leukemogenic AML1-ETO fusion protein increases carcinogen-DNA adduct formation with upregulated expression of cytochrome P450-1A1 gene." Exp Hematol **35**(8): 1249-1255.

Yeh, I. T. (2007). "Postmenopausal hormone replacement therapy: endometrial and breast effects." Adv Anat Pathol **14**(1): 17-24.

Yerushalmi, R., R. Woods, et al. "Ki67 in breast cancer: prognostic and predictive potential." Lancet Oncol **11**(2): 174-183.

Yoshida, T. and P. H. Stern (2012). "How vitamin D works on bone." Endocrinol Metab Clin North Am **41**(3): 557-569.

Yu, Z., C. V. Loehr, et al. (2006). "In utero exposure of mice to dibenzo[a,l]pyrene produces lymphoma in the offspring: role of the aryl hydrocarbon receptor." Cancer Res **66**(2): 755-762.

Yuan, X. and T. R. Malek (2012). "Cellular and molecular determinants for the development of natural and induced regulatory T cells." Hum Immunol **73**(8): 773-782.

Zhao, C., G. Toresson, et al. (2005). "Mouse estrogen receptor beta isoforms exhibit differences in ligand selectivity and coactivator recruitment." Biochemistry **44**(22): 7936-7944.