

From the National Institute of Environmental Medicine
Unit of Environmental Health Risk Assessment
Karolinska Institutet, Stockholm, Sweden

**MOLECULAR MECHANISMS
OF AHR MEDIATED
ENDOCRINE DISRUPTION
OF ESTROGEN AND
RETINOIC ACID SIGNALING
PATHWAYS**

David Wahlström



**Karolinska
Institutet**

Stockholm 2008

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Universitetservice US-AB

© David Wahlström, 2008
ISBN 978-91-7357-505-8

ABSTRACT

Dioxins and similar compounds are toxic substances ubiquitously present in the environment. The most potent dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is considered to be one of the most toxic compounds known to man. Dioxins have been shown to act as potent endocrine disruptor compounds (EDCs) by inducing dysregulation of several hormone signaling pathways, which include, but are not limited to, estrogens, retinoids and thyroid hormone signaling. Most, if not all, of the effects of dioxins are mediated through ligand binding and subsequent transcriptional activation of the intracellular aryl hydrocarbon receptor (AhR).

The present study aimed at investigating the mechanisms behind the endocrine disrupting effects of dioxins on the retinoid- and estrogen-hormone signaling pathways, utilizing mainly *in vitro* model systems. In addition, we have investigated the biological activity of a recently discovered retinoid metabolite.

In paper I, a microarray study was performed in order to screen for novel genomic effects of dioxin exposure. Male Sprague-Dawley rats were exposed with a single low or high dose of TCDD. After 6h-7days the animals were sacrificed and hepatic samples were analyzed using a global microarray chip. Approximately 185 genes were found to be differentially regulated ≥ 2 -fold by dioxins. Analysis of the differentially altered genes revealed that dioxin-AhR is directly or indirectly involved in regulating widespread cellular functions such as metabolism and excretion of endo- and xenobiotics (CYP1A1, 1A2, UGT1A6/7), cell cycle regulation (Cyclin D1), cellular signaling (RXR γ), steroid metabolism (Srd5a1), circadian regulation (Per2) and cellular differentiation (IGF1), to mention a few. In particular, decreased expression was seen for CYP7A1, SHP, FXR, Ntcp and Oatp2. Decreased expressions of this network of genes imply major deregulation of cholesterol metabolism, bile acid synthesis and transport.

Several mechanisms have been proposed to explain the antiestrogenic action of dioxins, including alterations of estrogen receptor (ER) levels, induction of estradiol-metabolizing enzymes that alter tissue levels of estrogens and competition for common DNA-binding sites. Recently, it was found that ARNT, which is an obligatory partner protein for AhR, acts as a potent coactivator for the ERs. In paper II, mechanisms of ARNT in ER dependent transcription was further characterized. These studies showed that reducing the levels of available ARNT by activation of the AhR- or HIF-pathways or targeted downregulation of ARNT using siRNA coincided with a decrease in ER transcriptional activity, especially of the ER β subtype. These findings demonstrate that competition for ARNT may contribute to the antiestrogenic effects of dioxins.

One of the most sensitive and early signs of dioxin exposure *in vivo* is disturbed retinoid homeostasis with hepatic depletion of retinoid stores and increased levels of all *trans*-retinoic acid (at-RA), the biological ligand for the nuclear retinoid receptors (RARs and RXRs). In paper III, the effects of cotreatment with dioxin on at-RA induced transcription *in vitro* was investigated. We found that TCDD repressed at-RA induced mRNA levels of the at-RA target genes *RAR β 2* and *CYP26a1*, using Realtime-PCR. Interestingly, at-RA was found to reciprocally repress TCDD induced transcription of AhR target genes *AhRR* and *CYP1A1*. In addition, a novel finding was that RXR β and ARNT can interact in the presence of both TCDD and at-RA. The importance of this interaction needs to be further investigated.

In paper IV, the recently identified retinoid metabolite S-4o9cDH-RA was found to be a biologically active retinoid with the ability to regulate RAR-dependent transcription, both *in vitro* and *vivo*. We suggest that S-4o9cDH-RA is a novel endogenous ligand for at least RAR α and RAR β .

LIST OF PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their roman numerals (I-IV)

- I. Nick Fletcher, **David Wahlström**, Rebecca Lundberg, Charlotte B Nilsson, Kerstin C Nilsson, Kenneth Stockling, Heike Hellmold and Helen Håkansson. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the mRNA expression of critical genes associated with cholesterol metabolism, bile acid biosynthesis, and bile transport in rat liver: A microarray study. *Toxicology and Applied Pharmacology* 207 (2005) 1 – 24
- II. Joëlle Rüegg, Elin Swedenborg, **David Wahlström**, Aurelie Escande, Patrick Balaguer, Katarina Pettersson and Ingemar Pongratz. The transcription factor ARNT functions as an estrogen receptor beta selective co-activator, and its recruitment to alternative pathways mediates anti-estrogenic effects of TCDD. *Molecular Endocrinology*, Feb 2008
- III. **David Wahlström**, Helen Håkansson and Katarina Pettersson. Transcriptional crosstalk between RAR and AhR signalling pathways in murine P19 embryonic carcinoma cells. *Submitted to Toxicology in vitro*
- IV. Jan Philipp Schuchardt*, **David Wahlström***, Norbert Giese, Malin Hedengran Faulds, Madalina Stefan, Henning Hopf, Helen Håkansson, Gregor Eichele, Katarina Pettersson and Heinz Nau (*chaired first authorship). Biological activity in vitro and in vivo by the endogenous retinoid metabolite S-9-cis-4-oxo-13,14-dihydro-retinoic acid. *Submitted to Endocrinology*

* These authors contributed equally to this study

CONTENTS

Molecular mechanisms of AhR mediated endocrine disruption of estrogen and retinoic acid signaling pathways.

1 DIOXINS	1
1.1 Introduction	1
1.2 Endocrine disruption	1
1.3 AhR/ARNT	2
1.4 Physiological role of AhR.....	4
2 NUCLEAR RECEPTORS	7
3 RETINOIDS	10
3.1 Introduction	10
3.2 The nuclear retinoid receptors	10
3.4 RAR/AhR interactions	12
4 ESTROGENS	17
4.1 Introduction	17
4.2 ER/AhR interactions	17
5 AIMS OF THE THESIS	20
Aims in summary	20
6 RESULTS IN SUMMARY	21
Paper I: Transcriptional response to TCDD exposure in rat liver	21
Paper II: interaction between arnt and er.....	22
Paper III: crosstalk between RAR & AhR pathways.....	22
Paper IV: S-4-oxo-9-cis-13,14-dihydro-retinoic acid	24
7 FUTURE PERSPECTIVES	25
8 ACKNOWLEDGEMENTS	27
9 REFERENCES	29

LIST OF ABBREVIATIONS

AF	Activation function
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
At-RA	All- <i>trans</i> retinoic acid
bHLH-PAS	Basic helix-loop-helix-PER ARNT SIM
bMAL	Brain and muscle ARNT-like protein
CYP	Cytochrome P450
DBD	DNA binding domain
DRE	Dioxin response element
E2	17 β -estradiol
EDC	Endocrine disruptor compound
ER	Estrogen receptor
FICZ	lindolo[3,2-b]carbazole
HIF	Hypoxia inducible factor
HRE	Hormone response element
Hsp90	Heat shock protein 90
LBD	Ligand binding domain
NR	Nuclear receptor
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RXR	Retinoid X receptor
SRC-1	Steroid receptor coactivator 1
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TIF-2	Transcriptional intermediary factor 2
3MC	3-methylcholantren
TRP	Tryptophan
XAP2	Hepatitis B virus X-associated protein 2
XRE	Xenobiotic response element

1 DIOXINS

1.1 Introduction

Dioxins¹ are widespread and very persistent environmental pollutants. They are formed unintentionally, through improper combustion processes of both industrial and household waste, and as unwanted byproducts in the industry working with structurally related chemicals (Ahlborg et al. 1992; Baker and Hites 2000). Dioxins are amongst the most toxic compounds known to man, and exposure to these kinds of chemicals induces a plethora of adverse effects in both experimental and wildlife animals. In the animals investigated, some major effects seen include endocrine disruption, cancer, reproductive failures in both males and females, disturbances in immune response, liver damage and a characteristic wasting syndrome with an immense loss of weight (Mukerjee 1998; Birnbaum and Tuomisto 2000; Bock and Kohle 2006). There is also data pointing out that prenatal and early postnatal exposure to low levels of dioxins may induce behavioral and learning deficits accompanied by more persistent developmental and reproductive failures. These effects are seen in experimental animals but are suspected to occur also in humans (Peterson et al. 1993; Brouwer et al. 1995). Most of the conditions induced by dioxin exposure can probably be related to the fact that dioxins are some of the most potent endocrine disruptor compounds (EDCs) known. This property of dioxins results in dysregulation of several hormonal signaling pathways and therefore a very wide panorama of effects.

Due to their great resistance to biological degradation (Mukerjee 1998), dioxins typically accumulate in the food chain, and are consequently present in the highest levels in top predators such as the polar bear. Owing to their lipophilicity, they are stored in the adipose tissues of exposed animals and humans.

The major route of exposure to dioxins in humans is through the diet, and we are usually exposed over the whole life span. Importantly, maternal exposure of the fetus may occur during pregnancy, as well as postnatally through breast feeding, which counts as the main human route of exposure besides food derived exposure. Accidental exposure to higher doses of dioxins is rare, and the most significant marker for acute exposure in humans is a condition known as chloracne, characterized by severe skin lesions.

1.2 Endocrine disruption

Generally, humans are exposed to a wide spectrum of different substances via our diet, which can be beneficial as well as bad to our overall health. Ordinary food sources such as different soy products, flax seed oil and diverse products that are sold in for example health shops are mostly thought of as harmless products containing nutrients and perhaps health promoting substances. Nevertheless, these often naturally derived products may actually contain substances with capacity to disturb hormone signaling, so called

¹ In this thesis, the term dioxin refers to the polychlorinated dibenzodioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like PCBs which are included in the toxicological equivalence factor (TEF) scheme (Van den Berg et al. 2006). These compounds bind to AhR and has similar chemical structure and toxicological effects as the most potent congener; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The toxicity of each different congener is usually expressed relative to TCDD.

endocrine disruption. An endocrine disruptor compound (EDC) is per definition an exogenous substance or mixture of substances that can interact with and disturb hormonal systems in a way that may cause adverse effects for the overall health in an individual or its offspring. The mechanistic reason behind this is that both diverse chemicals as well as substances from the nature can be structurally similar to the endogenous ligands for hormonal receptors, and thereby have the potential to bind and regulate the transcriptional activity of that receptor (Hilscherova et al. 2000). Importantly, dioxins are amongst the most potent EDCs known, with capacity to interrupt the signaling through several hormonal pathways. This is of great concern, since embryogenic as well as postnatal exposure to these kinds of EDCs can potentially disturb crucial developmental events since these are often regulated by finely tuned temporal and spatial hormonal signaling cascades. Until now, most concern has been focused on reproduction and early development, it is believed that some disorders in adult stages may result from EDC exposure early in life, when organ systems is developing and maturing. The hormone systems suspected to be dysregulated by dioxins are needed for normal development and function of several important organ systems in the body, such as reproductive organs, cardiovascular system, bone and teeth, immune system, brain and overall homeostasis. In line with this, the disorders believed to be caused by dioxin exposure includes a wide spectrum, such as hormone related cancers (breast, testicular, uterus and prostate), type II diabetes, cardiovascular disease and metabolic syndrome including obesity. In addition, biological differences between males and females are mostly regulated by hormone systems. The synthesis, levels and effects of different hormones varies between males and females. Therefore, the effects from dioxin exposure may differ depend on gender. The hormonal signaling pathways best characterized to be dysregulated by dioxins includes retinoid, estrogen and thyroid hormone signaling systems. It will be further discussed in coming chapters how TCDD and similar compounds act as endocrine disruptors for the retinoid and estrogen hormonal pathway.

1.3 AhR/ARNT

The exact mechanism underlying the toxicological effects mediated by dioxins like TCDD is not completely elucidated, yet, it is commonly accepted that the physiological effects of dioxins are mediated through ligand binding and subsequent activation of the intracellular aryl hydrocarbon receptor (AhR)-signaling pathway. Studies of AhR-deficient mice confirm this notion by presenting a phenotype refractory to the toxic effects of TCDD (FernandezSalguero et al. 1996). The AhR belongs to the bHLH-PAS (basic helix-loop-helix Per-ARNT-SIM) family of transcription factors (Gu et al. 2000; Kewley et al. 2004). The two structural domains, bHLH and PAS share considerable sequence homology between members of the family. The bHLH domain is required for dimerization and positions the basic region to allow specific DNA sequence recognition. The PAS domains contain interfaces involved in dictating dimerization specificity and, for selected members of the family, chaperone docking sites (Furness et al. 2007). The AhR is unique within the family, in the sense that it appears to be the only family member that requires ligand binding for its activation. Other members of the family include the AhR nuclear translocator (ARNT), the hypoxia inducible factor 1 alpha (HIF1 α) and the circadian factors CLOCK, bMal and PER, among others. ARNT functions as an obligatory dimerization partner for several of the bHLH-PAS proteins, for instance the AhR and the HIF1 α . In addition, ARNT has been shown to act as a

potent coactivator of estrogen receptor transcriptional activity (Brunnberg et al. 2003). Three isoforms of ARNT have been identified; ARNT, ARNT2 and ARNT3 or bMAL (brain and muscle ARNT like protein). Structurally, ARNT and ARNT 2 share extensive sequence homology whereas bMAL is less conserved, especially in the C-terminal part. ARNT and ARNT 2 appear to be functionally interchangeable to some extent, while bMAL selectively interact only with certain bHLH-PAS members such as CLOCK. ARNT and ARNT 2 demonstrate both unique and overlapping functions. *In vitro*, hypoxic response through HIF1 α could be mediated via both ARNT and ARNT 2, while AhR mediated transcriptional activity in the presence of ligand could only be regulated in presence of ARNT, not ARNT 2 (Sekine et al. 2006).

Among the diverse compounds that can bind to and activate the AhR, TCDD is the most potent one, wherefore TCDD is often used as a reference compound in assessment of AhR-activating substances. In its unliganded state, the AhR is located in the cytosol, bound by two molecules of the molecular chaperone hsp90 (heat shock protein 90) together with additional hsp90 associated factors, the immunophilin like protein called XAP2 (hepatitis B virus protein X associated protein 2) and the co-chaperone p23 (Kazlauskas et al. 1999; Petrusis and Perdew 2002; Hollingshead et al. 2004). Upon binding by TCDD (or other agonists), the AhR is released from the hsp90 complex and translocates to the nucleus, where it dimerizes with ARNT, forming a transcriptionally active complex, which regulate transcription by direct binding to specific regulatory DNA sequences, called XREs (xenobiotic responsive elements) located in the promoter regions of AhR target genes (see figure 1).

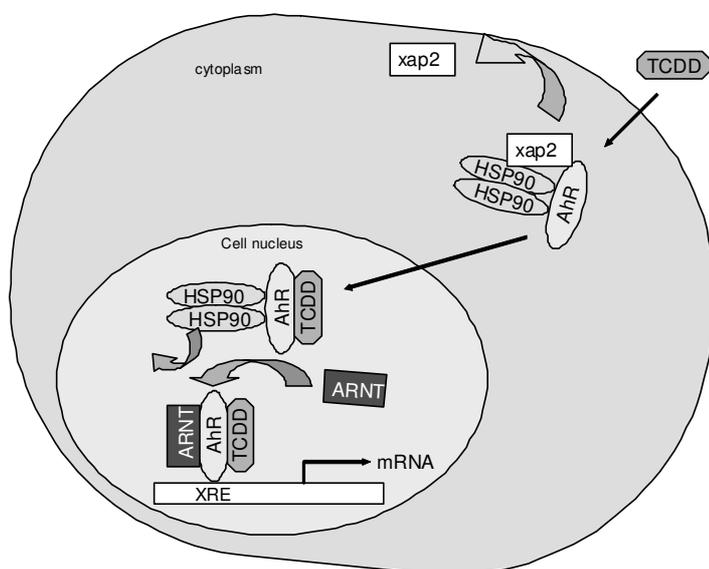


Figure 1. *Simplified scheme of the AhR-signaling pathway.* Unliganded AhR is localized in the cytosol, in complex with hsp90, xap2 and the cochaperon p23. Upon ligand binding, xap2 dissociates from the complex, which translocates to the nuclear compartment. Once in the nucleus, the ligand bound AhR dissociates from hsp90 and interacts with its obligatory partner protein ARNT. The ligand/AhR/ARNT-complex regulates the transcription of AhR target genes by the recognition of specific DNA sequences called XREs (xenobiotic/dioxin responsive elements).

Genes that are regulated by AhR are in many cases involved in metabolism and detoxification of endobiotics and xenobiotics, such as several members of the cytochrome P450 family of enzymes. Induction of the CYP1A1 enzyme is a classical and sensitive marker of dioxin exposure in the liver. Other genes that are regulated by the AhR/ARNT complex include enzymes involved in the clearance of reactive metabolites originating from biodegradation of for instance xenobiotics, such as glutathione-S-transferase Ya and UDP-glucuronosyltransferase form 6. Recent data from studies utilizing microarray technology demonstrate that the AhR also influences transcription of genes encoding proteins which regulate cell cycle, cell growth and differentiation events and apoptosis (Fletcher et al. 2005; Tijet et al. 2006).

1.4 Physiological role of AhR

The AhR is expressed in numerous murine tissues, both embryonic and adult. From gestational day 10-12, AhR expression is observed in the heart, liver, several neurons and the nasal pit, among others. From gestational day 13,5-15,5, the expression is greatly expanded, and AhR is expressed in the palatal shelf, adrenal gland, thymus, bone, muscle, gut, kidneys, developing pituitary, urogenital sinus. In adult mice (8-11 weeks old), the relative AhR expression is highest in the oocyte, epidermis, bladder, lung, digits, vomeronasal organ, liver, trachea, olfactory epithelium and retina (Mimura et al. 1997; Jain et al. 1998).

Several putative endogenous ligands for AhR have been identified during the last 15 years. These include light induced photoproducts of tryptophan (TRP), such as indolocarbazoles (Bjeldanes et al. 1991; Rannug et al. 1995; Wei et al. 1998; Wei et al. 2000), indirubin and indigo (Adachi et al. 2001), tryphthantoin and malassezin (Schrenk et al. 1997; Schrenk et al. 1999). Interestingly, one TRP derivative demonstrates a very high affinity for the AhR. The affinity of FICZ (lindolo[3,2-b]carbazole) towards AhR actually exceeds that of TCDD (Kd = 0.07nM vs. 0.48nM) (Rannug et al. 1987; Rannug et al. 1995). See figure 2 for the chemical structure between TRP, FICZ and TCDD. FICZ have been shown to bind to and activate AhR *in vitro*, illustrated by the induction of CYP1A1 in several cell lines (Wei et al. 1998; Wei et al. 2000). In more recent studies, both FICZ and other TRP derived photoproducts have been demonstrated to activate AhR also *in vivo* (Diani-Moore et al. 2006; Mukai and Tischkau 2007). These results suggest that it may be possible that AhR is activated not only by FICZ under physiological conditions, but rather by a mixture of multiple TRP metabolites. *In vivo*, these photoproducts may be synthesized not only as a result of light entering the retina, but also through dermal light exposure. This could possibly contribute to a large amount of synthesized photoproducts under conditions where dermal light exposure is high. It is further possible that they are transported from its site of synthesis in dermal parts via the blood stream to several organs where they may act as photo derived "light hormones", thus acting in a circadian fashion since the synthesized levels likely follows the day and night shifts. In comparison to exogenous AhR agonists, such as dioxins, FICZ are quickly degraded by CYP1A1, which it induces (Wei et al. 2000). In addition, TRP derived AhR ligands can be formed in ordinary cell culture medium kept under regular light conditions. In cell studies, the medium kept in light conditions induced CYP1A1 activity, in comparison to medium kept under dark conditions (Oberg et al. 2005). These results clearly show that

tryptophan derived AhR ligands, suggested to be endogenous AhR ligands, influence the background levels of CYP1A1 activity in cells in culture.

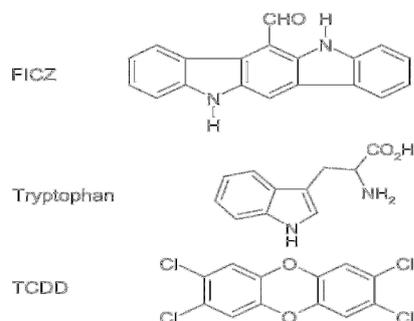


Figure 2: Chemical structure of the potential endogenous AhR ligand FICZ (6-formylindolo[3,2-b]carbazole), its mother compound tryptophan, and the most potent dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

In attempts to elucidate the physiological role of AhR, three independent laboratories have generated *AhR*-null (knock out) mice over the last decade. These mice were produced by deletion of either the first (Fernandezsalguero et al. 1995; Mimura et al. 1997) or the second (Schmidt et al. 1996) exon of the AhR gene. The phenotypes from the three strains appear to differ in some characteristics, but share several important features, such as resistance to TCDD toxicity and cardiovascular and reproductive defects. One of the most overt phenotype of the AhR knockout mice is the markedly decreased liver size compared to control mice, about 25-50 % smaller (Fernandezsalguero et al. 1995; Schmidt et al. 1996). The mechanism behind this seems to be related to the blood flow through the liver. During embryogenesis, the bloodflow partially bypasses the liver via a shunt known as the ductus venosus (DV). Normally, this shunt closes shortly after birth and consequently the blood is forced to find its way through the liver, providing it with nutrients and oxygen for its growth. In the absence of AhR though, the DV does not close, and the liver is basically nutrient deprived, causing reduced postnatal growth of the liver (Lahvis et al. 2000, 2005). The normal closure of DV has been shown to be regulated directly by AhR, since it is dependent of endothelial and/or hematopoietically expressed AhR (Bunger et al. 2003) and ARNT (Walisser et al. 2005). In addition to other less characterized defects related to the vascular system, several other pathologic conditions of the liver are observed. Of these, portal tract fibrosis represents the condition which is characterized in most detail. AhR deficiency in both cell cultures and *AhR*-null mice results in increased secretion of active TGF β , a profibrotic peptide, via a post-translational mechanism (Border and Noble 1994; Zaher et al. 1998; Branton and Kopp 1999; Santiago-Josefat et al. 2004). AhR-KO mice demonstrate abnormal accumulation of retinoids in the livers, due to a decrease in metabolism of retinoic acid, suggesting a prominent role for AhR in regulation of retinoid homeostasis (Andreola et al. 1997, 2004), which will be further discussed in the chapter about retinoids. Absence of retinoid metabolism has been shown to promote an increase in levels of TGF β activating enzymes (Kojima and Rifkin 1993; Okuno et al. 1997). Taken together, the data suggests that an interaction

between AhR, retinoic acid and TGF β pathways contribute to the hepatic fibrosis phenotype in the KO-mice.

In addition, AhR seems to have a role for proper function of multiple reproductive tissues in female mice, most notably the ovarian follicle. The number of mature follicles is markedly reduced in the AhR null-mice (Benedict et al. 2000, 2003; Baba et al. 2005). Recent data suggest that this reduction in mature follicles is due to a direct effect within the follicle itself due to an insufficient synthesis of estradiol (Baba et al. 2005; Barnett et al. 2007). It has been shown that AhR together with the orphan nuclear receptor Ad4BP (a.k.a. SF-1) is directly responsible for a peak in aromatase (CYP19) expression during preovulation, suggested to be required to drive the estrogen production necessary for the developing follicle (Honda et al. 1993; Baba et al. 2005). In addition to the AhR knockout mice, the function of its obligatory partner protein ARNT has also been investigated in transgenic animals. Targeted disruption of ARNT however, is embryonically lethal due to placental failure and ARNT-deficient embryos die *in utero* at embryonic day 10.5 (Kozak et al. 1997; Maltepe et al. 1997). This phenotype is significantly more severe than the one observed in AhR-*null* mice, demonstrating a role for ARNT in physiological processes not connected to AhR signaling. The disruption of HIF1 α signaling resulting from lack of ARNT has been postulated to cause the arrest in placental development, due to impaired angiogenesis (Fryer and Simon 2006). All three ARNT isoforms have been found to be expressed in the embryo, but in adult mice, expression of ARNT2 is limited to neuronal tissues and kidneys and bMAL to the brain and skeletal muscle (Drutel et al. 1996; Hirose et al. 1996). In contrast, ARNT is ubiquitously expressed, indicating that ARNT may have important roles in several tissues (Gu et al. 2000).

2 NUCLEAR RECEPTORS

Several hormones mediate their genomic action through ligand binding and subsequent activation of members from a group of intracellular transcription factors called nuclear receptors (NRs). The evolutionary ability to regulate biological events such as nutrient metabolism, reproduction and development in mammals and other multicellular organisms coincide with the evolution of nuclear receptors (Bertrand et al. 2004). Members of the nuclear receptor family share a common structural organization of functional domains (see fact box 1 and figure 3), primarily with a highly conserved DNA binding domain (DBD) and a less conserved ligand binding domain (LBD) (Schwabe and Teichmann 2004). The different nuclear receptors make up a superfamily of transcription factors working as sensors for a large set of lipid soluble molecules, such as steroid hormones (estrogens, androgens, corticosteroids and progesterone), vitamins (A, D) and dietary lipids (oxysterols, bile acids etc.). Thus, nuclear receptors are involved in transcriptional regulation of genes involved in a wide range of physiological events related to immune response, development, tissue homeostasis, reproduction and metabolism. In addition to the receptors with well characterized endogenous ligands (such as thyroid hormone, vitamin A and D, sex hormones etc.), almost half of the nuclear receptor superfamily consists of so called orphan receptors. Generally, the natural ligand and/or function for these nuclear receptors are unknown, at least was when the receptor was given its name (Benoit et al. 2006).

In the unliganded state, some of the nuclear receptors are bound to molecular chaperones such as hsp90, together with auxiliary proteins and immunophilins, in the same manner as AhR (see figure 1). Other nuclear receptor members are interacting with corepressor proteins in the unliganded state, actively repressing the transcription of target genes. When present in an hsp90 complex, the nuclear receptor-structure favors ligand binding and is prevented from heterodimerization with other nuclear receptors. Upon ligand binding, the receptor alters its conformation, resulting in dissociation from the chaperone complex or the corepressor proteins, and interacts with coactivator proteins, mediating the assembly of the basal transcription machinery components and subsequent transcriptional activation of target genes. Nuclear receptors regulate gene transcription through the recognition and binding to specific DNA sequences called hormone responsive elements (HREs) located in the regulatory parts of target genes. Nuclear receptors bind to their HREs either as monomers, homodimers or heterodimers. In this context, members from the retinoid X receptor family (RXRs) play a special role within the nuclear receptor superfamily, since they act as promiscuous heterodimerization partners for several other nuclear receptors, thus having roles in several signaling pathways (Laudet et al. 1992; Leid et al. 1992; Glass and McDonnell 2004). Steroid receptors like the estrogen receptors (ERs) almost exclusively bind their HREs as homodimers, whereas nonsteroidal receptors like the thyroid hormone receptor (TR), vitamin A and D receptors (RARs and VDR) preferentially bind their HREs as heterodimers, since heterodimerization with members from the RXR subfamily strongly enhances both the binding to DNA and the transcriptional activity (Aranda and Pascual 2001).

In addition to ligand binding and receptor dimerization, the transcriptional activity of nuclear receptors is regulated via interactions with coregulatory proteins. There are two

major kinds of coregulatory proteins, corepressors and coactivators. In the absence of ligand, some nuclear receptors can actively repress basal transcription of target genes via the interaction with corepressors, such as nuclear receptor corepressor (NCoR) or silencing mediators for retinoid and thyroid receptors (SMRT). These corepressors possess histone deacetylase activity, which keeps the chromatin inaccessible for transcription factor binding (Robyr et al. 2000).

Upon ligand binding, conformational changes of the receptor occurs, mainly in the C-terminal LBD domain. The conformational changes results in release of the corepressor proteins and favors binding of coactivator proteins instead. The coactivators function as bridging molecules that mediates the interaction between the ligand activated nuclear receptors and components of the basal transcription machinery. Numerous different coactivators have been described, where some are common to several signaling pathways, such as CREB binding protein (CBP) and its homologue p300 (Janknecht and Hunter 1996). The most abundant coactivator proteins that bind to the nuclear receptors are often members from the p160 family, such as steroid receptor coactivator 1 (SRC-1) and transcriptional intermediary factor 2 (TIF-2) (Klinge 2000). These coactivators also contain a highly conserved bHLH-PAS domain in their N-terminal part. Normally, this domain is a critical region for the heterodimerization with other bHLH-PAS proteins and DNA-binding, but seems to be less important for the interaction with nuclear receptors (Xu and Li 2003). Instead, the interaction with nuclear receptors is mediated via the so called NR (nuclear receptor)-box located in the central part of the coactivator, containing an LXXLL motif (where L corresponds to Leucine and X to any amino acid) through the AF-2 (activation function 2) located in the C-terminal part of the nuclear receptors (Hall and McDonnell 2005). One functional role for the coactivators is to recruit further coregulatory proteins and histone acetyltransferases to the target gene. These activities result in a more open chromatin structure and subsequent transcriptional activation after recruitment of the basal transcription machinery to the site.

In addition to ligand induced activity, several of the nuclear receptors can be modulated through phosphorylation. The outcome of phosphorylation of nuclear receptors are not fully understood yet, but is thought to be involved in various functions such as regulation of receptor stability, DNA binding, regulation of transcriptional activity both in absence and presence of ligand and interactions with coregulator proteins (Weigel and Moore 2007).

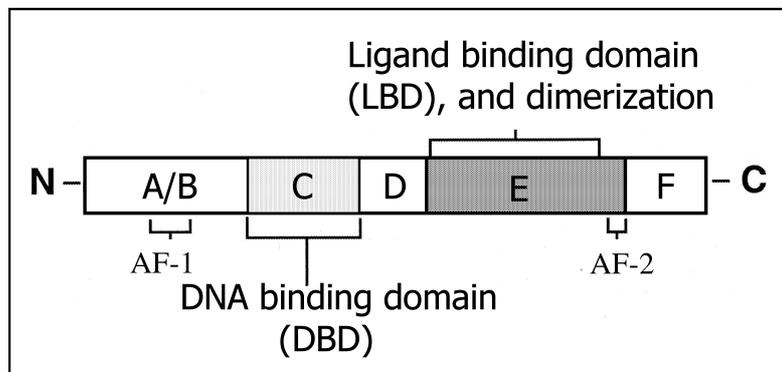


Figure 3: General structure of a nuclear receptor family member.

The prototypical nuclear receptor consists of several functionally well conserved domains. The region that varies the most between NRs is located in the N-terminal part; the A/B region, followed by the highly conserved C-region containing the DNA binding domain (DBD), a hinge (D-region) and finally the E/F-region including the ligand binding domain (LBD). Refer to fact box 1 for detailed information.

Fact box 1: The functional domains of a nuclear receptor

The A/B region

This part of the NRs is the most variable region in both size and sequence, and is likely to contribute to the differences between NR isoforms and responsible for their cell- and gene promoter-specific activity. It often contains a ligand independent AF-1 (activation function 1) transactivation domain.

The DNA binding domain

Region C, which confers the DBD, is the most conserved region in NRs. The DBD itself contains two “zinc fingers”, which recognizes specific NR responsive elements in the regulatory parts of the target gene. One of the zinc fingers contains the “P box” which is central for the recognition of the HRE by the NR, and the other zinc finger contains the so called “D box” which is involved in receptor dimerization. NR heterodimers can bind to HREs with diverse arrangements which are typically composed of two hexameric motifs configured either as direct repeats (DRs; » »), palindromes (» «) or inverted palindromes (« »). The orientation and spacing between the motifs confer the specificity of the HRE.

The hinge region

The hinge region between the DBD and LBD is not well conserved among the NRs. It often contains sequences involved in nuclear localization and interaction with corepressors. This region is also believed to enable conformational alterations and to give the receptor flexibility which allows rotation of the DBD.

The ligand binding domain

The ligand binding domain in the E/F region is a multifunctional domain important not only for ligand binding but also contain functional sites for dimerization, hsp-interactions and ligand dependent transactivation by the AF-2 (Wurtz et al. 1996; Imai et al. 1997). The general structure of this region is rather similar amongst the NRs (Moras and Gronemeyer 1998). This domain contains 12 conserved α -helical structures named H1-H12, packed together in a three layer sandwich-like structure. The size of the ligand binding pocket varies between the NRs, which decides the specificity of the ligands that can induce the receptor. Upon ligand binding, the receptor structure is generally altered to a more condensed form than in the unliganded state, and the alteration results in binding of coactivator proteins to the AF-2 (Aranda and Pascual 2001).

3 RETINOIDS

3.1 Introduction

In this thesis work, the term retinoids is used for natural and synthetic derivatives of Vitamin A. Vitamin A is an essential micro nutrient with many important roles in all mammals, from embryogenesis and throughout life. This fat soluble dietary component is derived from animal products such as eggs, milk and liver (mainly in the form of retinyl esters and retinol). It can also be digested in the form of provitamin A (carotenoids) from fruits and vegetables (Fisher and Voorhees 1996; Blomhoff and Blomhoff 2006). Retinoids are not produced endogenously, and must therefore be derived from the diet. However, digested retinoids can be stored in the liver, in the form of retinyl esters. During periods of insufficient amounts of retinoids from the diet, the retinyl ester stores in the liver are utilized to supply periphery tissues with sufficient amounts of retinoids.

Retinoids exists in several different metabolized forms, but only some of them have important biological functions. For example, retinal, the aldehyde form of retinol (ROH), is an essential component of the visual cycle in the retina, functioning as a chromophore. Consequently, an early sign of vitamin A deficiency is a poor vision in darkness. The most important retinoid is retinoic acid (RA), which is a lipid soluble non-steroid hormone, derived from an irreversible enzymatic conversion of retinal. RA has a vital role in processes such as embryogenesis, reproduction, growth and differentiation.

3.2 The nuclear retinoid receptors

The biological function of RA is to regulate gene expression. RA is the natural ligand to members from the nuclear receptor superfamily, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The major form of RA; all-*trans* retinoic acid (at-RA) is the primary ligand for the RAR family members, whereas the 9-*cis* form (9-*cis* RA) is the most potent endogenously derived ligand found for the RXR family members (Mangelsdorf et al. 1995; Chambon 1996). See figure 3 for the structure of different retinoid forms. RARs and RXRs exist in three different subtypes (α , β and γ), and in addition several isoforms (for example RAR β 2). RXRs in particular, but also RARs, are able to regulate gene transcription as homodimers, but RARs preferentially binds to members from the RXR family, thus forming a heterodimeric RAR/RXR complex. Due to the large amount of different RAR and RXR isoforms, a multitude of several different homo/hetero-dimers are theoretically possible. This serves a possibility for very specific at-RA-induced gene regulation. Even though the different RAR and RXR isoforms appear to have some tissue specific expression, it is unclear whether they differentially regulate gene transcription. Experiments in mice applying gene knockout technique implies that the RAR subtypes may be functionally redundant (Mark et al. 1999).

RAR/RXR heterodimers regulate transcription of retinoid target genes by recognizing HREs called retinoic acid response elements (RAREs), located in the gene promoter upstream of the transcription start site. The classical RARE in RA target genes are called DR5, and is typically composed of two direct repeats of a core hexameric motif spaced by 5 base pairs (thereof the name: 5-bp-spaced direct repeat). The DR5 element is found in the gene promoters of several classical RA target genes such as RAR β ,

CYP26 and several Hox and HNF genes. RAREs can also consist of DR1s or DR2s, but not as abundantly as the DR5 (Bastien and Rochette-Egly 2004). Over the last centuries, RA has been suspected to be involved in the regulation of several hundreds of genes, and identified direct/indirect RA target genes are reviewed in (Balmer and Blomhoff 2002).

RAR/RXR-dimers are able to regulate gene transcription also in the absence of ligand, and actively repress transcription of RA target genes (Weston et al. 2003). RAR/RXR-dimers can interact with corepressor proteins such as SMRT and NCoR, which recruits complexes containing histone deacetylases to the gene promoters, thus keeping the chromatin in an inaccessible state, preventing transcription (Li et al. 1997; Yoh and Privalsky 2001). Upon ligand binding, the RAR/RXR alters conformation, dissociates from the corepressor protein complex in favor for interaction with coactivator proteins such as SRC-1, TIF2 and CBP/p300, which initiate transcriptional activation of the gene (Chen and Li 1998).

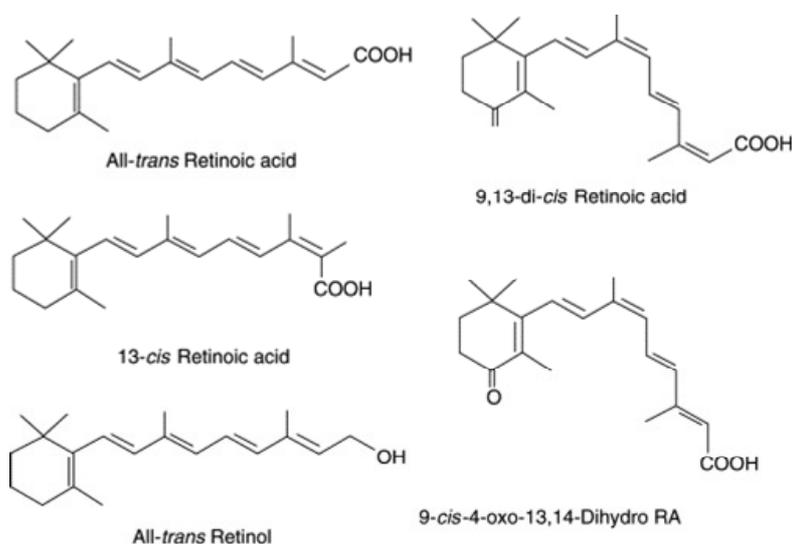


Figure 3: Structure and function of different retinoid forms

The non-steroid hormone at-RA is considered to be the foremost signaling molecule in the retinoid signaling system. It is derived in a two step enzymatic conversion of all-*trans* retinol to all-*trans* retinal to at-RA. However, other natural retinoid forms potentially contribute to retinoid signaling. Among those found, 9-*cis* RA has for a long time been accepted as the most potent endogenous ligand for the RXRs. In addition, 13-*cis* RA show biological activity, but is not able to regulate transcription through the retinoid receptors (Blaner 2001). 9,13-di-*cis*-RA is able to bind RAR α and induces fibrogenesis through the formation of TGF β (Imai et al. 1997; Okuno et al. 1999). Moreover, the 4-oxo forms of RA, ROH and retinaldehyde are thought to be important in *Xenopus* development through transcriptional activation of RARs and RXRs (Achkar et al. 1996; Blumberg et al. 1996; Pijnappel et al. 1998).

3.4 RAR/AhR interactions

The precise physiological functions of AhR are still not fully characterized, but one of the natural roles for the AhR seems to be in the regulation of retinoid homeostasis. This is evident from studies using both AhR knockout animals as well as wild type animals exposed to exogenous AhR agonists such as TCDD. Animals put under both of these extreme situations demonstrate pronounced effects on the tissue levels of different retinoids, including RA. In addition, several lines of evidence suggest that the putative crosstalk between the two signaling systems extends beyond AhR mediated effects on retinoid metabolism and also involves transcriptional interference between the two signaling systems (Janosek et al. 2006; Murphy et al. 2007)

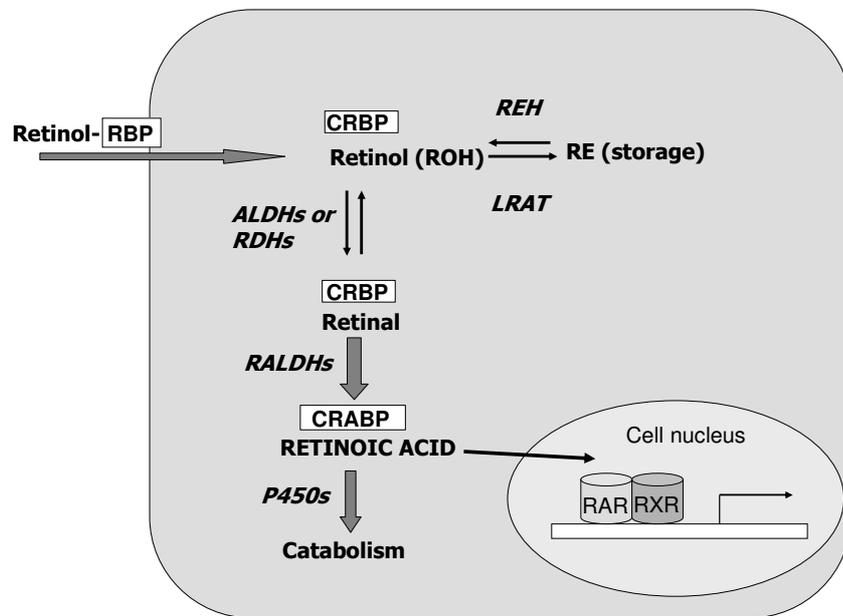


Figure 4: Simplified view of retinoid metabolism (see fact box 2 for details).

Fact box 2. Retinoid metabolism.

The regulation of gene transcription mediated by the nuclear retinoid receptors (RARs and RXRs) are believed to be partly regulated by the availability of the receptor ligand, i.e. at-RA. Therefore, the intracellular levels of at-RA are normally under strict regulation by finely tuned paths of biosynthesis and degradation. Retinoid homeostasis is maintained by a balance between storage, biosynthesis of biologically active at-RA followed by catabolic pathways for its excretion. The conversion between the different retinoid forms is mediated by an orchestra of different enzymes, together with specialized retinoid binding proteins. The expression and activity of several of these enzymes are regulated by retinoids. Thus, retinoid metabolism and homeostasis is regulated by both feedback/feed forward mechanisms. For example, RA directly induces the expression of CYP26, which is a major RA-catabolizing cytochrome P450 enzyme, involved in the excretion of excess RA. Upon high levels of at-RA, the expression of CYP26 is induced through several RARE in the gene promoter that is recognized by the ligand activated RAR/RXR dimer (Ray et al. 1997; Loudig et al. 2005).

The irreversible formation of RA from its precursor retinal is mediated by retinaldehyde dehydrogenases (RALDHs). RALDH2 seems to be a critical enzyme in RA formation, and is downregulated by elevated levels of at-RA (Niederreither et al. 1997). In addition, there are several types of retinoid binding proteins, such as retinol binding protein (RBP), cellular retinol binding protein (CRBP) and cellular retinoic acid binding protein (CRABP). These different binding proteins seem to have functional roles other than carrying retinoids. For example, CRBPI is recognized as a substrate by retinal dehydrogenase (RDH), which converts retinal to RA. Further on, CRABP, which binds RA, is also mediating RA catabolism in a similar mechanism. Thus, in addition to the retinoid metabolizing enzymes, also the retinoid binding proteins have pivotal roles in the regulation of the retinoid levels and action (Napoli et al. 1995, 1996; Noy 2000).

The first suggestion that TCDD and related compounds have an impact on retinoid metabolism and signaling came from observations that TCDD exposure results in conditions similar to vitamin A deficiency, such as reduced growth, abnormal development and immune function. A very representative effect of TCDD exposure in most investigated species is a dramatic decrease in hepatic retinoid levels, which is a well characterized effect in all investigated rodent models (Nilsson and Hakansson 2002). This early and sensitive sign of dioxin exposure is linked to an increased whole body retinoid turnover (Kelley et al. 1998) including a mobilization of retinoids from retinyl ester stores (Brouwer et al. 1989; Jurek et al. 1990; Vanbirgelen et al. 1995), altered retinol esterification (Nilsson et al. 1996; Hoegberg et al. 2003) and increased excretion of polar retinoid metabolites (Hakansson and Ahlborg 1985; Brouwer et al. 1989). Most importantly though, altered tissue levels of retinoic acid have the potential to alter the transcription of genes under the control of the retinoid receptors (Kelley et al. 1998; Nilsson et al. 2000; Hoegberg et al. 2003). The effects on hepatic retinoid

levels is seen not only in animals exposed to high acute doses of TCDD, but also as a result of single doses as low as 0.1 µg/kg bw (Nilsson et al. 2000). In addition, the effects are seen in animals exposed in utero (Morse and Brouwer 1995) as well as lactationally (Hakansson et al. 1987).

The disturbances in retinoid homeostasis from dioxin exposure may result from dysregulation of retinoid metabolizing enzyme(s) by the action of AhR. In line with this, the retinoid phenotype of AhR knockout mice suggests that enzymes important in the retinoid metabolism are reciprocally dysregulated in the absence of AhR. A large amount of enzymes are involved in the metabolism of retinoids (see figure 4 and fact box 2 for an overview of retinoid metabolism), such as several cytochrome P450 enzymes (CYPs), UDP-glucuronosyl-transferases (UGTs) and alcohol dehydrogenases (ALDHs). Consequently, there are several candidate enzymes to be potentially (dys)regulated by the AhR. For example, the irreversible conversion of retinal to RA is mediated by RALDHs (Duester 2000). Two XREs have been characterized in the mouse promoter of RALDH2, making it a possible target for AhR action (Wang et al. 2001). This suggests that the accumulation of at-RA seen in TCDD exposed animals could be caused by an induced RALDH2 expression. Further on, several of the CYP450s are acknowledged to mediate the same conversion both *in vitro* and *in vivo* (Roberts et al. 1992; Raner et al. 1996; Tomita et al. 1996; Zhang et al. 2000). Other CYPs are involved in the catabolism of RA, a process which starts with hydroxylation of RA followed by glucuronidation and excretion. A well known enzyme which normally contributes significantly in the catabolism of RA is CYP26, which is also a direct RA target gene. The levels of CYP26 are the same in wild type as well as AhR-null animals (Andreola et al. 1997; Ray et al. 1997; Loudig et al. 2000). The CYP2C39 on the other hand, which is suggested to be one of the primary murine retinoid hydroxylases, is expressed at lower level in AhR-null animals than in their wildtype counterparts, suggesting that this enzyme could be responsible for increased levels of at-RA in the AhR-null mice. Even though it seems to be downregulated in the absence of AhR, treatment with TCDD did not appear to alter its expression in wildtype mice (Andreola et al. 2004). However, the exact role for the novel CYP2C39 in RA-metabolism is not entirely clear yet. Similarly, the expression of cellular retinol binding protein I (CRBPI) is higher in the livers of null-mice (Andreola et al. 1997), but in a recent study where wildtype mice were exposed to TCDD the expression of CRBPI was not altered (Hoegberg et al. 2005). Thus, these two factors in retinoid handling seems dysregulated in AhR-null mice, but unaffected by TCDD treatment. Another CYP450 with activity towards at-RA that were recently discovered is CYP2S1. The expression of CYP2S1 is inducible by TCDD exposure since it has XREs in its gene promoter, but is also inducible by UV-light and retinoid exposure. Moreover, the only recognized endogenous substrates for CYP2S1 to date are retinoids. It has been suggested that CYP2S1 may play an important role in differentiating skin, since it is highly expressed in epithelial tissues (Du et al. 2006) and during developmental processes as it is expressed during all fetal stages (Choudhary et al. 2003; 2005). Thus, the AhR seems to be potentially involved in retinoid metabolism at several points, but the exact mechanisms underlying the disturbed retinoid homeostasis in the absence of AhR or due to dioxin exposure is not elucidated completely. Probably the AhR-mediated influence in retinoid handling can be both tissue and cell type specific, and perhaps differ between species. In addition to AhR-mediated alterations of RA

tissue levels, there may be interactions going on between RA and AhR signaling pathways also on the transcriptional/receptor level. Transcriptional interference between the two signaling systems may occur on different levels, and involve competition between common cofactor proteins, alterations in receptor availability and direct transcriptional inhibition.

One of the earliest indications that the AhR influences RA-dependent transcription originates from a study by Rubin and Rice (1988). Treatment of SCC-4 keratinocytes of human origin with AhR agonist such as TCDD, 3 methylcholantren (3MC) and benzo(a)pyren were found to inhibit at-RA induced activation of tissue transglutaminase, which is an important enzyme for proper differentiation of the skin. This repression by AhR-agonists was later found to be mediated on the transcriptional level. The exact mechanism behind this is still unclear, since the repression by TCDD treatment did not seem to result from either a change in mRNA stability, or altered binding or activation of a RARE-luciferase construct that were transfected (Krig and Rice 2000). After this, more RA target genes have been found to be inhibited by TCDD treatment, such as cellular retinoic acid binding protein II (CRABP II), RAR β and RDH9 (Weston et al. 1995; Tijet et al. 2006). Interactions between the AhR- and RA-pathways have been further established for several other genes with various functions. For example, at-RA as well as TCDD modulates the expression of matrix metalloproteinases (MMPs) (Murphy et al. 2004; Villano et al. 2006). The proteins in this family have functions involved in diverse important cellular processes such as tissue structure, angiogenesis and proliferation (Brinckerhoff and Matrisian 2002). In a variety of cell lines, at-RA treatment changes the expression of MMPs, mostly by transcriptional repression by interfering with the AP-1 signaling pathway (Vincenti et al. 1996). TCDD induces the expression of MMP-1 in normal human keratinocytes, but cotreatment with at-RA leads to an even more increased expression than TCDD alone. The mechanism behind this was found to be transactivation mediated by TCDD in combination with increased stability of the MMP-1 mRNA mediated by at-RA (Murphy et al. 2004). Thus, this interaction was not a matter of transcriptional interference, rather a subject of synergism between the AhR and RA pathways. The balance in retinoid metabolism is regulated on several levels, and involves feedback and feed forward regulation via the levels of diverse retinoid forms. Since AhR is likely to be directly involved in regulating retinoid metabolism at one or several points, it is not unexpected to find signs of transcriptional crosstalk in relation to genes directly involved in retinoid metabolism and handling. For instance, the expression of retinal oxidase, which is converting retinal to RA, is induced by TCDD treatment. Interestingly, during simultaneous treatment with at-RA and TCDD the expression was instead downregulated (Yang et al. 2005).

Some of the genes that are regulated by both AhR and RA pathways contain both XREs and RAREs in their promoters, thus providing a ground for steric interference between RAR/RXR-protein complexes and AhR/ARNT mediated action. This is the case for the P450 CYP1A1, which is highly inducible by AhR ligands, endogenous as well as exogenous. The CYP1A1 gene promoter contains multiple XREs, but also an RARE. For long, it has been speculated whether this RARE is inducing CYP1A1 expression as this was initially suggested after cell studies using reporter constructs (Vecchini et al. 1994). However, today it is commonly accepted that this RARE is inhibitory to CYP1A1 expression upon at-RA exposure (Wanner et al. 1996; Soprano et al. 2001).

In addition to transcriptional interference over common target genes and regulation of receptor ligand levels, another potential way to regulate gene expression is through modulating the receptor levels. There are studies showing the capacity of the AhR pathway to modulate the levels of RARs and RXRs, as well as it is considered that targeted down regulation of the AhR is an important mechanism in regulating the activity via this pathway (Pollenz 2002).. As an example for the later, treatment with at-RA repressed transcription from the AhR promoter in a murine epidermal cell line (FitzGerald et al. 1996). However, treatment of human keratinocytes with at-RA did not alter either AhR or ARNT mRNA expression (Murphy et al. 2004). In the opposite situation, treatment of the cells with TCDD resulted in elevated mRNA levels for both RAR γ and RXR α . In contrast, RAR β expression was inhibited by TCDD treatment in embryonic palate mesenchymal cells (Weston et al. 1995). Thus, the capacity of AhR to regulate the transcriptional levels of RARs and RXRs seems to be receptor and cell type specific. The difference in AhR expression after at-RA treatment of the murine and the human epidermal cell types may depend on species specific differences between the human and murine AhR promoter and/or different responsiveness to at-RA (i.e. different RAR/RXR isoforms present and/or different at-RA metabolism). In addition, yet another line of interference between the signaling systems could involve a competition for common co-factor proteins. Indeed, there are several co-activator proteins utilized by both pathways; RIP140 (Vincenti et al. 1996; Kumar et al. 1999; Nguyen et al. 1999), CBP/p300 and SRC-1 (Yao et al. 1996; Tohkin et al. 2000; Beischlag et al. 2002). In addition, the co-repressor SMRT seems to be involved also in AhR mediated transcription, and recent studies suggest a possible role for SMRT in the crosstalk between AhR and RAR pathways since it modulate the transcriptional activity in both systems (Nguyen et al. 1999; Rushing and Denison 2002; Widerak et al. 2006). In summary, the crosstalk between the two signaling systems may occur on several levels as described above. Anyway, there is no doubt that both the absence of AhR as well as the non-physiological hyperactivation of AhR by persistent ligands such as TCDD is leading to an affected retinoid homeostasis. In both cases, this is connected with altered levels of the biologically active ligand for the nuclear retinoid receptors, RA. It is very likely that this may lead to downstream effects on RA-mediated gene regulation, given that the transcriptional regulation by these nuclear receptors is thought to be regulated partly by the abundance of its ligand. The disturbed retinoid homeostasis is probably caused by direct or indirect dysregulation of several factors such as enzymes and binding proteins important in the handling, storage and metabolism of retinoids. Thus, the AhR receptor seems to have a natural role in regulating retinoid homeostasis. Beside this, there are substantial data indicating that a transcriptional interference occurs between the systems. No one has yet revealed any general molecular mechanism for this, and so far, it seems that several mechanisms mediate different types of crosstalk between the systems, often dependent on cell or tissue type and/or species differences.

4 ESTROGENS

4.1 Introduction

Estrogens, like many other hormones, elicit a large spectrum of important biological functions. In particular, estrogens are a key factor in female hormone regulation and signaling, as they are the primary “female” steroid hormones. Anyway, estrogens have important functions in both males and females, even though estrogens are produced endogenously in lower amounts in males. The major endogenous estrogens are 17- β -estradiol (E_2), estrone and estriol, which are produced mainly in the ovaries, but also in placenta, fatty tissues and the adrenal gland cortex. Estrogens have important functions for a proper development and function of reproductive organs in both sexes. They are responsible for morphological, behavioral and metabolic changes during diverse stages of reproduction. In addition, estrogens have also important roles in the maintenance of bone mass, regulation of organism homeostasis and behavior and is protecting against cardiovascular disease (Hess et al. 2001).

Similar to retinoic acid, the cellular response to estrogens is mainly mediated by members from the superfamily of nuclear receptors, which in this case is the estrogen receptor (ER). One decade ago, it was discovered that the estrogen receptor occurs in two similarly structured subforms, the ER α and ER β (Kuiper et al. 1996).

Functionally, current knowledge indicate that ER β seems to play its most important role in the ovaries, cardiovascular system and brain, and a less important role in more classical estrogen target tissues such as bone, uterus and the hypothalamus/pituitary (Harris 2007).

Structurally, the two ER forms contain a highly conserved DBD, but the LBDs show only 58% homology. ER α contains two distinct AFs, the constitutively active and ligand independent AF-1 in the N-terminus. In the C-terminal part, both ER α and ER β harbors a ligand dependent AF-2 (Damdimopoulos et al. 2008).

Upon binding of ligand, the subsequent receptor conformational change differs whether the receptor is bound by an agonist or an antagonist. In contrast to binding of an antagonist such as tamoxifen or raloxifen, binding of an agonist such as E_2 , will result in a repositioning of helix 12 that enables binding of coactivators (Nilsson et al. 2001). ERs regulate gene transcription of estrogen target genes either as homo- or hetero-dimers that recognize specific estrogen responsive elements (EREs) in the regulatory parts of target genes. Similar to the retinoid receptors, in the absence of ligand, the ERs can interact with corepressor proteins such as SMRT or NCoR that actively represses transcription from ER target genes.

4.2 ER/AhR interactions

In general, dioxin exposure results in antiestrogenic effects (Ahlborg et al. 1995), but recent studies also points out that dioxin exposure may induce estrogenic effects in some cases (Watanabe et al. 2004; Boverhof et al. 2006). There are indications that dioxins act antiestrogenic in mature females but estrogenic in immature females and males (Brunnberg 2007). Thus, the different response from dioxin exposure is probably dependent on different factors such as gender and developmental stage. An example of sex related differences from dioxin exposure are hepatic tumors. In rats, hepatic tumors are one of the most sensitive markers of dioxin exposure. These are strongly estrogen

dependent and occur primarily in female rats. Anyhow, the anti-estrogenic effects of dioxins are particularly well-characterized, and evidence for disruption of estrogen-mediated functions includes for example inhibition of estrogen-induced responses in rodent uterus and mammary tumors, and in human breast cancer cells (Wormke et al. 2003).

In addition to dioxins, other compounds exist that can act as agonists to AhR, and thus potentially influence estrogen dependent signaling. One such compound is 3-methylcholantren (3MC). Similar to dioxins, 3-MC is a potent agonist to AhR, and has been reported to act both antiestrogenic as well as estrogenic. Interestingly, in AhR knockout mice, 3-MC was able to induce cyclin D1 mRNA expression and increased uterine wet weight, thus acting estrogenic *in vivo*, in the absence of AhR (Abdelrahim et al. 2006). In a recent study, 3-MC acted antiestrogenic in some cell types and estrogenic in others. This could be related to the capacity of the different cell types to metabolize 3MC. In comparison to TCDD, 3-MC can quite easily be metabolized, and as a consequence of this, one or several of the 3-MC-metabolites act as ER ligand(s) (Swedenborg et al. 2007). Thus, 3-MC can act as an agonist for both AhR and ERs, dependent on situation.

In contrast to the relatively unknown details about RAR-AhR crosstalk, there is more detailed knowledge about ER-AhR interactions. In fact, several possible mechanisms have been suggested to mediate the anti estrogenic effects of AhR ligands:

- *Degradation of ER proteins.* It has been suggested that E₂-activated degradation of the ER protein by proteasomes may be an important mechanism that limits the duration of estrogenic responses in target tissues. It has been demonstrated that this pathway is also induced through liganded activation of the AhR by TCDD. In contrast, E₂ treatment did not alter the levels of AhR protein, demonstrating that proteasome-dependent inhibitory crosstalk between the two signaling pathways is only working in one direction. It was further manifested that this mechanism by TCDD was highly selective on the ER α subform (Wormke et al. 2003).
- *Increased metabolism of steroids.* Circulating estrogens are primarily metabolized in the liver. Since dioxins are potent inducers of several CYPs putatively involved in the degradation of estrogens, it is thought that this might contribute to decreased levels of ligand for the ERs. All data from animal studies do not support this idea though, since circulating levels of 17 β -estradiol did not alter upon TCDD treatment in rodents (Ahlborg et al. 1995; Safe and Wormke 2003), but then again, no antiestrogenic activity was induced by TCDD in transgenic mice lacking either AhR or CYP1B1, supporting a role for CYP1B1 in altering the estrogen levels (Takemoto et al. 2004).
- *Competition for common cofactors.* Recently, ARNT was found to be a very potent coactivator for both ER subforms (Brunnberg et al. 2003). This provided evidence for a direct link between the AhR and ER pathways on the receptor level, not seen before. Even though ARNT is ubiquitously expressed, the protein levels might be limited in certain cell/tissue types. This could result in competition between signaling pathways over ARNT as a cofactor. For example, sequestering of ARNT by AhR upon dioxin treatment could provide a

mechanistic explanation for repressed ER transcriptional activity if ARNT is an important factor for the ERs.

- *Competition for common target genes.* Some ER target genes may contain recognition sites also for both AhR/ARNT. Consequently, these genes might be actively regulated by both signaling pathways, which may provide a basis for transcriptional interference. Two examples of this kind of interaction is provided with the *Cathepsin D* and *pS2* genes, suggested to be regulated in a similar way by AhR/dioxins and ERs. In the promoter of a *Cathepsin D*, there is an ERE responsible for transcriptional activation of the gene by estrogens. In the same region, a negative DRE is also located. Binding of AhR/ARNT to this negative responsive element results in disrupted formation of the sp1/ER-DNA complex, thus repressing ER transcriptional activity (Wang et al. 1998; Safe and Wormke 2003).

5 AIMS OF THE THESIS

Exposure to dioxins such as TCDD and similarly potent exogenously derived agonists to the AhR induces a range of adverse effects in most animal species. One of the main adverse effects from these substances is the non-physiological “hyperactivation” of the AhR signaling pathway, causing endocrine disruption. The main objectives of this thesis work were to investigate and characterize the mechanism(s) behind the disruptive effects seen on the retinoid and estrogen signaling systems. Alterations in retinoid metabolism and homeostasis are among the most sensitive and early markers of exposure to dioxins. The effects include decreased hepatic storage of retinoids, increased retinoid metabolism and excretion, and altered levels of at-RA. In addition, tissue levels of the recently discovered retinoid metabolite S-4o9cDH-RA is drastically decreased by dioxin exposure in both mice and rats.

Aims in summary

- To investigate effects from low- and high-dose TCDD exposure in rat liver on the transcriptional level, by applying microarray technique. The main objective was to identify genes differentially regulated by TCDD that may be involved in retinoid metabolism and signaling. The use of a “global” microarray chip with a large quantity of different genes also gave the opportunity to identify novel genes and gene networks differentially regulated by TCDD not necessarily connected to the retinoid pathway.
- To further investigate the mechanism behind the disruptive effect of TCDD exposure on estrogen hormone signaling and to advance the characterization of ARNT as a co-activator for the nuclear estrogen receptors, ER α and ER β .
- To identify possible points of interaction in the putative crosstalk between the RAR/RXR and AhR/ARNT pathways. The idea was to apply a coherent approach in different cell lines to investigate the mechanism(s) behind the disruptive effects of dioxin exposure on retinoid signaling. Thus, the aim was to investigate aspects of transcriptional interference and possible protein-protein interactions between components of the respective signaling pathway *in vitro*, after exposure to AhR agonists such as TCDD and 3-MC alone or in combination with at-RA.
- To characterize the possible biological activity of the novel and major retinoid metabolite S-4o9cDH-RA, found to be related to both dioxin exposure as well as dietary conditions.

6 RESULTS IN SUMMARY

Paper I: Transcriptional response to TCDD exposure in rat liver

The first paper in this thesis work is based on investigation of microarray data achieved from livers of dioxin exposed rats. The study was performed in collaboration with Astra Zeneca which supported us with the study design and collection of the microarray data. With the microarray chip used in this study around 180 transcripts were differentially regulated than 2.0-fold or more due to TCDD exposure. Not all of these altered transcripts represent known gene products though, and several genes that were altered on the chip were represented by several probes. Even though, the results provided us with a massive amount of microarray data to analyze. Primarily we aimed at identify gene changes that may influence retinoid metabolism or signaling, and thereafter genes or gene networks associated with other signaling pathways, intermediary metabolism and hepatotoxicity. The investigated liver samples were collected from male Sprague–Dawley rats exposed to single oral doses of TCDD (0.4 or 40 µg/kg bw). All the animals were treated simultaneously, and killed after 6, 24 hours or 7 days. This gave the possibility to investigate early/transient gene changes as well as later and more persistent alterations in gene transcription due to dioxin exposure.

Generally, low dose exposure resulted in induction of a battery of phase I and phase II metabolizing enzymes, such as CYP1A1, CYP1A2, NAD(P)H dehydrogenase and UGT1A6/7. The induction seen for this group of genes was present at all time points, after both TCDD doses. In addition, low dose exposure altered the expression of genes important also for other type of activities, exemplified by the transcripts for DNA-damage-inducible Gadd45 α and Cyclin D1. These genes were altered already at the shortest time point, suggesting that AhR/ARNT might be directly involved in events related to cellular stress/DNA damage as well as cell cycle control.

Following high dose exposure, widespread alterations in gene expression were observed for genes encoding cellular signaling proteins, cellular adhesion, cytoskeletal and membrane transport proteins, as well as transcripts coding for proteins involved in lipid, carbohydrate and nitrogen metabolism. In addition, transcripts coding for proteins associated with steroid metabolism, immune function, and intermediary metabolism were markedly affected as well.

Interestingly, we found a decreased expression of CYP7A1, SHP (short heterodimer partner), FXR (Farnesoid X Receptor), Ntcp, and Slc21a5 (oatp2), which were also confirmed by Realtime PCR analyses in independent rat liver samples. Altered expression of these genes implies major deregulation of cholesterol metabolism and bile acid synthesis and transport. In summary, while low-dose TCDD exposure appeared mainly to induce enzymes associated with metabolism and excretion of xenobiotics, high-dose TCDD exposure resulted in more widespread changes in gene expression. Together, the number of probesets affected by TCDD exposure in these rat livers represented approximately 180 genes with a known or inferred function. In relation to the endocrine disruptive effect by dioxin exposure, it is interesting to analyze changes in genes related to cellular signaling. As such, a group of probesets representing transcription factors were altered. Among these, RXR γ , Nrf2 (NF-E2-related factor), Onecut1, Kruppel-like factor 9, Nuclear factor I/X and Tgfb1i4 (TGF β 1 induced transcript 4) were altered already at the shortest time point. This suggests that

these genes may be direct target genes for the AhR, but this needs to be further analyzed.

Paper II: interaction between arnt and er

This study was intended to further characterize the endocrine disruptive effect of dioxin exposure on estrogen hormone signaling.

The earlier identification of ARNT as a potent co-activator for the estrogen hormone receptors (ER α and β) gave evidence that the AhR/ARNT pathway is involved in crosstalk with other signaling systems via direct interactions on the receptor level. Additionally, it gave a reasonable explanation for the anti estrogenic effects from dioxin exposure since it seems that ARNT could be an important player in ER activity, and withdrawal of ARNT could decrease this activity.

In paper II, we aimed at elucidate further details behind the co-activating function of ARNT on the ER's. In this study we show that activation of either AhR or HIF-1 α through the recruitment of ARNT, results in a repressed ER transcriptional activity. Similarly, limiting the pool of available ARNT in the cells by using siRNA to downregulate the ARNT levels also results in a repressed ER transcriptional activity. Performing chromatin immunoprecipitation (ChIP)-analysis showed that the decrease in ER activity coincided with a decreased ability to recruit ARNT to ER regulated promoters upon TCDD treatment. We also found that dioxin mediated repression of ER activity is significantly stronger on ER β than ER α . The same difference was evident for the co-activation of ERs by ARNT, which was more pronounced on ER β compared to ER α . Further on, the area responsible for the difference between the ER β -ER α responses to ARNT was identified to be present in the N-terminal A/B domain of ER β , using different receptor mutants together with a ERE-luciferase reporter construct in transfection experiments. Finally, to confirm the results using an endogenous promoter, the mRNA levels of the E2-responsive gene pS2 were measured with Realtime-PCR. In presence of different combinations of receptor mutants, the cells were treated with TCDD and/or E2. TCDD alone had no effect on the pS2 transcription, but repressed E2 induced pS2 transcription by ER α with about 40% and by ER β with 80%.

Paper III: crosstalk between RAR & AhR pathways

As mentioned in the introduction, dioxin exposure affects the retinoid homeostasis in most investigated species. These well characterized effects on retinoid homeostasis and turnover results from even very low concentrations of dioxin exposure. Normally, the endogenous levels of at-RA, the retinoid metabolite with strongest biological activity, are strictly regulated, but dioxin exposure results in altered levels of this metabolite in several investigated tissues. It has been suggested that the AhR may have a natural role in regulating the retinoid levels, including the biologically active metabolite at-RA. Since the main consequence from dioxin exposure seems to be an inappropriate hyper-activity of the AhR/ARNT signaling cascade, this may have severe consequences for the multitude of genes regulated by retinoids since they could potentially be dysregulated due to the "hypervitaminosis A state" (with very high levels of at-RA) induced by dioxin exposure. This is of major concern especially during embryogenesis and developmental stages, since at-RA in high doses is known to act teratogenic. In paper three we aimed at investigate the mechanism behind the putative crosstalk between the retinoid and AhR/ARNT signaling pathways on the transcriptional level. The experiments were carried out in the murine P19 embryonic carcinoma cells. In the

literature, these cells have been utilized for investigating several retinoid dependent events. Since they also contain a functional AhR/ARNT pathway, they make up a nice cell model system for investigating the putative crosstalk between these pathways. The first thing to bring about was to evaluate if transcriptional interference occurs over RARE-regulated genes, i.e. if treatment of cells with TCDD could disturb endogenous transcription of genes regulated by at-RA. After treatment of cultured P19 cells with either at-RA or TCDD, or in combination, the mRNA levels of the direct RAR/RXR target genes CYP26a1 and RAR β 2 were quantified assessing Real-Time PCR. Interestingly, we found that co-treatment with TCDD repressed the mRNA levels compared to in cells treated with at-RA only. This result indicates that a potent AhR ligand as TCDD in fact has the potency to directly disturb gene regulatory events induced by at-RA, i.e. transcriptional interference seems to occur over RARE regulated genes as a direct consequence of dioxin exposure.

Commonly, one way to interrupt an intracellular signaling cascade is through so called negative feed back regulation, which may be facilitated through several different mechanisms. Since it is likely that the AhR/ARNT pathway have a natural role in regulating the retinoid homeostasis, the existence of a feed back regulation on the AhR activity induced by the RAR/RXR pathway could be expected.

In this context, the next move in this paper became naturally to turn the initial question around to see if retinoid treatment can disturb AhR mediated gene regulation.

Interestingly, in this situation, cotreatment of P19 cells with at-RA reciprocally repressed AhR-regulated gene transcription compared to TCDD treatment only. This was shown using both quantitative Real-Time PCR (with primers recognizing the AhR responsive genes AhRR and CYP1A1), as well as transfected luciferase reporter constructs (regulated by a XRE; xenobiotic responsive element). This indicates that transcriptional interference can occur also over AhR regulated genes, upon retinoid treatment. These findings suggest that the two signaling systems interact with each other on the transcriptional level, in both directions.

In addition, when investigating protein-protein interactions in immunoprecipitations we found that RXR β binds to the AhR obligatory partner protein ARNT. ARNT was found to be co-precipitated strongly with RXR β in extracts from cells treated with at-RA, but not as strongly in the presence of both at-RA and TCDD. The interaction became stronger the more at-RA present, and together with the notion that at-RA does not bind the RXRs, this strongly suggests that RARs is involved in the interaction. This novel finding forced us to investigate if the interaction between ARNT/RXR β could be of any relevance for RAR-regulated signaling. When co-transfecting increasing amounts of ARNT together with a RARE-luciferase reporter plasmid (containing the simple DR5 RA-responsive element) in CV-1 cells, ARNT imparted a slight dose-dependent increase in the RA induced transcriptional activity. This indicated that ARNT may have some ability to enhance RA-dependent transcription. To investigate this further, P19 cells were transiently transfected with a luciferase reporter construct containing the minimal naturally RA-responsive RAR β 2 promoter segment, together with increasing amounts of the ARNT-expression plasmid. Again, ARNT was found to modestly, although significantly, increase the RA induced luciferase activity from the RAR β 2-promoter. The reasons for the quite limited co-activating effect of ARNT on RA-dependent transcription could be several. One reasonable explanation can be that the RAR/RXR complex may preferentially employ other coactivators than ARNT, such as TIF2. Another reasonable cause could be that the binding of ARNT by the RAR/RXR

complex may serve primarily as a natural mechanism whereby the transcriptional activity of AhR is down regulated upon elevated levels of at-RA.

Paper IV: S-4-oxo-9-cis-13,14-dihydro-retinoic acid

During investigations of tissue levels of different retinoids following TCDD exposure, a novel retinoid metabolite was found. This metabolite was characterized as S-4-oxo-9-cis-13,14-dihydro-retinoic acid (S-4o9cDH-RA). The endogenous levels of S-4o9cDH-RA in wild type mice and rats showed to be significantly higher than at-RA, especially in the liver. Since the transcriptional activity of the retinoid receptors are believed to be regulated a in large part by the abundance of ligand, tissue levels of at-RA are generally strictly regulated. In contrast, the level of S-4o9cDH-RA increases dramatically following vitamin A supplementation in mice, especially in the liver (Schmidt et al 2003). This suggests that this metabolite is highly regulated by dietary intake. Compared to at-RA, for which the levels increases in several investigated tissues after dioxin exposure, S-4o9cDH-RA is nearly untraceable after the same treatment (Hoegberg et al 2005). Thus, S-4o9cDH-RA has a potential role as an extremely sensitive marker for dioxin exposure. The aim of paper IV was to evaluate if S-4o9cDH-RA has any biological function in gene regulation, i.e. is acting as a ligand for the nuclear retinoid receptors RARs and/or RXRs. In this study we employed several cell based model systems where we transfected luciferase reporter plasmids regulated by different RARE constructs. S-4o9cDH-RA showed an ability to activate RAR-dependent transcription from all of these luciferase reporter plasmids, as well as increasing endogenous mRNA levels of RARE regulated genes employing Real-Time PCR. In addition to luciferase reporter assays and quantitative PCR studies in several cell types, an avian developmental model was used. In order to investigate the biological activity in a more *in vivo*-like model, the developing chick model were utilized for studying morphology and gene regulation upon S-4o9cDH-RA treatment. Indeed, this model showed evidence that S-4o9cDH-RA can induce morphological changes in the developing chick wing bud in a similar way as at-RA, as well as inducing endogenous gene transcription, evident by a group of selected RA-target genes.

Further on, limited proteolysis assays were applied in order to investigate the ligand properties of S-4o9cDH-RA. We found that S-4o9cDH-RA acts as a ligand for both RAR α and RAR β . This became apparent in view of the fact that S-4o9cDH-RA induced the same conformational changes to the *in vitro*-translated receptors as at-RA. Interestingly, S-4o9cDH-RA did not activate any of the investigated RXR isoforms as we initially suspected. This was a bit unpredicted, since S-4o9cDH-RA has high structural similarity to 9-cis retinoic acid (9c-RA), the best characterized endogenous RXR ligand found so far. In conclusion, S-4o9cDH-RA seems to be a novel ligand for some (if not all) of the RAR's, and perhaps play a so far unknown physiological role in gene regulation. In that case, the gene regulatory function is likely to be connected to the dietary intake of vitamin A. This would be in great contrast to at-RA, for which the endogenous levels are very stringently regulated regardless of dietary fluctuations.

7 FUTURE PERSPECTIVES

The use of microarray technology as in paper I can be a considerable good tool to screen genomic effects mediated via AhR, especially since this pathway seems to be engaged in a natural crosstalk with several other signaling systems, thereby mediating a wide range of dysregulation upon dioxin exposure. This makes screening with for example Realtime-PCR a more time consuming and effort taking alternative to microarray. Today it is possible to choose a more “to the point” set of genes on an array than when paper I was performed, enabling a more tailored investigation focusing on the direct target genes of dioxin/AhR, i.e. to identify the key players causing the plethora of long term effects by dioxin.

Obviously, the mechanism behind effects of dioxin exposure on other signaling pathways can not be explained only by finding direct target genes for AhR that are expressed in a non-physiological fashion by a “hyper activated” AhR/ARNT-signaling by dioxin. Ours and others investigations over the crosstalk between AhR/RAR and AhR/ER signaling have revealed that multiple mechanisms may be involved. Such as 1) Competition over common target genes or 2) limited pools of common cofactors, or 3) regulating the levels of receptors or 4) ligands for the receptors.

1. Several genes seem to be commonly regulated by ERs and AhR, as well as RAR/RXR and AhR. Future investigations using for example Realtime-PCR to investigate such transcriptional interference over ER-, RAR/RXR- or AhR/ARNT regulated genes could help to obtain better understanding for the crosstalk
2. There is now evidence that the obligatory AhR partner protein ARNT interacts with both ERs and RXR/(RAR?). The later needs to be further characterized and by utilizing ChIP- assay it will be possible to reveal if ARNT is present over RAR/RXR regulated genes, i.e. has a functional role for the transcriptional activity of RAR/RXR.
3. AhR induced proteosomal degradation of ERs has been shown to be a mechanism whereby dioxins can act antiestrogenic.
4. As well as AhR mediated induction of enzymes that alters the metabolism of both steroid hormones as well as retinoids.

Thus, in order to achieve detailed mechanistic explanations behind the crosstalk between AhR-ER and AhR-RAR, several kinds of methodology must be used. As an example, by using immunoprecipitation and/or ChIP-assay one can further characterize which proteins are present when ARNT and RXR β are coprecipitated. Another way to reveal the possible importance of ARNT for RAR dependent activities could be to use siRNA to downregulate intracellular levels of ARNT and compare to a situation where there is plenty of ARNT present (by for example overexpressing ARNT in cells).

The novel retinoid S-4o9cDH-RA showed to be transcriptionally active in both cell studies as well as in an avian developmental model. The transcriptional activity was not as strong as seen for at-RA, in a direct comparison of the concentrations used, but there remain several question marks about this metabolite. For instance, we don't know

which enzymes are responsible for its synthesis or degradation. This could be revealed by targeted disruption of potential enzymes. It is also unknown how S-4o9cDH-RA is transported into the cells and the possible importance of retinoid binding proteins in the cellular handling of S-4o9cDH-RA. Thus, it is too early to claim that this retinoid is weaker as a ligand for the RARs from this study, since the effects we see can be dependent on several still unknown factors as the ones mentioned. Maybe S-4o9cDH-RA is not taken up by the cells to the same degree as at-RA, or perhaps it is more rapidly degraded by cellular enzymes. To draw conclusions about S-4o9cDH-RA's efficiency compared to at-RA and its biological relevance, more studies must be performed. Most importantly, we show that S-4o9cDH-RA is functioning as a novel ligand for at least two of the RAR family members, both in an *in vivo*-like model, and all cell types investigated.

8 ACKNOWLEDGEMENTS

Efter ett antal väldigt lärorika år som doktorand på KI kan jag nu se tillbaka på det hela och inse att jag träffat massor av trevliga, roliga människor som jag både kunnat bolla idéer med och som blivit vänner även utanför KI's väggar.

Jag vill först och främst tacka mina handledare **Helen Håkansson** och **Katarina Pettersson!** Tack för att ni hjälpt till att göra hela det här projektet genomförbart! Jag vill också tacka **Pi**, handledare under mitt examensarbete som visade hur forskning på riktigt går till, utanför universitetskurserna. Du var nog mycket du som fick mig att trivas så bra på labbet, från dag 1! **Kristina T**, vi lyckades tillsammans få de gamla HPLC-maskinerna att gå runt ett par varv till, på övertid. Tack **Inga-Lill** för att allt runtomkring alltid funkade så bra på enheten, utan dig skulle allt ha gått betydligt långsammare.

Jag har massor av roliga minnen från IMM, från vardagsgrejor i jobbet till alla sköna fester med roliga människor. Om jag inte minns fel så var det **Ian C, Ralf M, Bengt J** och **Roland G** som utgjorde IMM's luciatåg 2003, galet och helt oförglömligt. Det finns utöver dessa trevliga herrar en rad roliga människor som jag lärt känna under tiden på IMM: **Mattias Ö, Patrik A, Nick F, Natasha S, Rebecca L, Monica L, Krister H, Emma, Lina, Malin, Kristian D** och **Martin V, Rickard, Ilona** och **Louise L, Daniel B** och **Mattias P, Sara B, Terese, Katarina** och **Åse, Fredrik R.**

Sist men inte minst **Salomon**, din gamle stekare, som alltid är lika norrländskt cool-lugn oavsett vad som händer runtomkring, du är en förebild. Du var inte ens det minsta nervös när vi skickade upp dig på ett tandemhopp förra sommaren, otroligt! Synd bara att du envisas med att klamra dig fast vid Uppsala, man hinner ju ses så sällan tyvärr! Och **Daniel**, du ska nog få fler möjligheter att visa vad du går för i köket efter att vi skickade dig på italiensk matlagingskurs, jag kommer inte att missa nästa tillfälle!

När mitt projekt tog en lite ny inriktning hamnade jag hos IPO-gruppen i Huddinge, och fick då lära känna ännu fler härliga människor. Inte minst **Ingemar P** själv, tack för allt stöd! Även fast du inte varit min handledare officiellt så har du funnits tillhands vid behov och hjälpt till med såväl ekonomi som manusrevidering. Från att ha befunnit mig i en vardag med många grabbar på IMM hamnade jag nu i en grupp bestående av enbart tjejer, och Ingemar, vilket var lite annorlunda. Jag har bara trivts bättre och bättre för var dag i ert sällskap tjejer, och det har varit kul att lära känna er; **Petra, Elin, Mimmi, Joelle, Malin, Katta, Wen, Maria** och **Maria, Tanja, Paulina** och **Krista**, samt **Sara, Jan-Philip** och **Fabrice**, som kom dit en kortare period.

Petra, Elin och Mimmi, tack för att ni ställt upp som bollplank och stöttat, era råd har betytt mycket!

Joelle och **Ivan**, tack för alla trevliga hemmafester i er underbara lya i Gamla stan!

Tack **Ingemar** och **Katta** för att ni bjudit oss alla till ert hem vid flertalet tillfällen!

Johan H, tänk att världen är så liten jämt! I alla fall Bromma och Sollentuna! Kul att lära känna dig, **Ylva** och **Markus**! Synd bara att du valde fel förband en gång i tiden, annars hade vi nog känt varann längre ;-) Hoppas vi får tid att fortsätta träna lite ihop även framöver.

Patrik, du lyckades väl aldrig få med mig på att cykla från Sollentuna till Novum? Det var synd, jag hade säkert mått gott av några långrepor!

Tomas, jag väntar fortfarande på en invigningsgrogg på er beryktade altan! När blir det?

På Novum i övrigt finns många trevliga människor jag lärt känna under resor, luncher och runt på de olika labben: **Kirsten, Inger x 3, Cristophoros, Tassos, Knut, Eckardt, Karolina, Anders, Pia, Lovisa** och **Maria, Per, Gudrun, Karin D, Hui, David, Marika, Lotta, Anna, Milica, Patricia, Agneta, Lars-Arne**, och andra som jag kanske missat!

Till alla er utanför jobbet, tack för att ni finns där och påminner om att det finns annat att göra i livet än att pipettera prover!

Tack alla i min familj med bonussyskon och ungar för stöd och support, fastän ni aldrig riktigt förstått vad det är jag håller på med på jobbet trots mina försök till pedagogiska förklaringar; **pappa** och **Gona, mamma och Björn, Micke, Lotta, Anna-Lisa, Elin, Karin** och **Emma**, med **Frippe, Fredrik** och **Anders. Mormor** och **morfar**, jag är så glad att ni fortfarande hänger med. Jag kommer snart och hälsar på!

Nezze, Pepe, Stefan och **Tobias**, det är värdefullt att lyckas pussla ihop något galet upptåg åtminstone en gång per år, trots karriärer och små barn! Forspaddling, fallskärmshoppning, skärmflygning, dykning och annat skoj är sånt som man får perspektiv på tillvaron av! Snart ska jag väl lyckas få med er till crossbanan också hoppas jag!

Till kärntruppen: **Kråkan, Adam** och **Quicken**, tack för att ni är ett sånt skönt gäng goa vänner, och för alla gångna och kommande oförglömliga fester, middagar, hojåkningar och annat bus ihop med oss!

Och sist men inte minst, **Marie**, min stora kärlek, tack för att du finns! Jag fattar fortfarande knappt vad som händer! Hur livet kan vända och fyllas av så mycket lycka så plötsligt, så oväntat! Du har också varit enormt stöttande och förstående med mitt doktorerande, tack för det! Jag har nog aldrig varit så långvarigt stressad som på sistone, men nu kommer jag snart bli mig själv igen så du känner igen mig! Jag älskar dig!

9 REFERENCES

- Abdelrahim, M., Ariazi, E., Kim, K., Khan, S., Barhoumi, R., Burghardt, R., Liu, S. X., Hill, D., Finnell, R., Wlodarczyk, B., Jordan, V. C. and Safe, S. (2006). "3-Methylcholanthrene and other aryl hydrocarbon receptor agonists directly activate estrogen receptor alpha." *Cancer Research* **66**(4): 2459-2467.
- Achkar, C. C., Derguini, F., Blumberg, B., Langston, A., Levin, A. A., Speck, J., Evans, R. M., Bolado, J., Nakanishi, K., Buck, J. and Gudas, L. J. (1996). "4-Oxoretinol, a new natural ligand and transactivator of the retinoic acid receptors." *Proceedings of the National Academy of Sciences of the United States of America* **93**(10): 4879-4884.
- Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., Miller, C. A., Kato, T., Saeki, K. and Matsuda, T. (2001). "Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine." *Journal of Biological Chemistry* **276**(34): 31475-31478.
- Ahlborg, U. G., Brouwer, A., Fingerhut, M. A., Jacobson, J. L., Jacobson, S. W., Kennedy, S. W., Ketrup, A. A. F., Koeman, J. H., Poiger, H., Rappe, C., Safe, S. H., Seegal, R. F., Tuomisto, J. and Vandenberg, M. (1992). "Impact of Polychlorinated Dibenzo-P-Dioxins, Dibenzofurans, and Biphenyls on Human and Environmental-Health, with Special Emphasis on Application of the Toxic Equivalency Factor Concept." *European Journal of Pharmacology-Environmental Toxicology and Pharmacology Section* **228**(4): 179-199.
- Ahlborg, U. G., Lipworth, L., Tituserstoff, L., Hsieh, C. C., Hanberg, A., Baron, J., Trichopoulos, D. and Adami, H. O. (1995). "Organochlorine Compounds in Relation to Breast-Cancer, Endometrial Cancer, and Endometriosis - an Assessment of the Biological and Epidemiologic Evidence." *Critical Reviews in Toxicology* **25**(6): 463-531.
- Andreola, F., Calvisi, D. F., Elizondo, G., Jakowlew, S. B., Mariano, J., Gonzalez, F. J. and De Luca, L. M. (2004). "Reversal of liver fibrosis in aryl hydrocarbon receptor null mice by dietary vitamin A depletion." *Hepatology* **39**(1): 157-66.
- Andreola, F., FernandezSalguero, P. M., Chiantore, M. V., Petkovich, M. P., Gonzalez, F. J. and De Luca, L. M. (1997). "Aryl hydrocarbon receptor knockout mice (AHR(-/-)) exhibit liver retinoid accumulation and reduced retinoic acid metabolism." *Cancer Research* **57**(14): 2835-2838.
- Andreola, F., Hayhurst, G. P., Luo, G., Ferguson, S. S., Gonzalez, F. J., Goldstein, J. A. and De Luca, L. M. (2004). "Mouse liver CYP2C39 is a novel retinoic acid 4-hydroxylase. Its down-regulation offers a molecular basis for liver retinoid accumulation and fibrosis in aryl hydrocarbon receptor-null mice." *J Biol Chem* **279**(5): 3434-8.
- Aranda, A. and Pascual, A. (2001). "Nuclear hormone receptors and gene expression." *Physiol Rev* **81**(3): 1269-304.
- Baba, T., Mimura, J., Nakamura, N., Harada, N., Yamamoto, M., Morohashi, K. and Fujii-Kuriyama, Y. (2005). "Intrinsic function of the aryl hydrocarbon (Dioxin) receptor as a key factor in female reproduction." *Molecular and Cellular Biology* **25**(22): 10040-10051.

- Baker, J. I. and Hites, R. A. (2000). "Is combustion the major source of polychlorinated dibenzo-p-dioxins and dibenzofurans to the environment? A mass balance investigation." *Environmental Science & Technology* **34**(14): 2879-2886.
- Balmer, J. E. and Blomhoff, R. (2002). "Gene expression regulation by retinoic acid." *Journal of Lipid Research* **43**(11): 1773-1808.
- Barnett, K. R., Tomic, D., Gupta, R. K., Miller, K. P., Meachum, S., Paulose, T. and Flaws, J. A. (2007). "The aryl hydrocarbon receptor affects mouse ovarian follicle growth via mechanisms involving estradiol regulation and responsiveness." *Biology of Reproduction* **76**(6): 1062-1070.
- Bastien, J. and Rochette-Egly, C. (2004). "Nuclear retinoid receptors and the transcription of retinoid-target genes." *Gene* **328**: 1-16.
- Beischlag, T. V., Wang, S., Rose, D. W., Torchia, J., Reisz-Porszasz, S., Muhammad, K., Nelson, W. E., Probst, M. R., Rosenfeld, M. G. and Hankinson, O. (2002). "Recruitment of the NCoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex." *Molecular and Cellular Biology* **22**(12): 4319-4333.
- Benedict, J. C., Lin, T. M., Loeffler, I. K., Peterson, R. E. and Flaws, J. A. (2000). "Physiological role of the aryl hydrocarbon receptor in mouse ovary development." *Toxicological Sciences* **56**(2): 382-388.
- Benedict, J. C., Miller, K. P., Lin, T. M., Greenfeld, C., Babus, J. K., Peterson, R. E. and Flaws, J. A. (2003). "Aryl hydrocarbon receptor regulates growth, but not atresia, of mouse preantral and antral follicles." *Biology of Reproduction* **68**(5): 1511-1517.
- Benoit, G., Cooney, A., Giguere, V., Ingraham, H., Lazar, M., Muscat, G., Perlmann, T., Renaud, J. P., Schwabe, J., Sladek, F., Tsai, M. J. and Laudet, V. (2006). "International Union of Pharmacology. LXVI. Orphan nuclear receptors." *Pharmacological Reviews* **58**(4): 798-836.
- Bertrand, W., Brunet, F. G., Escriva, H., Parmentier, G., Laudet, V. and Robinson-Rechavi, M. (2004). "Evolutionary genomics of nuclear receptors: From twenty-five ancestral genes to derived endocrine systems." *Molecular Biology and Evolution* **21**(10): 1923-1937.
- Birnbaum, L. S. and Tuomisto, J. (2000). "Non-carcinogenic effects of TCDD in animals." *Food Additives and Contaminants* **17**(4): 275-288.
- Bjeldanes, L. F., Kim, J. Y., Grose, K. R., Bartholomew, J. C. and Bradfield, C. A. (1991). "Aromatic Hydrocarbon Responsiveness-Receptor Agonists Generated from Indole-3-Carbinol In vitro and In vivo - Comparisons with 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin." *Proceedings of the National Academy of Sciences of the United States of America* **88**(21): 9543-9547.
- Blaner, W. S. (2001). "Cellular metabolism and actions of 13-cis-retinoic acid." *Journal of the American Academy of Dermatology* **45**(5): S129-S135.
- Blomhoff, R. and Blomhoff, H. K. (2006). "Overview of retinoid metabolism and function." *Journal of Neurobiology* **66**(7): 606-630.
- Blumberg, B., Bolado, J., Derguini, F., Craig, A. G., Moreno, T. A., Chakravarti, D., Heyman, R. A., Buck, J. and Evans, R. M. (1996). "Novel retinoic acid receptor ligands in *Xenopus* embryos." *Proceedings of the National Academy of Sciences of the United States of America* **93**(10): 4873-4878.

- Bock, K. W. and Kohle, C. (2006). "Ah receptor: Dioxin-mediated toxic responses as hints to deregulated physiologic functions." *Biochemical Pharmacology* **72**(4): 393-404.
- Border, W. A. and Noble, N. A. (1994). "Transforming Growth-Factor-Beta in Tissue Fibrosis." *New England Journal of Medicine* **331**(19): 1286-1292.
- Boverhof, D. R., Kwekel, J. C., Humes, D. G., Burgoon, L. D. and Zacharewski, T. R. (2006). "Dioxin induces an estrogen-like, estrogen receptor-dependent gene expression response in the murine uterus." *Molecular Pharmacology* **69**(5): 1599-1606.
- Branton, M. H. and Kopp, J. B. (1999). "TGF-beta and fibrosis." *Microbes and Infection* **1**(15): 1349-1365.
- Brinckerhoff, C. E. and Matrisian, L. M. (2002). "Timeline - Matrix metalloproteinases: a tail of a frog that became a prince." *Nature Reviews Molecular Cell Biology* **3**(3): 207-214.
- Brouwer, A., Ahlborg, U. G., Vandenberg, M., Birnbaum, L. S., Boersma, E. R., Bosveld, B., Denison, M. S., Gray, L. E., Hagmar, L., Holene, E., Huisman, M., Jacobson, S. W., Jacobson, J. L., Koopmanesseboom, C., Koppe, J. G., Kulig, B. M., Morse, D. C., Muckle, G., Peterson, R. E., Sauer, P. J. J., Seegal, R. F., Smitsvanprooijje, A. E., Touwen, B. C. L., Weisglaskuperus, N. and Winneke, G. (1995). "Functional-Aspects of Developmental Toxicity of Polyhalogenated Aromatic-Hydrocarbons in Experimental-Animals and Human Infants." *European Journal of Pharmacology-Environmental Toxicology and Pharmacology Section* **293**(1): 1-40.
- Brouwer, A., Hakansson, H., Kukler, A., Vandenberg, K. J. and Ahlborg, U. G. (1989). "Marked Alterations in Retinoid Homeostasis of Sprague-Dawley Rats Induced by a Single Ip Dose of 10-Mu-G/Kg of 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin." *Toxicology* **58**(3): 267-283.
- Brunnberg, S. (2007). Ah Receptor mediated toxicity-Studies in vitro and in a transgenic mouse model. *Institute of environmental medicine*. Solna, Karolinska institutet: 1-47.
- Brunnberg, S., Pettersson, K., Rydin, E., Matthews, J., Hanberg, A. and Pongratz, I. (2003). "The basic helix-loop-helix-PAS protein ARNT functions as a potent coactivator of estrogen receptor-dependent transcription." *Proceedings of the National Academy of Sciences of the United States of America* **100**(11): 6517-6522.
- Bunger, M. K., Moran, S. M., Glover, E., Thomae, T. L., Lahvis, G. P., Lin, B. C. and Bradfield, C. A. (2003). "Resistance to 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and abnormal liver development in mice carrying a mutation in the nuclear localization sequence of the aryl hydrocarbon receptor." *Journal of Biological Chemistry* **278**(20): 17767-17774.
- Chambon, P. (1996). "A decade of molecular biology of retinoic acid receptors." *Faseb Journal* **10**(9): 940-54.
- Chen, J. D. and Li, H. (1998). "Coactivation and corepression in transcriptional regulation by steroid/nuclear hormone receptors." *Critical Reviews in Eukaryotic Gene Expression* **8**(2): 169-190.
- Choudhary, D., Jansson, I., Schenkman, J. B., Sarfarazi, M. and Stoilov, I. (2003). "Comparative expression profiling of 40 mouse cytochrome P450 genes in

- embryonic and adult tissues." *Archives of Biochemistry and Biophysics* **414**(1): 91-100.
- Choudhary, D., Jansson, I., Stoilov, I., Sarfarazi, M. and Schenkman, J. B. (2005). "Expression patterns of mouse and human CYP orthologs (families 1-4) during development and in different adult tissues." *Archives of Biochemistry and Biophysics* **436**(1): 50-61.
- Damdimopoulos, A. E., Spyrou, G. and Gustafsson, J. A. (2008). "Ligands Differentially Modify the Nuclear Mobility of Estrogen Receptors {alpha} and." *Endocrinology* **149**(1): 339-45.
- Diani-Moore, S., Labitzke, E., Brown, R., Garvin, A., Wong, L. and Rifkind, A. B. (2006). "Sunlight generates multiple tryptophan photoproducts eliciting high efficacy CYP1A induction in chick hepatocytes and in vivo." *Toxicological Sciences* **90**(1): 96-110.
- Drutel, G., Kathmann, M., Heron, A., Schwartz, J. C. and Arrang, J. M. (1996). "Cloning and selective expression in brain and kidney of ARNT2 homologous to the Ah receptor nuclear translocator (ARNT)." *Biochemical and Biophysical Research Communications* **225**(2): 333-339.
- Du, L. P., Neis, M. M., Ladd, P. A. and Keeney, D. S. (2006). "Differentiation-specific factors modulate epidermal CYP1-4 gene expression in human skin in response to retinoic acid and classic aryl hydrocarbon receptor ligands." *Journal of Pharmacology and Experimental Therapeutics* **319**(3): 1162-1171.
- Duester, G. (2000). "Families of retinoid dehydrogenases regulating vitamin A function - Production of visual pigment and retinoic acid." *European Journal of Biochemistry* **267**(14): 4315-4324.
- Fernandezsalguero, P., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S. T., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M. and Gonzalez, F. J. (1995). "Immune-System Impairment and Hepatic-Fibrosis in Mice Lacking the Dioxin-Binding Ah Receptor." *Science* **268**(5211): 722-726.
- FernandezSalguero, P. M., Hilbert, D. M., Rudikoff, S., Ward, J. M. and Gonzalez, F. J. (1996). "Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity." *Toxicology and Applied Pharmacology* **140**(1): 173-179.
- Fisher, G. J. and Voorhees, J. J. (1996). "Molecular mechanisms of retinoid actions in skin." *Faseb Journal* **10**(9): 1002-1013.
- FitzGerald, C. T., FernandezSalguero, P., Gonzalez, F. J., Nebert, D. W. and Puga, A. (1996). "Differential regulation of mouse Ah receptor gene expression in cell lines of different tissue origins." *Archives of Biochemistry and Biophysics* **333**(1): 170-178.
- Fletcher, N., Wahlstrom, D., Lundberg, R., Nilsson, C. B., Nilsson, K. C., Stockling, K., Hellmold, H. and Hakansson, H. (2005). "2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the mRNA expression of critical genes associated with cholesterol metabolism, bile acid biosynthesis, and bile transport in rat liver: A microarray study." *Toxicology and Applied Pharmacology* **207**(1): 1-24.
- Fryer, B. H. and Simon, M. C. (2006). "Hypoxia, HIF and the placenta." *Cell Cycle* **5**(5): 495-498.
- Furness, S. G. B., Lees, M. J. and Whitelaw, M. L. (2007). "The dioxin (aryl hydrocarbon) receptor as a model for adaptive responses of bHLH/PAS transcription factors." *Febs Letters* **581**(19): 3616-3625.

- Glass, C. K. and McDonnell, D. P. (2004). "The Jensen Symposium: A tribute to a pioneer in the field of nuclear receptor biology." *Molecular Cell* **13**(4): 459-467.
- Gu, Y. Z., Hogenesch, J. B. and Bradfield, C. A. (2000). "The PAS superfamily: Sensors of environmental and developmental signals." *Annual Review of Pharmacology and Toxicology* **40**: 519-561.
- Hakansson, H. and Ahlborg, U. G. (1985). "The Effect of 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin (Tcdd) on the Uptake, Distribution and Excretion of a Single Oral Dose of 11,12-H-3 Retinyl Acetate and on the Vitamin-a Status in the Rat." *Journal of Nutrition* **115**(6): 759-771.
- Hakansson, H., Waern, F. and Ahlborg, U. G. (1987). "Effects of 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin (Tcdd) in the Lactating Rat on Maternal and Neonatal Vitamin a Status." *Journal of Nutrition* **117**(3): 580-586.
- Hall, J. M. and McDonnell, D. P. (2005). "Coregulators in nuclear estrogen receptor action - From concept to therapeutic targeting." *Molecular Interventions* **5**(6): 343-357.
- Harris, H. A. (2007). "Estrogen receptor-beta: Recent lessons from in vivo studies." *Molecular Endocrinology* **21**(1): 1-13.
- Hess, R. A., Bunick, D. and Bahr, J. (2001). "Oestrogen, its receptors and function in the male reproductive tract - a review." *Molecular and Cellular Endocrinology* **178**(1-2): 29-38.
- Hilscherova, K., Machala, M., Kannan, K., Blankenship, A. L. and Giesy, J. P. (2000). "Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples." *Environmental Science and Pollution Research* **7**(3): 159-171.
- Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., Saijo, Y., Gotoh, O., Sogawa, K. and FujiiKuriyama, Y. (1996). "cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt)." *Molecular and Cellular Biology* **16**(4): 1706-1713.
- Hoegberg, P., Schmidt, C. K., Fletcher, N., Nilsson, C. B., Trossvik, C., Schuur, A. G., Brouwer, A., Nau, H., Ghyselinck, N. B., Chambon, P. and Hakansson, H. (2005). "Retinoid status and responsiveness to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking retinoid binding protein or retinoid receptor forms." *Chemico-Biological Interactions* **156**(1): 25-39.
- Hoegberg, P., Schmidt, C. K., Nau, H., Ross, A. C., Zolfaghari, R., Fletcher, N., Trossvik, C., Nilsson, C. B. and Hakansson, H. (2003). "2,3,7,8-tetrachlorodibenzo-p-dioxin induces lecithin: retinol acyltransferase transcription in the rat kidney." *Chemico-Biological Interactions* **145**(1): 1-16.
- Hollingshead, B. D., Petrusis, J. R. and Perdew, G. H. (2004). "The aryl hydrocarbon (Ah) receptor transcriptional regulator hepatitis B virus X-associated protein 2 antagonizes p23 binding to Ah receptor-Hsp90 complexes and is dispensable for receptor function." *Journal of Biological Chemistry* **279**(44): 45652-45661.
- Honda, S., Morohashi, K., Nomura, M., Takeya, H., Kitajima, M. and Omura, T. (1993). "Ad4bp Regulating Steroidogenic P-450 Gene Is a Member of Steroid-Hormone Receptor Superfamily." *Journal of Biological Chemistry* **268**(10): 7494-7502.
- Imai, S., Okuno, M., Moriwaki, H., Muto, Y., Murakami, K., Shudo, K., Suzuki, Y. and Kojima, S. (1997). "9,13-di-cis-retinoic acid induces the production of tPA and

- activation of latent TGF-beta via RAR alpha in a human liver stellate cell line, LI90." *Febs Letters* **411**(1): 102-106.
- Jain, S., Maltepe, E., Lu, M. M., Simon, C. and Bradfield, C. A. (1998). "Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse." *Mechanisms of Development* **73**(1): 117-123.
- Janknecht, R. and Hunter, T. (1996). "Transcription - A growing coactivator network." *Nature* **383**(6595): 22-23.
- Janosek, J., Hilscherova, K., Blaha, L. and Holoubek, I. (2006). "Environmental xenobiotics and nuclear receptors - Interactions, effects and in vitro assessment." *Toxicology in Vitro* **20**(1): 18-37.
- Jurek, M. A., Powers, R. H., Gilbert, L. G. and Aust, S. D. (1990). "The Effect of Tcdd on Acyl Coa-Retinoil Acyltransferase Activity and Vitamin-a Accumulation in the Kidney of Male Sprague-Dawley Rats." *Journal of Biochemical Toxicology* **5**(3): 155-160.
- Kazlauskas, A., Poellinger, L. and Pongratz, I. (1999). "Evidence that the Co-chaperone p23 regulates ligand responsiveness of the dioxin (aryl hydrocarbon) receptor." *Journal of Biological Chemistry* **274**(19): 13519-13524.
- Kelley, S. K., Nilsson, C. B., Green, M. H., Green, J. B. and Hakansson, H. (1998). "Use of model-based compartmental analysis to study effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vitamin A kinetics in rats." *Toxicological Sciences* **44**(1): 1-13.
- Kewley, R. J., Whitelaw, M. L. and Chapman-Smith, A. (2004). "The mammalian basic helix-loop-helix/PAS family of transcriptional regulators." *International Journal of Biochemistry & Cell Biology* **36**(2): 189-204.
- Klinge, C. M. (2000). "Estrogen receptor interaction with co-activators and co-repressors." *Steroids* **65**(5): 227-251.
- Kojima, S. and Rifkin, D. B. (1993). "Mechanism of Retinoid-Induced Activation of Latent Transforming Growth-Factor-Beta in Bovine Endothelial-Cells." *Journal of Cellular Physiology* **155**(2): 323-332.
- Kozak, K. R., Abbott, B. and Hankinson, O. (1997). "ARNT-deficient mice and placental differentiation." *Developmental Biology* **191**(2): 297-305.
- Krig, S. R. and Rice, R. H. (2000). "TCDD suppression of tissue transglutaminase stimulation by retinoids in malignant human keratinocytes." *Toxicological Sciences* **56**(2): 357-364.
- Kuiper, G., Enmark, E., PeltoHuikko, M., Nilsson, S. and Gustafsson, J. A. (1996). "Cloning of a novel estrogen receptor expressed in rat prostate and ovary." *Proceedings of the National Academy of Sciences of the United States of America* **93**(12): 5925-5930.
- Kumar, M. B., Tarpey, R. W. and Perdew, G. H. (1999). "Differential recruitment of coactivator RIP140 by Ah and estrogen receptors. Absence of a role for LXXLL motifs." *J Biol Chem* **274**(32): 22155-64.
- Lahvis, G. P., Lindell, S. L., Thomas, R. S., McCuskey, R. S., Murphy, C., Glover, E., Bentz, M., Southard, J. and Bradfield, C. A. (2000). "Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice." *Proceedings of the National Academy of Sciences of the United States of America* **97**(19): 10442-10447.
- Lahvis, G. P., Pyzalski, R. W., Glover, E., Pitot, H. C., McElwee, M. K. and Bradfield, C. A. (2005). "The aryl hydrocarbon receptor is required for developmental

- closure of the ductus venosus in the neonatal mouse." *Molecular Pharmacology* **67**(3): 714-720.
- Laudet, V., Hanni, C., Coll, J., Catzeflis, F. and Stehelin, D. (1992). "Evolution of the Nuclear Receptor Gene Superfamily." *Embo Journal* **11**(3): 1003-1013.
- Leid, M., Kastner, P. and Chambon, P. (1992). "Multiplicity Generates Diversity in the Retinoic Acid Signaling Pathways." *Trends in Biochemical Sciences* **17**(10): 427-433.
- Li, H., Leo, C., Schroen, D. J. and Chen, J. D. (1997). "Characterization of receptor interaction and transcriptional repression by the corepressor SMRT." *Molecular Endocrinology* **11**(13): 2025-2037.
- Loudig, O., Babichuk, C., White, J., Abu-Abed, S., Mueller, C. and Petkovich, M. (2000). "Cytochrome P450RAI(CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism." *Mol Endocrinol* **14**(9): 1483-97.
- Loudig, O., Maclean, G. A., Dore, N. L., Luu, L. and Petkovich, M. (2005). "Transcriptional co-operativity between distant retinoic acid response elements in regulation of Cyp26A1 inducibility." *Biochem J* **392**(Pt 1): 241-8.
- Maltepe, E., Schmidt, J. V., Baunoch, D., Bradfield, C. A. and Simon, M. C. (1997). "Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT." *Nature* **386**(6623): 403-407.
- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. (1995). "The Nuclear Receptor Superfamily - the 2nd Decade." *Cell* **83**(6): 835-839.
- Mark, M., Ghyselinck, N. B., Wendling, O., Dupe, V., Mascrez, B., Kastner, P. and Chambon, P. (1999). "A genetic dissection of the retinoid signalling pathway in the mouse." *Proceedings of the Nutrition Society* **58**(3): 609-613.
- Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T. N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M. and FujiiKuriyama, Y. (1997). "Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor." *Genes to Cells* **2**(10): 645-654.
- Moras, D. and Gronemeyer, H. (1998). "The nuclear receptor ligand-binding domain: structure and function." *Current Opinion in Cell Biology* **10**(3): 384-391.
- Morse, D. C. and Brouwer, A. (1995). "Fetal, Neonatal, and Long-Term Alterations in Hepatic Retinoid Levels Following Maternal Polychlorinated Biphenyl Exposure in Rats." *Toxicology and Applied Pharmacology* **131**(1): 175-182.
- Mukai, M. and Tischkau, S. A. (2007). "Effects of tryptophan photoproducts in the circadian timing system: Searching for a physiological role for aryl hydrocarbon receptor." *Toxicological Sciences* **95**(1): 172-181.
- Mukerjee, D. (1998). "Health impact of polychlorinated dibenzo-p-dioxins: A critical review." *Journal of the Air & Waste Management Association* **48**(2): 157-165.
- Murphy, K. A., Quadro, L. and White, L. A. (2007). "The Intersection Between the Aryl Hydrocarbon Receptor (AhR)- and Retinoic Acid-Signaling Pathways." *Vitamins and Hormones-Advances in Research and Applications* **75**: 33-67.
- Murphy, K. A., Villano, C. M., Dorn, R. and White, L. A. (2004). "Interaction between the aryl hydrocarbon receptor and retinoic acid pathways increases matrix metalloproteinase-1 expression in keratinocytes." *Journal of Biological Chemistry* **279**(24): 25284-93.

- Napoli, J. L. (1996). "Retinoic acid biosynthesis and metabolism." *Faseb Journal* **10**(9): 993-1001.
- Napoli, J. L., Boerman, M. H., Chai, X., Zhai, Y. and Fiorella, P. D. (1995). "Enzymes and binding proteins affecting retinoic acid concentrations." *J Steroid Biochem Mol Biol* **53**(1-6): 497-502.
- Nguyen, T. A., Hoivik, D., Lee, J. E. and Safe, S. (1999). "Interactions of nuclear receptor coactivator/corepressor proteins with the aryl hydrocarbon receptor complex." *Archives of Biochemistry and Biophysics* **367**(2): 250-257.
- Niederreither, K., McCaffery, P., Drager, U. C., Chambon, P. and Dolle, P. (1997). "Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development." *Mechanisms of Development* **62**(1): 67-78.
- Nilsson, C. B. and Hakansson, H. (2002). "The retinoid signaling system--a target in dioxin toxicity." *Crit Rev Toxicol* **32**(3): 211-32.
- Nilsson, C. B., Hanberg, A., Trossvik, C. and Hakansson, H. (1996). "2,3,7,8-tetrachlorodibenzo-p-dioxin affects retinol esterification in rat hepatic stellate cells and kidney." *Environmental Toxicology and Pharmacology* **2**(1): 17-23.
- Nilsson, C. B., Hoegberg, P., Trossvik, C., Azais-Braesco, V., Blaner, W. S., Fex, G., Harrison, E. H., Nau, H., Schmidt, C. K., van Bennekum, A. M. and Hakansson, H. (2000). "2,3,7,8-tetrachlorodibenzo-p-dioxin increases serum and kidney retinoic acid levels and kidney retinol esterification in the rat." *Toxicology and Applied Pharmacology* **169**(2): 121-131.
- Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M. and Gustafsson, J. A. (2001). "Mechanisms of estrogen action." *Physiological Reviews* **81**(4): 1535-1565.
- Noy, N. (2000). "Retinoid-binding proteins: mediators of retinoid action." *Biochemical Journal* **348**: 481-495.
- Oberg, M., Bergander, L., Hakansson, H., Rannug, U. and Rannug, A. (2005). "Identification of the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole, in cell culture medium, as a factor that controls the background aryl hydrocarbon receptor activity." *Toxicol Sci* **85**(2): 935-43.
- Okuno, M., Moriwaki, H., Imai, S., Muto, Y., Kawada, N., Suzuki, Y. and Kojima, S. (1997). "Retinoids exacerbate rat liver fibrosis by inducing the activation of latent TGF-beta in liver stellate cells." *Hepatology* **26**(4): 913-921.
- Okuno, M., Sato, T., Kitamoto, T., Imai, S., Kawada, N., Suzuki, Y., Yoshimura, H., Moriwaki, H., Onuki, K., Masushige, S., Muto, Y., Friedman, S. L., Kato, S. and Kojima, S. (1999). "Increased 9,13-di-cis-retinoic acid in rat hepatic fibrosis: implication for a potential link between retinoid loss and TGF-beta mediated fibrogenesis in vivo." *Journal of Hepatology* **30**(6): 1073-1080.
- Peterson, R. E., Theobald, H. M. and Kimmel, G. L. (1993). "Developmental and Reproductive Toxicity of Dioxins and Related-Compounds - Cross-Species Comparisons." *Critical Reviews in Toxicology* **23**(3): 283-335.
- Petrulis, J. R. and Perdew, G. H. (2002). "The role of chaperone proteins in the aryl hydrocarbon receptor core complex." *Chemico-Biological Interactions* **141**(1-2): 25-40.
- Pijnappel, W. W. M., Folkers, G. E., de Jonge, W. J., Verdegem, P. J. E., de Laat, S. W., Lugtenburg, J., Hendriks, H. F. J., van der Saag, P. T. and Durston, A. J. (1998). "Metabolism to a response pathway selective retinoid ligand during

- axial pattern formation." *Proceedings of the National Academy of Sciences of the United States of America* **95**(26): 15424-15429.
- Pollenz, R. S. (2002). "The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation." *Chemico-Biological Interactions* **141**(1-2): 41-61.
- Raner, G. M., Vaz, A. D. N. and Coon, M. J. (1996). "Metabolism of all-trans, 9-cis, and 13-cis isomers of retinal by purified isozymes of microsomal cytochrome P450 and mechanism-based inhibition of retinoid oxidation by citral." *Molecular Pharmacology* **49**(3): 515-522.
- Rannug, A., Rannug, U., Rosenkranz, H. S., Winqvist, L., Westerholm, R., Agurell, E. and Grafstrom, A. K. (1987). "Certain Photooxidized Derivatives of Tryptophan Bind with Very High-Affinity to the Ah Receptor and Are Likely to Be Endogenous Signal Substances." *Journal of Biological Chemistry* **262**(32): 15422-15427.
- Rannug, U., Rannug, A., Sjoberg, U., Li, H., Westerholm, R. and Bergman, J. (1995). "Structure elucidation of two tryptophan-derived, high affinity Ah receptor ligands." *Chemistry & Biology* **2**(12): 841-845.
- Ray, W. J., Bain, G., Yao, M. and Gottlieb, D. I. (1997). "CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family." *Journal of Biological Chemistry* **272**(30): 18702-18708.
- Roberts, E. S., Vaz, A. D. N. and Coon, M. J. (1992). "Role of Isozymes of Rabbit Microsomal Cytochrome-P-450 in the Metabolism of Retinoic Acid, Retinol, and Retinal." *Molecular Pharmacology* **41**(2): 427-433.
- Robyr, D., Wolffe, A. P. and Wahli, W. (2000). "Nuclear hormone receptor coregulators in action: Diversity for shared tasks." *Molecular Endocrinology* **14**(3): 329-347.
- Rubin, A. L. and Rice, R. H. (1988). "2,3,7,8-Tetrachlorodibenzo-Para-Dioxin and Polycyclic Aromatic-Hydrocarbons Suppress Retinoid-Induced Tissue Transglutaminase in Sc-4 Cultured Human Squamous Carcinoma-Cells." *Carcinogenesis* **9**(6): 1067-1070.
- Rushing, S. R. and Denison, M. S. (2002). "The silencing mediator of retinoic acid and thyroid hormone receptors can interact with the aryl hydrocarbon (Ah) receptor but fails to repress Ah receptor-dependent gene expression." *Arch Biochem Biophys* **403**(2): 189-201.
- Safe, S. and Wormke, M. (2003). "Inhibitory aryl hydrocarbon receptor-estrogen receptor a cross-talk and mechanisms of action." *Chemical Research in Toxicology* **16**(7): 807-816.
- Santiago-Josefat, B., Mulero-Navarro, S., Dallas, S. L. and Fernandez-Salguero, P. M. (2004). "Overexpression of latent transforming growth factor-beta binding protein 1 (LTBP-1) in dioxin receptor-null mouse embryo fibroblasts." *Journal of Cell Science* **117**(6): 849-859.
- Schmidt, J. V., Su, G. H. T., Reddy, J. K., Simon, M. C. and Bradfield, C. A. (1996). "Characterization of a murine Ahr null allele: Involvement of the Ah receptor in hepatic growth and development." *Proceedings of the National Academy of Sciences of the United States of America* **93**(13): 6731-6736.
- Schrenk, D., Riebinger, D., Till, M., Vetter, S. and Fiedler, H. P. (1997). "Tryptanthrins: A novel class of agonists of the aryl hydrocarbon receptor." *Biochemical Pharmacology* **54**(1): 165-171.

- Schrenk, D., Riebinger, D., Till, M., Vetter, S. and Fiedler, H. P. (1999). Tryptanthrins and other tryptophan-derived agonists of the dioxin receptor. Tryptophan, Serotonin and Melatonin. **467**: 403-408.
- Schwabe, J. W. and Teichmann, S. A. (2004). "Nuclear receptors: the evolution of diversity." *Sci STKE* **2004**(217): pe4.
- Sekine, H., Mimura, J., Yamamoto, M. and Fujii-Kuriyama, Y. (2006). "Unique and overlapping transcriptional roles of arylhydrocarbon receptor nuclear translocator (Arnt) and Arnt2 in xenobiotic and hypoxic responses." *Journal of Biological Chemistry* **281**(49): 37507-37516.
- Soprano, D. R., Gambone, C. J., Sheikh, S. N., Gabriel, J. L., Chandraratna, R. A. S., Soprano, K. J. and Kochhar, D. M. (2001). "The synthetic retinoid AGN 193109 but not retinoic acid elevates CYP1A1 levels in mouse embryos and Hepa-1c1c7 cells." *Toxicology and Applied Pharmacology* **174**(2): 153-159.
- Swedenborg, E., Ruegg, J., Hillenweck, A., Rehnmark, S., Hedengran Faulds, M., Zalko, D., Pongratz, I. and Pettersson, K. (2007). "3-methyl cholanthrene displays dual effects on ER{alpha} and ER{beta} signaling in a cell-type specific fashion." *Mol Pharmacol*.
- Takemoto, K., Nakajima, M., Fujiki, Y., Katoh, M., Gonzalez, F. J. and Yokoi, T. (2004). "Role of the aryl hydrocarbon receptor and Cyp1b1 in the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin." *Archives of Toxicology* **78**(6): 309-315.
- Tijet, N., Boutros, P. C., Moffat, I. D., Okey, A. B., Tuomisto, J. and Pohjanvirta, R. (2006). "Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries." *Molecular Pharmacology* **69**(1): 140-153.
- Tohkin, M., Fukuhara, M., Elizondo, G., Tomita, S. and Gonzalez, F. J. (2000). "Aryl hydrocarbon receptor is required for p300-mediated induction of DNA synthesis by adenovirus E1A." *Molecular Pharmacology* **58**(4): 845-851.
- Tomita, S., Okuyama, E., Ohnishi, T. and Ichikawa, Y. (1996). "Characteristic properties of a retinoic acid synthetic cytochrome P-450 purified from liver microsomes of 3-methylcholanthrene-induced rat." *Biochimica Et Biophysica Acta-General Subjects* **1290**(3): 273-281.
- Walisser, J. A., Glover, E., Pande, K., Liss, A. L. and Bradfield, C. A. (2005). "Aryl hydrocarbon receptor-dependent liver development and hepatotoxicity are mediated by different cell types." *Proceedings of the National Academy of Sciences of the United States of America* **102**(49): 17858-17863.
- Van den Berg, M., Birnbaum, L. S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N. and Peterson, R. E. (2006). "The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds." *Toxicological Sciences* **93**(2): 223-241.
- Vanbirgelen, A., Vanderkolk, J., Fase, K. M., Bol, I., Poiger, H., Brouwer, A. and Vandenberg, M. (1995). "Subchronic Dose-Response Study of 2,3,7,8-Tetrachlorodibenzo-P-Dioxin in Female Sprague-Dawley Rats." *Toxicology and Applied Pharmacology* **132**(1): 1-13.
- Wang, F., Hoivik, D., Pollenz, R. and Safe, S. (1998). "Functional and physical interactions between the estrogen receptor Sp1 and nuclear aryl hydrocarbon receptor complexes." *Nucleic Acids Research* **26**(12): 3044-3052.

- Wang, X., Sperkova, Z. and Napoli, J. L. (2001). "Analysis of mouse retinal dehydrogenase type 2 promoter and expression." *Genomics* **74**(2): 245-50.
- Wanner, R., Panteleyev, A., Henz, B. M. and Rosenbach, T. (1996). "Retinoic acid affects the expression rate of the differentiation-related genes aryl hydrocarbon receptor, ARNT and keratin 4 in proliferative keratinocytes only." *Biochimica et Biophysica Acta-Molecular Basis of Disease* **1317**(2): 105-11.
- Watanabe, H., Suzuki, A., Goto, M., Ohsako, S., Tohyama, C., Handa, H. and Iguchi, T. (2004). "Comparative uterine gene expression analysis after dioxin and estradiol administration." *Journal of Molecular Endocrinology* **33**(3): 763-771.
- Vecchini, F., Lenoir-Viale, M. C., Cathelineau, C., Magdalou, J., Bernard, B. A. and Shroot, B. (1994). "Presence of a retinoid responsive element in the promoter region of the human cytochrome P4501A1 gene." *Biochemical and Biophysical Research Communications* **201**(3): 1205-12.
- Wei, Y. D., Bergander, L., Rannug, U. and Rannug, A. (2000). "Regulation of CYP1A1 transcription via the metabolism of the tryptophan-derived 6-formylindolo 3,2-b carbazole." *Archives of Biochemistry and Biophysics* **383**(1): 99-107.
- Wei, Y. D., Helleberg, H., Rannug, U. and Rannug, A. (1998). "Rapid and transient induction of CYP1A1 gene expression in human cells by the tryptophan photoproduct 6-formylindolo 3,2-b carbazole." *Chemico-Biological Interactions* **110**(1-2): 39-55.
- Weigel, N. L. and Moore, N. L. (2007). "Steroid receptor phosphorylation: A key modulator of multiple receptor functions." *Molecular Endocrinology* **21**(10): 2311-2319.
- Weston, A. D., Blumberg, B. and Underhill, T. M. (2003). "Active repression by unliganded retinoid receptors in development: less is sometimes more." *Journal of Cell Biology* **161**(2): 223-228.
- Weston, W. M., Nugent, P. and Greene, R. M. (1995). "Inhibition of retinoic-acid-induced gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin." *Biochem Biophys Res Commun* **207**(2): 690-4.
- Widerak, M., Ghoneim, C., Dumontier, M. F., Quesne, M., Corvol, M. T. and Savouret, J. F. (2006). "The aryl hydrocarbon receptor activates the retinoic acid receptor alpha through SMRT antagonism." *Biochimie* **88**(3-4): 387-397.
- Villano, C. M., Murphy, K. A., Akintobi, A. and White, L. A. (2006). "2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces matrix metalloproteinase (MMP) expression and invasion in A2058 melanoma cells." *Toxicology and Applied Pharmacology* **210**(3): 212-224.
- Vincenti, M. P., White, L. A., Schroen, D. J., Benbow, U. and Brinckerhoff, C. E. (1996). "Regulating expression of the gene for matrix metalloproteinase-1 (collagenase): Mechanisms that control enzyme activity, transcription, and mRNA stability." *Critical Reviews in Eukaryotic Gene Expression* **6**(4): 391-411.
- Wormke, M., Stoner, M., Saville, B., Walker, K., Abdelrahim, M., Burghardt, R. and Safe, S. (2003). "The aryl hydrocarbon receptor mediates degradation of estrogen receptor alpha through activation of proteasomes." *Molecular and Cellular Biology* **23**(6): 1843-1855.

- Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D. and Gronemeyer, H. (1996). "A canonical structure for the ligand-binding domain of nuclear receptors." *Nature Structural Biology* **3**(1): 87-94.
- Xu, J. M. and Li, Q. T. (2003). "Review of the in vivo functions of the p160 steroid receptor coactivator family." *Molecular Endocrinology* **17**(9): 1681-1692.
- Yang, Y. M., Huang, D. Y., Liu, G. F., Zhong, J. C., Du, K., Li, Y. F. and Song, X. H. (2005). "Inhibitory effects of vitamin A on TCDD-induced cytochrome P-450 1A1 enzyme activity and expression." *Toxicological Sciences* **85**(1): 727-34.
- Yao, T. P., Ku, G., Zhou, N. D., Scully, R. and Livingston, D. M. (1996). "The nuclear hormone receptor coactivator SRC-1 is a specific target of p300." *Proceedings of the National Academy of Sciences of the United States of America* **93**(20): 10626-10631.
- Yoh, S. M. and Privalsky, M. L. (2001). "Transcriptional repression by thyroid hormone receptors - A role for receptor homodimers in the recruitment of SMRT corepressor." *Journal of Biological Chemistry* **276**(20): 16857-16867.
- Zaher, H., Fernandez-Salguero, P. M., Letterio, J., Sheikh, M. S., Fornace, A. J., Roberts, A. B. and Gonzalez, F. J. (1998). "The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis." *Molecular Pharmacology* **54**(2): 313-321.
- Zhang, Q. Y., Dunbar, D. and Kaminsky, L. (2000). "Human cytochrome P-450 metabolism of retinals to retinoic acids." *Drug Metabolism and Disposition* **28**(3): 292-297.