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INDREK TEINO

Studies on aryl hydrocarbon receptor in the mouse granulosa cell model

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Chair of Cell Biology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

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Supervisor: Prof. Toivo Maimets, PhD
Chair of Cell Biology
Institute of Molecular and Cell Biology
University of Tartu, Estonia

Reviewer Prof. Ants Kurg
Chair of Biotechnology
Institute of Molecular and Cell Biology
University of Tartu, Estonia

Opponent: Prof. Jason Matthews, PhD
Department of Nutrition
Institute of Basic Medical Sciences
University of Oslo, Norway

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LIST OF ORIGINAL PUBLICATIONS

This thesis includes the following original publications that are referred to in the text by their Roman numerals:

I. Matvere, A., **Teino, I.**, Varik, I., Kuuse, S., Tiido, T., Kristjuhan, A., Maimets, T. (2019) FSH/LH-Dependent Upregulation of Ahr in Murine Granulosa Cells Is Controlled by PKA Signaling and Involves Epigenetic Regulation. Int. J. Mol. Sci. 20(12):3068

II. **Teino, I.**, Matvere, A., Kuuse, S., Ingerpuu, S., Maimets, T., Kristjuhan, A., Tiido, T. (2014) Transcriptional repressioon of the Ahr gene by LHCGR signaling in preovulatory granulosa cells is controlled by chromatin accessibility. Mol. Cell. Endocrinol. 382(1):292–301

III. **Teino, I.**, Kuuse, S., Ingerpuu, S., Maimets, T., Tiido, T. (2012) The aryl hydrocarbon receptor regulates mouse Fshr promoter activity through an E-box binding site. Biol. Reprod. 86(3):77

My contributions to these articles are as follows:

Ref. I I participated in designing and performing the experiments, data analysis and manuscript preparation.

Ref. II I participated in designing and performing the experiments, data analysis and manuscript preparation.

Ref. III I participated in experiment design, performed the experiments, contributed to data analysis and manuscript preparation

LIST OF ABBREVIATIONS

AC	adenylate cyclase
ActD	actinomycin D
AHR	aryl hydrocarbon receptor
AHRR	AHR repressor
AIP	AHR-interacting protein
AP-1	activator protein 1
ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH	basic helix-loop helix
CA-AHR	constitutively active AHR
cAMP	cyclic adenosine monophosphate
CHART-PCR	chromatin accessibility by real-time PCR
CHX	cycloheximide
CTL	cytotoxic T-lymphocyte
CYP19A1	cytochrome P450 family 19 subfamily A member 1, aromatase
CYP1A1	cytochrome P450 family 1 subfamily A member 1
CYP1B1	cytochrome P450 family 1 subfamily B member 1
DAPA	DNA affinity precipitation assay
DES	diethylstilbestrol
E2	oestradiol
E2F1	E2F transcription factor 1
EB	embryoid body
ERK1/2	extracellular signal-regulated kinase 1/2
FICZ	6-formylindolo[3,2-*b*]carbazole
FSH	follicle stimulating hormone
Fshr	follicle stimulating hormone receptor
FSK	forskolin
GC	granulosa cell
HAH	halogenated aromatic hydrocarbon
hCG	human chorionic gonadotropin
HDAC	histone deacetylase
hES cell	human embryonic stem cell
HSP90	heat shock protein 90
IDO1	indoleamine-2,3-dioxygenase 1
ISX	intestine-specific homeobox transcription factor
KLF6	Kruppel like factor 6
Kyn	kynurenine
LH	luteinising hormone
Lhcgr	luteinising hormone/chorionic gonadotropin receptor
mES cell	mouse embryonic stem cell

NFκB	nuclear factor kappa B
PAH	polycyclic aromatic hydrocarbon
PAS domain	Per/Arnt/Sim domain
p-Creb	phosphorylated cAMP responsive element binding protein
PD-1	programmed death protein 1
PD-L1	programmed death ligand 1
PKA	protein kinase A
PMSG	pregnant mare serum gonadotropin
RB	retinoblastoma protein
TCDD	2,3,7,8-tetrachlorodibenzo-para-dioxin
TDO2	tryptophan-2,3-dioxygenase 2
TiPARP	TCDD-inducible poly(ADP-ribose) polymerase
TSA	trichostatin A
TSS	transcriptional start site
USF1	upstream stimulatory factor 1
XRE	xenobiotic response element

1. INTRODUCTION

For decades, scientists have concentrated on determining how various environmental pollutants affect organisms. The adverse effects of these chemicals on health have been established, among others, in tumorigenesis, impairments in immune system and reproduction, although investigations on the precise mechanisms are still ongoing. Many of these contaminants were identified to act as agonists of the aryl hydrocarbon receptor (AHR), which is widely expressed in the organism. Upon ligand binding, AHR translocates to the nucleus and modulates the expression of its target genes, including those responsible for the degradation of these chemicals. Initially, AHR was considered to act solely as a mediator of environmental stimuli. However, later studies using knockout models have attributed AHR a role in cellular homeostasis clarifying that it is more than a xenobiotic-interacting protein. To date, a large number of AHR modulators, both exogenous and endogenous, has been identified. Importantly, published data provide evidence that different ligands exert distinct effects in terms of gene expression and this may additionally depend on cell type and model organism. Apart from activity modulation, the expression of AHR itself is subject to various signals. Although this has gained less attention, the need for research in this specific field is evident, as overexpression of AHR has been observed in various tumours. Hence, alteration of AHR expression, in addition to activity modulation, has potential therapeutic value.

The role of AHR in female reproduction has been elucidated concerning xenobiotic AHR agonists acting as endocrine disruptors. Ahr is also known to modulate the expression of *aromatase* (*Cyp19a1*) and *follicle stimulating hormone receptor* (*Fshr*) in the ovarian granulosa cells (GCs) endogenously i.e. without exogenous ligands. Importantly, *Ahr* expression itself is fluctuating largely in rat granulosa cells during oestrous cycle, referring to the importance of *Ahr* regulation. Considering this, the present thesis is focused on delineating the mechanisms that control *Ahr* expression in mouse GCs. Both upregulation during follicular maturation as well as downregulation following luteinising hormone (LH) surge are addressed. Moreover, the interactions between Ahr and *Fshr* promoter are characterised in detail.

The first part of the thesis demonstrates that Ahr is upregulated *in vivo* in GCs during ovarian follicle maturation and this requires both FSH and LH signalling. The increase of Ahr levels is intrinsic to large antral follicles. This involves reduction of protein kinase A (PKA) signalling, as exemplified by decreased p-Creb levels, and is regulated by chromatin remodelling. The second part focuses on how Ahr is downregulated in preovulatory GCs following LH-surge. The mechanism includes PKA dependent decrease of *Ahr* transcription resulting from reduced chromatin accessibility. The third part concentrates on identifying

the region of *Fshr* promoter that is responsive to Ahr. Binding of Ahr to the E-box motif is determined. Further, Ahr interacts directly with *Fshr* promoter rather than via other proteins, supported by the fact that its DNA binding ability is crucial to activate *Fshr* promoter. Finally, evidence is provided that TCDD regulates *Fshr* positively.

2. LITERATURE OVERVIEW

2.1. Aryl hydrocarbon receptor

The ligand-dependent transcription factor – aryl hydrocarbon receptor (AHR) – was initially identified as the mediator by which 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD), an environmental pollutant, exerts its toxic effects. In 1976 it was established that TCDD, along with 23 another chemicals, induced the hepatic CYP1A1 activity through a protein, which was named aromatic/aryl hydrocarbon receptor – AHR (Poland & Glover 1976). Since then, there are numerous publications investigating the toxicological role of AHR in various tissue and cell types, including cancer. The identification of novel xenobiotic ligands as well as investigations in their mode of action are still ongoing. Recent advances in the field have expanded our knowledge on the role of AHR. The finding of endogenous AHR ligands has paved way in our understanding that AHR is more than a xenobiotic interacting protein and it has essential roles in the organism without activation by exogenous ligands. AHR has been shown to be important in normal cellular homeostasis and aberrations in its signalling pathway are associated with various health conditions.

To date, the expression of AHR has been determined in most cell types, suggesting it has a central role in cellular processes. Apart from its role in the induction of xenobiotic metabolizing enzymes such as classical targets CYP1A1 and CYP1B1, AHR has been shown to regulate numerous genes involved in cellular homeostasis (e.g. p27, p16) but also interact with proteins like RB, E2F1, NFκB etc. (Frauenstein et al. 2013; Ge & Elferink 1998; Marlowe et al. 2008; Ray & Swanson 2004; Tian et al. 1999; Vogel et al. 2013). The importance of AHR has been underscored in development as embryonic and hematopoietic stem cells as well as and neural progenitors are affected by AHR activity (Boitano et al. 2010; Ko et al. 2016; Latchney et al. 2011). Further publications have, among others, described the role of AHR in female reproduction, liver homeostasis and immune system (Baba et al. 2005; Barnett et al. 2007a; Lu et al. 2015; Quintana et al. 2008). More importantly, latest advances in the field have emphasised the modulation of AHR activity in cancer treatments involving immunomodulatory checkpoint inhibitors.

2.2. The structure of AHR

Human *AHR* is located on the seventh chromosome (chromosome 12 in mouse). It consists of 11 exons coding for a 6243 bp mRNA (5548 bp in mouse) (Ensembl.org a, b). This mRNA is translated into 848 (805 in mouse) amino acid residue protein with the approximate molecular weight of 96 kDa (~90 kDa in

mouse) (Uniprot.org a, b). AHR belongs to the basic helix-loop-helix-PER/ARNT/SIM (bHLH-PAS) family of proteins. The highly conserved N-terminus contains nuclear localisation and export sequences (Figure 1) (Ikuta et al. 1998, 2000). The bHLH domain includes basic residues responsible for AHR interaction with DNA and a HLH motif required for dimerization with ARNT (Schulte et al. 2017). Adjacent to these sequences are serine residues, which are targets of protein kinase C (PKC). Phosphorylation of these amino acids has been reported to inhibit AHR nuclear import (Ikuta et al. 2004). The PAS domain, also present in PER, ARNT and SIM proteins, consists of two inverted repeats – PAS-A and PAS-B. In general, PAS-A binds HSP90 in the cytoplasm. In the nucleus, however, this domain is responsible for heterodimerisation with ARNT. PAS-B, in turn, is essential for ligand binding and for interactions with its cytoplasmic binding partners HSP90, AIP/XAP2/ARA9 (AHR-interacting protein/X-associated protein 2/AHR-associated protein 9) and p23 (Bell & Poland 2000; Kazlauskas et al. 1999; Perdew 1988). Next to PAS domains lies the inhibitory domain (ID) that suppresses target gene activation (Ma et al. 1995). The C-terminus of AHR varies among species accounting for differences in protein length. It contains a glutamine rich domain responsible for transactivation of its target genes as well as binding sites for various cofactors like p300, RIP140 and SMRT (Kumar & Perdew 1999; Rushing & Denison 2002; Tohkin et al. 2000).

For a long time AHR was refractory to crystallisation. Thus, homology studies were performed utilising the similarity with other proteins containing PAS domains. Recently, however, there have been publications describing the AHR/ARNT dimer in complex with DNA (Schulte et al. 2017; Seok et al. 2017). This advancement allows identification of novel AHR agonists/antagonists as the need for identification of novel AHR ligands is growing in both toxicological aspects as well as therapy.

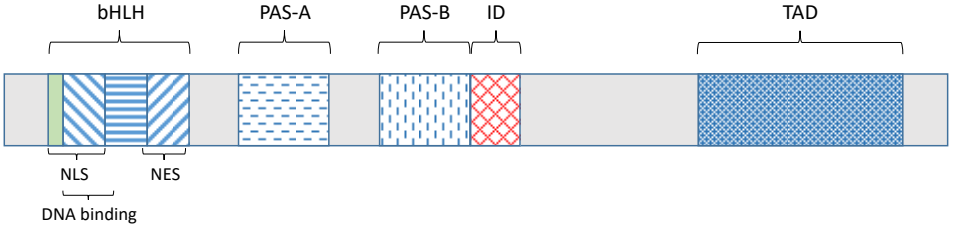

Figure 1. Schematic representation of AHR. The N-terminal bHLH domain harbours nuclear localisation sequence (NLS), nuclear export sequence (NES) and DNA binding sequence. PAS-A domain is responsible for heterodimerisation, whereas PAS-B contains the ligand-binding pocket and interacts with HSP90. Next to the PAS domains lies the inhibitory domain (ID), which suppresses target gene activation. The C-terminal transactivation domain (TAD) is responsible for target gene activation and cofactor binding.

2.3. AHR signalling pathway

The unliganded or resting AHR resides in the cytoplasm in complex with AIP, p23 and two molecules of HSP90. Activation of AHR by ligands results in conformational changes exposing the nuclear localisation signal to importin β (Petrulis et al. 2003). Subsequently, AHR translocates to the nucleus where it is freed from its cytoplasmic chaperones, which inhibit AHR binding to DNA (Heid et al. 2000; Lees & Whitelaw 1999). HSP90 is displaced by ARNT forming a heterodimer (McGuire et al. 1994; Soshilov & Denison 2008). This complex is capable to recognise and bind specific sequences in the regulatory region of AHR target genes. The AHR/ARNT dimer then recruits additional cofactors to modulate target gene expression, followed by ubiquitination, nuclear export and degradation in 26S proteasome pathway (Figure 2) (Ma & Baldwin 2000). Apart from this cascade of events, termed genomic AHR pathway, the existence of non-genomic pathway has been described. Although it has gained less attention, studies indicate that the non-genomic pathway includes elevated intracellular calcium levels, release of the tyrosine kinase c-Src from the activated AHR, focal adhesion kinase activation and subsequent increase in cell migration (Tomkiewicz et al. 2013).

The classical consensus sequence for AHR/ARNT binding – XRE/DRE/AHRE (xenobiotic/dioxin/AHR response element) – consists of the nucleotides 5'-TNGCGTG-3' (Denison et al. 1988). It has been shown that the TNGC half-site is bound by AHR, whereas ARNT binds GTG. Additional publications have emphasised the importance of adjacent nucleotides in the flanking regions of the consensus binding site (Shen & Whitlock 1992). More precisely, A and T have been shown to be important in target gene transactivation at positions +1 and +3 downstream of XRE, respectively as mutations in these positions rendered the promoter uninducible by TCDD (Matikainen et al. 2001; Shen & Whitlock 1992). In addition, recent publications have described novel AHR binding sites as genes regulated by AHR lack the classical XRE motif. One study has provided evidence that AHR binds directly a tetranucleotide motif 5'-GGGA-3' without ARNT (Huang & Elferink 2012). It was later established that AHR heterodimerises with the tumour suppressor Kruppel-Like Factor 6 (KLF6) expanding the list of mechanisms by which AHR influences gene expression (Wilson et al. 2013). Additional ChIP-Seq experiments have revealed that indeed AHR and ARNT binding sites do not overlap completely, indicating that AHR influences target gene activation independently (Lo & Matthews 2012). Apart from binding DNA directly, AHR has also been demonstrated to influence target gene expression via other proteins. It has been shown that AHR binds rat *Cyp1a2* enhancer via an unknown protein (Sogawa et al. 2004). Supporting this, AHR/ARNT complex was found to bind DNA motifs previously known as AZF1, FOXA1 and SP1 response elements possibly exerting its effect via these proteins (Lo & Matthews 2012).

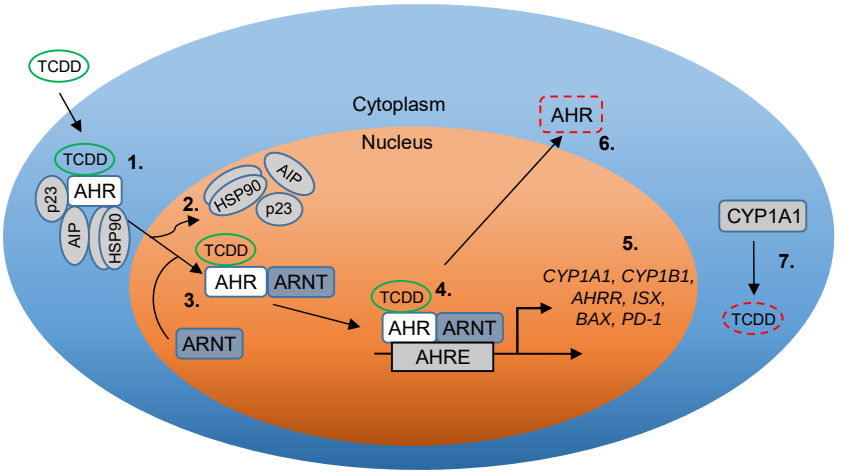

Figure 2. Genomic AHR signalling pathway. In an inactive state, the AHR is located in the cytoplasm in complex with its molecular chaperones p23, AIP and two units of HSP90 (1). Upon ligand (TCDD) binding, the AHR translocates to the nucleus, sheds its chaperones (2) and dimerises with its nuclear partner ARNT (3). This heterodimer then binds AHR response elements (AHRE) located in the regulatory regions of its target genes (4), thereby inducing their expression (5). Subsequently the 26S proteasome pathway degrades AHR (6). The AHR-induced CYP1A1 is responsible for degradation of AHR ligands (7).

2.4. Regulation of AHR

Initially, the majority of studies regarding AHR concentrated on its activation by various environmental chemicals having affinity to AHR. With the identification of endogenous ligands, the role of AHR in the organism has expanded significantly. However, this has also resulted in many contradicting findings as the effects on target gene expression are often dependent on the specific ligand but more importantly vary between distinct cell types and organisms. Apart from the modulation of AHR activity, the expression of AHR itself, although ubiquitous, varies in time and across tissues. High AHR expression has been determined, among others, in lung, liver, placenta, thymus and kidney, ascribing it an important role in cellular homeostasis (Manchester et al. 1987; Mason & Okey 1982).

The mechanisms regulating AHR signalling are highly dependent on cell type and the modulator itself. However, the importance of AHR repressor (AHRR) has been underscored in AHR-dependent gene expression. AHRR expression is induced by AHR ligands like TCDD and 3-methylcholanthrene through direct, although low-affinity, binding of AHR/ARNT to its response elements. The low affinity was reported to be due to varying nucleotides flanking the XRE (Baba et

al. 2001). The N-terminus of AHRR is comparable to AHR but it does not bind ligands (Korkalainen et al. 2004; Mimura et al. 1999). Additionally, it was found that the transactivation domain varies significantly, rendering AHRR unable to activate target gene expression. AHRR competes with AHR for mutual cofactors like ARNT, binds the XRE motif, inhibits AHR target gene expression and results in nuclear export, ubiquitination and degradation of AHR via 26S proteasome pathway (Ma & Baldwin 2000; Mimura et al. 1999). This mechanism, however, is also tissue-specific, as AHRR induction has been reported to vary in different cell types and its expression does not necessarily correlate with xenobiotic-induced CYP1A1 levels (Bernshausen et al. 2006).

Previous publications have reported that AHR expression was elevated in rat and mouse liver, prostate and hepatocytes *in vivo* after TCDD administration, although this tended to vary depending on the dose and duration of treatment (Chang et al. 2005; Franc et al. 2001a,b; Sommer et al. 1999). On the other hand, there are data that TCDD significantly reduces the amount of AHR protein e.g. in mouse hepatoma cells and human keratinocytes *in vitro* (Chen et al. 2005; Ray & Swanson 2004). The downregulation of AHR protein was dependent on a cycloheximide-sensitive factor, influencing the half-life of AHR (Ma et al. 2000). It was later established that the TCDD-inducible poly(ADP-ribose) polymerase (TiPARP) was responsible for the decline of AHR protein levels (MacPherson et al. 2013). TiPARP expression is induced by activated AHR. It binds AHR directly and enhances its proteolytic degradation thereby silencing AHR target gene expression.

Apart from regulation of AHR by its ligands, there are data emphasising the role of other factors modulating AHR expression. For example, TGF-β, a multi-functional cytokine expressed in a variety of cells, has been shown to influence AHR expression. More precisely, TGF-β-dependent downregulation of AHR in lung carcinoma cells and upregulation in hepatoma cells by downstream SMAD 2, 3, 4 and TGIF were observed, respectively (Wolff et al. 2001). In addition, IL-4, IL-13, lipopolysaccharides (LPS) and WNT/β-catenin have been shown to induce AHR expression in B cells and prostate cancer, respectively (Chesire et al. 2004; Marcus et al. 1998; Tanaka et al. 2005). A landmark phenotype resulting in foetal TCDD exposure is cleft palate (Mimura et al. 2003). This, however, has been shown to be reversed by TGF-β, which may be due to downregulation of AHR expression and ablation of the toxic effect by its ligand TCDD (Thomae et al. 2005). AHR expression has also been reported to decline during aging. Published studies have indicated that AHR expression is downregulated in older rat liver and prostate, compared to younger animals indicating that this may play a role in diseases during aging (Mikhailova et al. 2005; Sommer et al. 1999). Additional dynamic fluctuations in AHR expression have been observed by Chaffin and colleagues in rat oestrous cycle (Chaffin et al. 2000). By analysing ovarian AHR expression, they noticed initial upregulation during maturation of

ovarian follicles followed by downregulation at later stages after luteinising hormone (LH) surge. The latter downregulation was also evident in liver samples, emphasising that the response to xenobiotics, their degradation and thus toxic effects may severely depend on hormonal signalling.

Recent advances in investigating the regulation of AHR have identified that its expression is regulated through epigenetic mechanisms. Mulero-Navarro and colleagues identified that *AHR* promoter contains CpG islands that are targets of DNA methylation (Mulero-Navarro et al. 2006). They found that AHR is down-regulated in acute lymphoblastic leukaemia (ALL) and chronic myeloid leukaemia (CML) cell lines and this was due to methylation of AHR promoter as treatment with the DNA methyl transferase inhibitor 5-Aza-2'-deoxycytidine restored AHR expression. Importantly, *AHR* was significantly methylated in primary ALL similarly to tumour suppressors like p53, p16 and p73. Additional mechanisms regulating AHR have been identified at the post-transcriptional level. In addition to AHR regulating the expression of miRNAs, AHR itself has been shown to be downregulated by miR-124 (Huang et al. 2011). AHR expression reversely correlated with the expression of miR-124 in neuroblastoma, one of the most common solid tumours in children, and miR-124 was shown to directly supress AHR expression by targeting its 3'-UTR. Inhibition of miR-124 resulted in cell differentiation, cell cycle arrest and apoptosis, suggesting a potential AHR-dependent therapeutic avenue in neuroblastoma therapy as AHR activation has additionally been shown to impair neuroblastoma progression and metastasis (Wu et al. 2019). Efforts in uncovering the regulation of AHR have discovered another post-transcriptional mechanism. The adenosine-to-inosine RNA editing elicited by adenosine deaminase acting on RNA 1 (ADAR1) has been shown to edit the 3'-UTR of *AHR* mRNA in hepatocellular carcinoma cells (Nakano et al. 2016). This created a *de novo* binding site for miR-378, which bound *AHR* mRNA resulting in downregulation of AHR protein levels. Importantly, AHR protein and mRNA levels did not correlate positively in a panel of human liver samples, whereas miR-378 showed an inverse correlation with AHR protein levels.

Collectively, the evidence on the control of AHR expression underscores a diversity in AHR regulation, depending on the cell type, experimental conditions and the biological factors used. Thus, there seems to be no rigorous or unified mechanism that governs AHR expression across the organism.

2.5. AHR ligands

Initially, AHR was considered to interact solely with environmental contaminants. Thus, early studies concentrated mainly on the toxicological aspects of AHR activation. Later studies have established several phytochemicals and medical

drugs as AHR ligands. The analyses of AHR-dependent gene expression as well as the use of AHR knockout models also indicated existence of endogenous ligands. The identification of these endogenous ligands enabled characterisation and broadening of the AHR role in normal homeostasis as well as disease. In addition to ligands acting as AHR agonists, there are data about antagonists – compounds that have been determined to typically inhibit TCDD-dependent AHR target gene activation. Importantly, the modulation of AHR activity is not always straightforward, as distinct ligands may have different effects in terms of target gene expression and this is dependent on the animal model as well as cell type tested.

The best-characterised environmental contaminant AHR ligands belong to the groups of halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) generated as side products in the chemical industry as well as during combustion (Figure 3). HAHs that have affinity to AHR are dibenzofurans (e.g. 2,3,7,8-tetrachlorobibenzofuran, TCDF), biphenyls (3,3',4,4',5-pentachlorobiphenyl, PCB126) and dibenzo-p-dioxins, including TCDD, which has the highest known affinity for AHR (Farrell et al. 1987; Jones & Anderson 1999; Poland & Glover 1976). PAHs comprise of a large group of chemicals containing at least four benzene rings, including well-characterised AHR ligands benzo[a]pyrene, 3-methylcholanthrene, 7,12-dimethylbenzanthracene (Jones & Anderson 1999; Piskorska-Pliszczynska et al. 1986). Although activation of AHR by its ligands results in induction of several xenobiotic degrading enzymes and subsequent elimination from cells, the metabolised ligands are capable to produce PAH-DNA adducts and are thus potent carcinogens. In addition, several drug compounds in use and in clinical trials have been shown to modulate AHR activity. The anti-allergy drug Tranilast and neuroimmunomodulator Laquinimod activate AHR (Hu et al. 2013; Kaye et al. 2016; Ott et al. 2019). The anti-malarial drug primaquine has been shown to activate AHR (Backlund & Ingelman-Sundberg 2004). In contrast Vemurafenib, which is used to treat late-stage melanoma has been shown to antagonise AHR (Hawerkamp et al. 2019).

Apart from the anthropogenic chemicals, many natural and endogenous AHR ligands have been determined to date. Indigo and indirubin, compounds that have been historically used for colouring but also found in humans, are reported to activate AHR (Adachi et al. 2001). The arachidonic acid metabolite lipoxin A4 as well as prostaglandin G2, heme metabolites bilirubin and biliverdin have been shown to activate AHR (Phelan et al. 1998; Schaldach et al. 1999; Seidel et al. 2001). In addition, tryptophan metabolites are known to activate AHR. Ultraviolet (UV) radiation has been shown to generate 6-formylindolo[3,2-*b*]carbazole (FICZ), which bound AHR with high affinity (Fritsche et al. 2007). Another tryptophan metabolite kynurenine (Kyn), generated by tryptophan-2,3-dioxygenase 2 (TDO2) and indoleamine-2,3-dioxygenase 1 (IDO1), has been found to activate AHR and has recently gained much attention for its role in tumour

promotion and immune escape (Mezrich et al. 2010; Opitz et al. 2011). In addition, several metabolites of the downstream Kyn metabolism pathway, like kynurenic acid, xanthurenic acid and cinnabarinic acid, act as AHR agonists (DiNatale et al. 2010; Lowe et al. 2014). The dietary uptake of glucobrassicin, abundant in cruciferous vegetables like broccoli and cauliflower, results in production of indole-3-carbinol, indole-3-acetonitrile and downstream 3,3'di-indolylmethane and indolo[3,2-b]carbazole (ICZ), all of which activate AHR (Bjeldanes et al. 1991; Ito et al. 2007). Further, ITE (2-(19H-indole-39-carbonyl)-thiazole-4-carboxylic acid methyl ester) has been identified to activate AHR (Song et al. 2002). Although the origin of ITE, whether dietary, endogenous or a by-product of its extraction, remains elusive, its potential as an anticancer drug has been established in endometrial, ovarian and triple-negative breast cancer (Bian et al. 2019; Piwarski et al. 2020; Wang et al. 2013).

A lot of effort has been put into identifying AHR antagonists, which inhibit AHR nuclear import and target gene activation. Common methods for this are inhibition of TCDD-induced *CYP1A1* induction and competitive binding to TCDD-bound AHR. Although initial studies have determined a number of compounds acting as antagonists, later reports have revealed that the actual mechanism is much more complicated, as TCDD displacement and *CYP1A1* induction do not completely reflect the AHR status. Studies have shown that AHR can be selectively modulated i.e. it translocates to the nucleus and influences gene expression but does not bind the canonical XRE or induce *CYP1A1* expression (Murray et al. 2010). Accordingly, the plant polyphenol resveratrol, abundant in grapes and red wine, has been demonstrated to suppress TCDD-induced *CYP1A1* expression, although AHR translocated to the nucleus and bound XRE (Casper et al. 1999). There is also data about the agonist-selectivity of AHR antagonists as CH223191 inhibited HAH-, but not PAH- or indirubin-dependent AHR signalling (Zhao et al. 2010). Importantly, it was revealed that the known antagonists 3′-methoxy-4′-nitroflavone and 6,2′,4′,-trimethoxyflavone also exhibit species-selectivity. In contrast, the synthetic antagonist GNF351 has been reported to act as a complete antagonist and silence the effects of various AHR ligands (Smith et al. 2011). Additionally, StemRegenin 1 (SR1) has been shown to antagonise AHR and promote hematopoietic stem cell proliferation *in vitro* (Boitano et al. 2010).

Collectively, the diverse repertoire of AHR ligands is broad and expanding constantly. The agonistic and antagonistic effects that various compounds exert on AHR signalling are complicated and thus need to be confirmed by versatile experimental procedures in more detail. Considering the important role AHR plays in health and disease, it is, however, important to continue identifying novel AHR modulators, both exogenous as well as endogenous.

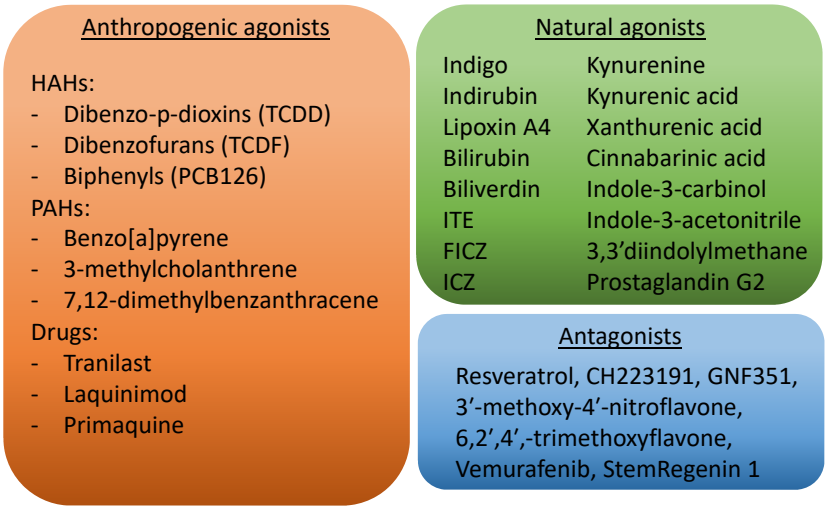

Figure 3. Examples of classes of chemicals and specific compounds known to act as AHR ligands.

2.6. AHR in cancer

As stated above, the repertoire of chemicals with AHR activity modulation characteristics is broad. Since the identification of AHR as a target of environmental contaminants with tumour-promoting potential, the role of AHR in cancer has been studied extensively in various cancer types in both animal models as well as humans. The constitutive activity of AHR has been reported to promote hepatocarcinogenesis and induce stomach tumours (Andersson et al. 2002; Moennikes et al. 2004). Despite, there seems to be no uniform role of AHR in tumorigenesis as both pro- and anti-tumour effects have been reported. Thus, AHR seems to have a dual role in carcinogenesis, which is highly dependent on the ligand, cancer type, as well as conditions in which the function of AHR is tested.

Breast cancer is one of the leading cancer types diagnosed in women. In mammary tumours, the tumour suppressor gene *BRCA1* has often acquired mutations or is downregulated(Wilson et al. 1999). In addition, several factors like diet and environmental contaminants have been implicated in the onset of breast cancer linking it to the environmental sensor AHR. Indeed, AHR has been demonstrated to positively regulate the expression of tumour suppressor *BRCA1* without xenobiotic AHR ligands (Hockings et al. 2006). AHR was shown to bind DNA directly via XREs present in the promoter region and interact with ERα. More importantly, the toxic AHR ligands TCDD and benzo(a)pyrene displaced coactivators from *BRCA1* promoter and reduced its expression. Interestingly,

similar results were obtained when using an AHR antagonist, implying a diverse ligand-dependent regulation of gene expression by AHR.

Skin cancer is one of the most common cancer types in Caucasians and the incidence is increasing (Leiter et al. 2014). In addition to the established role of ultraviolet (UV) radiation, various carcinogenic chemicals (e.g. PAHs) present in particulate matter and ambient air pollution are reported to cause skin cancer (Siddens et al. 2012). Many of these chemicals are shown to activate AHR, implicating a role for AHR in skin cancer (Gualtieri et al. 2011; Matsumoto et al. 2007). Previous reports have shown that UVB radiation induces the expression of AHR target genes CYP1A1/1B1 (Katiyar et al. 2000; Mukhtar et al. 1986). The underlying mechanisms were characterised in a study showing UVB-dependent formation of the tryptophan catabolite 6-formylindolo[3,2-*b*]carbazole (FICZ), an AHR agonist, and subsequent AHR activation (Fritsche et al. 2007). The activated AHR was further shown to downregulate the expression of the cell cycle inhibitor p27, resulting in survival of UVB-damaged cells (Frauenstein et al. 2013). In contrast, when AHR activity was inhibited, p27 expression increased leading to apoptosis of damaged cells. Moreover, in a recent work it was established that AHR activation represses the repair of mutagenic DNA photoproducts induced by UVB radiation (Pollet et al. 2018). Silencing of AHR, in turn, enhanced DNA repair coupled with an increase in DNA double-strand breaks and apoptosis. Thus, it seems that AHR activation enhances UVB- and possibly PAH-induced skin carcinogenesis. Inhibition of AHR activity, in turn, may have therapeutic values.

Glioblastoma is a neurological cancer with limited treatment options and high lethality. Although the aetiology of glioblastomas is unclear, the role of AHR in this type of disorder has been characterised. One study identified two single nucleotide polymorphisms in the *AHR* gene correlating with PAH-DNA adduct formation contributing to the risk of glioblastoma (Gu et al. 2012). Consistent with this, these SNPs also correlated with higher incidence of lung cancer in tobacco smokers (Chen et al. 2009). Additionally, nuclear accumulation of AHR has been detected in human glioblastomas *in vivo* and silencing of AHR signalling resulted in reduced clonogenic survival and invasiveness (Gramatzki et al. 2009; Guastella et al. 2018). Further investigations identified kynurenine (Kyn), a tryptophan metabolite generated by the neuron- and liver-specific enzyme tryptophan-2,3-dioxygenase (TDO), as a tumour-promoting AHR agonist (Opitz et al. 2011). The Kyn-AHR pathway was active in glioblastomas and contributed to tumour cell survival, motility and suppression of anti-tumour immune response.

The immunosuppression elicited by tumour cells via Kyn-AHR signalling has been further elucidated in hepatocellular carcinoma (HCC). The IL-6-inducible intestine-specific homeobox transcription factor (ISX), implicated in HCC, was found to promote a feedforward mechanism thereby enhancing tumourigenicity and evasion of immune cell surveillance (Wang et al. 2017). More specifically,

ISX was found to induce the expression of indoleamine-2,3-dioxygenase 1 (IDO1) and TDO2 resulting in elevated levels of Kyn and subsequent AHR activation, which in turn enhanced ISX expression (Figure 4). Additionally, ISX elevated the expression of programmed death ligand 1 (PD-L1) and programmed death 1 (PD-1) proteins in HCC. Accordingly, HCC patient samples with high ISX expression also had enhanced expression of IDO1, TDO2, AHR and PD-L1. More importantly, high expression of these genes correlated with shorter survival time of patients after surgical resection of HCC. PD-L1 and PD-1 interaction acts as an "off switch" keeping cytotoxic T-lymphocytes (CTLs) from attacking other cells in the body. Importantly, some cancers express high levels of PD-L1 thereby enabling them to hide from immunosurveillance.

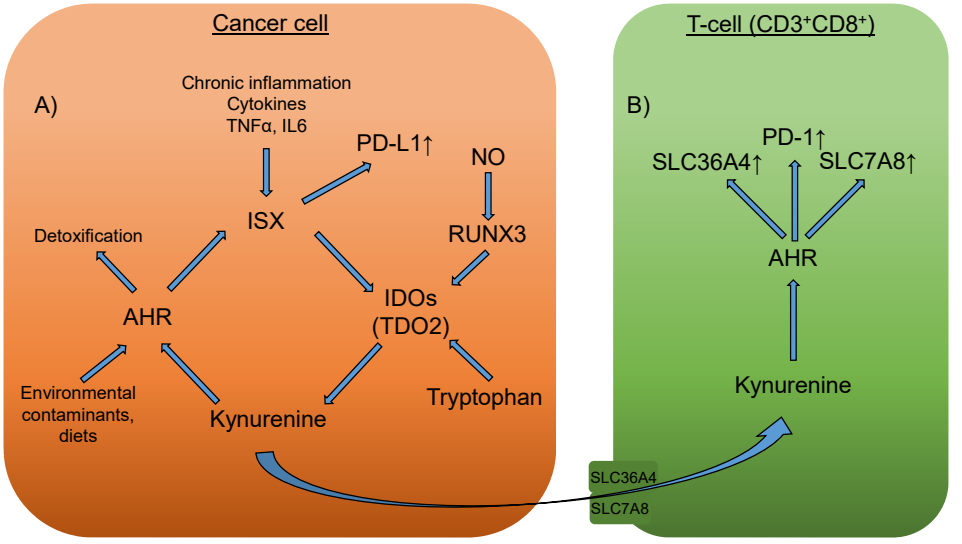

Figure 4. An established schematic model of Kyn-dependent immune escape of cancer cells. A) ISX-IDO-Kyn-AHR positive feedforward mechanism in cancer cells, resulting in PD-L1 upregulation. **B)** Kyn produced in cancer cells is transported into $CD3^{+}CD8^{+}$ immune cells via SLC36A4 and SLC7A8 transporters, resulting in AHR-dependent upregulation of PD-1, SLC36A4 and SLC7A8.

Recently, the Kyn-dependent crosstalk between tumour and immune cells has been further elucidated. Liu and colleagues found, that CTLs stimulate tumour cells to release high levels of Kyn, which was transported into CTLs via SLC7A8 (solute carrier family 7 member 8) and SLC36A4 (solute carrier family 36 member 4) transporters resulting in activation of AHR (Liu et al. 2018b). AHR then bound to the regulatory regions of PD-1, SLC7A8 and SLC36A4 genes thereby inducing their expression and resulting in suppression of immune response on tumour cells (Figure 4). Conversely, inhibition of AHR activity

downregulated these genes and enhanced the anti-tumour effect of CTLs. This regulatory network was confirmed in both animal models and human lung, colon and breast cancer patient samples providing evidence for future immunotherapy strategies. The impact of Kyn signalling has also been implicated in pancreatic cancer. It was established, that nitric oxide, produced by nitric oxide synthase 2 (NOS2), regulated RUNX3 transcription factor (Wang et al. 2020). RUNX3 then bound the promoter of *IDO1* upregulating its expression resulting in elevated Kyn levels. Kyn activated AHR thereby promoting cancer growth and invasiveness. These results correlated well with poor patient survival and are in accordance with a previous publication reporting NOS2-dependent enhancement of pancreatic cancer progression (Wang et al. 2016).

2.7. AHR in stem cells

The function of AHR has been characterised, by using its ligands or knockout models, in many cell types with high developmental potential i.e. stem cells. In mouse, AHR is expressed already at 1-cell stage (Wu et al. 2002). At 2- and 8-cell stage AHR expression is silenced followed by upregulation in later stages (Peters & Wiley 1995; Wu et al. 2002). The expression pattern of AHR was further characterised by Ko and colleagues, who found that AHR is basically absent in pluripotent mouse embryonic stem (mES) cells and its expression was recovered during non-directed differentiation into embryonic bodies (EB) (Ko et al. 2014). They uncovered that AHR is repressed by the core pluripotency factors Oct4, Nanog and Sox2, which bound Ahr distal promoter thereby inhibiting gene expression. Indeed it is known, that in ES cells, the transcription factors Oct4, Nanog and Sox2 act on their target genes by promoting self-renewal and silencing differentiation thus implicating a role of Ahr in differentiation (Boyer et al. 2005; Loh et al. 2006). It was later established that AHR regulates pluripotency, cell fate decision by restricting cardiogenesis and commitment towards neuroglia (Ko et al. 2016). Additionally, AHR repression was found to promote mES cell mitotic progression, thereby maintaining pluripotency.

Data about the role of AHR in pluripotent human embryonic stem (hES) cells is scarce. A recent article reported that AHR is indeed expressed in hES cells (Yamamoto et al. 2019). Moreover, they found that AHR is activated by its endogenous ligand kynurenine (Kyn), a metabolite of tryptophan, produced by indoleamine 2,3-dioxygenase 1 (IDO1). Inhibition of IDO1 activity and tryptophan catabolism resulted in reduced proliferation but not differentiation. Indeed it has been shown that tryptophan deprivation from cell culture media results in inhibition of cell growth and decreased cell numbers (Shiraki et al. 2014). Importantly, they observed diminished AHR expression during neural differentiation. Kyn levels and thus probably AHR activity were also reduced

during ectodermal differentiation underscoring the role of AHR in hES cell differentiation. Accordingly, in our experiments using the hES cell line H9, we observed that AHR is indeed expressed (Teino et al., manuscript in preparation). During non-directed differentiation into EBs, we observed downregulation of AHR, demonstrating a profound difference in AHR expression pattern and thus function between human and mouse ES cells. Further, during directed neural differentiation, AHR expression is initially downregulated followed by upregulation at later time points. Endodermal differentiation resulted in more profound repression of AHR expression in all time points tested. Mesodermal cells, in turn, seemed to lack AHR. This differential expression of AHR between lineages emphasises that AHR indeed has an important and distinct role during hES cell differentiation.

Nowadays, the therapeutic potential of pluripotent stem cells is widely accepted. However, the use of hES cell lines has many disadvantages as they are not autologous and more importantly accumulate a burden of cancer-associated mutations in the cell culture (Merkle et al. 2017). By whole exome sequencing of 140 hES cell lines collected from laboratories around the world, mutations in *TP53* were identified that conferred selective advantage. Thus, there is a need for patient-specific induced pluripotent stem (iPS) cells, generation of which, however, has been expensive and relatively inefficient. The reprogramming of somatic cells to pluripotency is under thorough investigation. Previously, the use of miRNAs has been described in cellular reprogramming. More precisely, exogenous expression of hES cell-specific miRNA cluster 302–367 resulted in efficient generation of iPS cells (Anokye-Danso et al. 2011; Suh et al. 2004). The miRNA-302, however, has been shown to be inducible by AHR (Hu et al. 2013). Activation of AHR by an anti-allergy drug Tranilast as well as other AHR agonists facilitated the miR-302-dependent reprogramming of mouse embryonic fibroblasts to pluripotency. In accordance with this, we detected AHR binding in close proximity upstream of miRNA-302 in hES cell genome by ChIP-Seq experiments (Teino et al., manuscript in preparation).

In addition to embryonic stem cells, the role of AHR has also been established in adult stem cells with lower developmental potential. Hematopoietic stem cells (HSC) are one of the best-characterised adult stem cells. Although they have high therapeutic value, their expansion *in vitro* is limited. Advances in this field have identified a small molecule compound StemRegenin 1 (SR1), which affects HSCs (Boitano et al. 2010). It was uncovered that SR1 antagonises AHR, thereby promoting proliferation and maintaining their undifferentiated status. Accordingly, Musashi-2 (MSI2), an RNA binding protein, induced HSC self-renewal (Hope et al. 2010). It was later established that MSI2 exerts its effect via attenuation of AHR signalling with downregulation of CYP1B1 being the key mechanism (Rentas et al. 2016). Thus, the role of AHR in HSC is well established. To date,

the SR1 expanded HSC (MGTA-456) are in clinical trials for patients undergoing hematopoietic stem cell transplantation (ClinicalTrials.gov).

Toxicological studies have provided evidence on adverse outcomes following TCDD exposure in mesodermal differentiation. TCDD-dependent AHR signalling resulted in impaired mesoderm formation as outlined by reduced mesoderm-specific marker gene expression (Fu et al. 2019; Neri et al. 2011). It was also found that TCDD disrupts differentiation embryonic stem cells into cardiomyocytes, concordant with the adverse effects of dioxins on heart development and function (Carreira et al. 2015; Humblet et al. 2008; Jokinen et al. 2003). Interestingly, the derailment of mesoderm differentiation was evident only if hES cells were treated with TCDD prior differentiation (Fu et al. 2019). This indicates that activation of AHR may influence the epigenetic landscape of hES cells consistent with its interaction with the nucleosome remodelling complex NuRD (nucleosome remodelling and deacetylation) and modulation of gene methylation (Gialitakis et al. 2017; Wu et al. 2004). The neurotoxic effects of TCDD have been investigated for decades. Studies on laboratory animals have shown several defects in brain development (Collins et al. 2008; Hays et al. 2002; Jiang et al. 2014; Mitsuhashi et al. 2010). Recently, it was found that TCDD treatment influenced human neuronal differentiation from hES cells (Sarma et al. 2019). More precisely, TCDD led to increased tyrosine hydroxylase-positive neuronal cell differentiation with concomitant downregulation of endo- and mesoderm-specific marker genes.

Collectively, the data emphasise that AHR has a crucial role in the regulation of cells with high developmental potential. However, much more work needs to be done in order to further elucidate the impact of AHR activity modulation during cell differentiation, whether by endogenous or exogenous ligands.

2.8. AHR in ovarian granulosa cells

Initial studies identifying several environmental contaminants acting as endocrine disrupting chemicals led to the investigation of the role of AHR in ovarian homeostasis using known xenobiotic AHR ligands. With the establishment of AHR knockout mouse models, the endogenous role of AHR was further characterised ascribing it a central role in the development of ovary as well as regulation of ovarian processes.

During embryonic development, the primordial germ cells proliferate rapidly forming germ cell nests that are surrounded by somatic cells called pre-granulosa cells (Figure 5) (Guigon & Magre 2006). Following mitotic arrest and meiotic entry, the germ cells are called oocytes (McLaren 2000). Subsequently, approximately 70% of oocytes undergo apoptosis, which is thought to aid breakdown of germ cell nest and formation of the surviving oocytes into primordial follicles

(Pepling & Spradling 2001). These primordial follicles comprise of an oocyte surrounded by a single layer of flattened granulosa cells and constitute the limited reproductive pool of the female organism.

The next steps in follicle maturation are categorised as initial and cyclic recruitments (McGee 2000). The initial recruitment is thought to be a continuous process during life and comprises of different stages of follicle maturation. First, a number of primordial follicles grow into primary follicles. This process encompasses growth of the oocyte, whereas the granulosa cells gain a cuboidal shape (Makabe et al. 2006). During formation of the preantral follicle, granulosa cells proliferate and the oocyte acquires *zona pellucida* – a ring of proteins secreted by the oocyte. Additionally, stromal cells surround the follicle and form a flattened layer of theca cells. The continuing growth of the follicle results in influx of fluids forming the antrum and antral follicles. The primary pathway of these antral follicles is to undergo degeneration, a process called atresia. However, after pubertal onset, a number of follicles are saved from apoptosis and enter the cyclic recruitment controlled by gonadotropins FSH (follicle stimulating hormone) and LH (luteinising hormone). (McGee 2000)

During cyclic recruitment, the FSH is responsible for rescuing a number of antral follicles from atresia. Among this cohort of growing follicles emerge the dominant follicles (one in humans), which grow faster and are more sensitive to FSH, due to increased Fsh receptor (Fshr) and Lhcgr expression (Bao et al. 1997; Evans & Fortune 1997; Xu et al. 1995). The dominant follicles express higher levels of oestradiol (E2) and inhibins, which downregulate pituitary-expressed FSH, thereby suppressing the growth of the remaining antral follicles that eventually undergo atresia (Chetkowski et al. 1986; Farnworth et al. 1988). By the end of the growth, the follicles reach the preovulatory stage and are mature for ovulation. Ovulation results from rapid increase in LH levels (termed LH surge) secreted by the pituitary followed by breakdown of the follicle and release of the oocyte and its surrounding cumulus granulosa cells. The remaining theca and mural granulosa cells, which surrounded the antrum, constitute the *corpus luteum* and express progesterone to primarily maintain pregnancy.

The maturation of ovarian follicles is under tight control of the hypothalamus-pituitary-ovary axis. Gonadotropin releasing hormone (GnRH) is synthesised and secreted in the hypothalamus. GnRH acts on gonadotroph cells located in the anterior pituitary. These cells, upon GnRH stimulation, express and secrete the gonadotropins FSH and LH. The secreted FSH and LH are transported to the ovary where they bind their corresponding receptors FSH receptor (Fshr) and LH/chorionic gonadotropin receptor (Lhcgr). Fshr expression is confined to granulosa cells at all stages of follicular maturation. Lhcgr, in turn, is expressed on granulosa cells of antral follicles and theca cells (Richards et al. 1976). The theca cells form a layer surrounding the follicular granulosa cells and the oocyte. These cells produce androgens, which diffuse into granulosa cells and are then

converted into the major female sex hormone E2 by Cyp19a1. The expression of Cyp19a1 is controlled by FSH that, through regulating E2 levels, supports the proliferation of granulosa cells and growth of follicles (Fitzpatrick & Richards 1991; Hsueh et al. 1984). Binding of FSH to its receptor Fshr activates adenylate cyclase to elevate cAMP levels. This, in turn, results in activation of the protein kinase A (PKA) pathway, subsequent phosphorylation of cAMP response element binding protein (Creb) and modulation of gene expression (Mukherjee et al. 1996). Similar events take place during LH binding to Lhcgr following LH surge prior to ovulation, although the outcome in terms of gene expression regulation differs drastically.

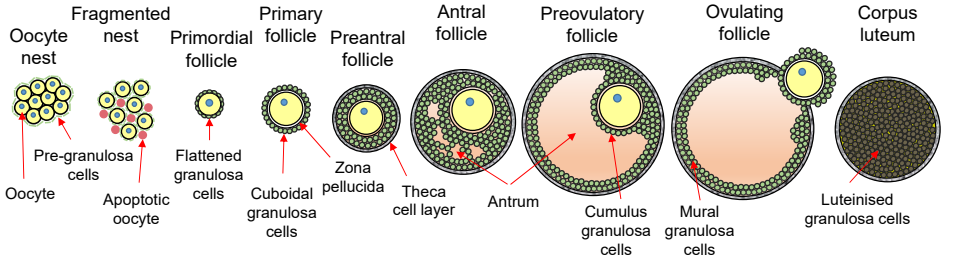

Figure 5. Stages of ovarian follicle maturation.

The role of AHR in the ovary has been established in different cell types and various developmental stages. AHR-KO mice, although fertile, had difficulties in maintaining conceptuses and fewer pups (Abbott et al. 1999; Baba et al. 2005). Additionally, it was found that significantly less pups survived lactation. During early stages of follicle formation AHR is expressed and seems to be responsible for inducing apoptosis. It has been found that foetal ovaries from AHR-KO mice have increased numbers of non-apoptotic oocytes in germ cell nests (Robles et al. 2000). In accordance with this, the AHR-KO neonatal ovaries harboured more primordial follicles compared to wild-type littermates (Benedict 2000; Robles et al. 2000).

Several environmental contaminants are known to cause oocyte destruction, ovarian failure and early menopause. Accordingly, the classical PAH and AHR ligand dimethylbenz(α)anthracene, but not TCDD, was shown to induce apoptosis of both mouse and human primordial follicles through induction of the pro-apoptotic *Bax* gene expression (Matikainen et al. 2001, 2002). It has also been reported that treatment with the Ahr agonist 3-methylcholanthrene results in degradation of oocyte DNA, reduced numbers of follicles in each stage of maturation and an Ahr antagonism can reverse this effect (Rhon-Calderón et al. 2016).

Further studies have broadened the role of AHR in follicular maturation. Experiments using adult AHR-KO mice have found that the lack of AHR results in slower growth and fewer number of antral follicles, but does not influence

atresia, compared to their wild type littermates (Benedict 2000; Benedict et al. 2003). These findings were corroborated by Barnett and colleagues, who also noted slower growth of AHR-KO follicles (Barnett et al. 2007b). This was reported to be due to decreased granulosa cell proliferation, resulting from reduced levels of cell cycle regulators cyclin-dependent kinase 4 (CDK4) and cyclin D2 (CCND2). CCND2 is known to be crucial for FSH-induced granulosa cell proliferation, as disruption of this gene results in infertility (Sicinski et al. 1996). Both CCND2 and CDK4 have, however, been shown to be influenced by AHR (Barhoover et al. 2010; Jung et al. 2010).

In addition, the AHR-KO mice had reduced levels of E2, but not its precursor testosterone, and treatment of these mice with E2 restored the growth comparable to wild type mice (Baba et al. 2005; Barnett et al. 2007b). E2, which stimulates granulosa cell proliferation, is synthesised by Cyp19a1 in the granulosa cells. *Cyp19a1*, in turn, has been shown to be directly regulated by AHR and is decreased in AHR-KO mice (Baba et al. 2005). Similar results were obtained *in vitro* using an Ahr antagonist (Bussmann et al. 2006). As proper E2 levels are required for normal oestrous cycle, reduced levels of E2 seem to account for follicular disturbances observed in AHR-KO mice (Baba et al. 2005). Further investigations have attributed AHR a role in gonadotropin responsiveness. AHR-KO mice had reduced ovarian weight, less *corpora lutea* and ovulated ova when immature mice were induced to ovulate by the classical superovulation scheme using pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) (Barnett et al. 2007a). This was found to be due to decreased expression of Fshr and Lhcgr. Importantly, AHR was shown to bind the promoter region of Fshr, but not Lhcgr, implicating direct regulation.

Although many studies have concentrated on the role of Ahr in ovarian processes, less attention has been paid on if and how Ahr itself is regulated. An earlier study established that AHR is downregulated in ovaries of immature rats following maternal TCDD exposure *in vivo*, probably accounting from one side for the adverse effects of xenobiotic Ahr ligands on reproduction (Chaffin & Hutz 1997). Accordingly, ligand-dependent downregulation of Ahr protein levels was observed *in vitro* in granulosa cells and this included the 26S proteasome pathway (Bussmann & Barañao 2006). More importantly, this study found that Ahr expression is reduced in cultured granulosa cells following FSH and E2 treatment. During the oestrous cycle, Ahr expression has been reported to fluctuate in the ovary (Chaffin et al. 2000). The levels of AHR showed a positive increasing trend as follicular maturation proceeded followed by downregulation at the time of LH surge and ovulation. Interestingly, similar pattern was observed in hepatic Ahr expression expanding the significance of Ahr regulation in organ homeostasis.

3. AIMS OF THE STUDY

Initially the majority of studies investigating Ahr focused on its role in toxicity by various environmental contaminants i.e. its ligands. Later, the importance of Ahr was established in cancer as well as homeostasis of various cell and tissue types, including reproductive tissue. To date, a variety of endogenous Ahr ligands have been ascertained broadening our understanding on the role of Ahr in the organism. In the face of numerous publications and ongoing research projects, relatively little attention has been paid on how Ahr itself is regulated. One previous study has delineated the differential expression of Ahr during the maturation of rat granulosa cells in the ovarian follicle (Chaffin et al. 2000). Another has implicated the regulation of *Fshr* expression by Ahr (Barnett et al. 2007a). Thus, we aimed to expand the knowledge about Ahr in the granulosa cells of mice further. Considering this, the aims of this thesis were:

1. To determine the regulation of mouse Ahr during maturation of granulosa cells and elucidate the possible mechanisms.
2. To investigate if and how Ahr is regulated during the preovulatory stage following Lhcgr stimulation.
3. To characterise the interplay between Ahr and *Fshr* promoter.

4. RESULTS AND DISCUSSION

4.1. The upregulation of Ahr during follicular maturation involves silencing of PKA signalling and chromatin remodelling (I)

Although attention has been paid on the role of Ahr in the female reproductive system, there is little data looking into the regulation of *Ahr* itself. Chaffin and colleagues demonstrated that in normally cycling rats the expression of *Ahr* fluctuates (Chaffin et al. 2000). More precisely, they observed a modest upregulation of ovarian *Ahr* mRNA during granulosa cell (GC) differentiation. Regarding this, we aimed to investigate, whether similar pattern can be observed in a mouse model with the perspective of elucidating the underlying mechanisms.

4.1.1. Both FSH and LH are required for upregulation of Ahr

Pregnant mare serum gonadotropin (PMSG) is a known surrogate classically used to induce maturation of high numbers ovarian follicles. PMSG has intrinsic FSH activity, but additionally its residual LH activity has been reported (Combarnous et al. 1984). In order to clarify, if PMSG has any effect on *Ahr* expression and whether it is due to its FSH-like activity, we injected immature female mice with 5 IU PMSG, 5 IU FSH or vehicle. After 48 h, ovaries were excised, granulosa cells harvested and gene expression patterns analysed. Western blot analyses showed that following PMSG treatment Ahr protein levels are significantly elevated compared to vehicle treatment. Similar pattern was observed in *Ahr* mRNA expression concordant with previously published data on rats (Chaffin et al. 2000). Injection of FSH, however, failed to influence the amount of Ahr protein in GCs. Interestingly, FSH was able to increase the expression of *Ahr* mRNA significantly, though to a smaller extent compared to PMSG (I, Figure 1 a–c). This referred to the importance of LH signalling in *Ahr* regulation. Indeed, it is known that LH is important in follicle maturation and its actions on GCs differ from FSH (Ruman et al. 2005). We also assessed the effects of LH and hCG (human chorionic gonadotropin – LH analogue) on *Ahr* expression but observed none (I, Supplementary Figure S1 a). This was somewhat expected as FSH is the main initiator of GC differentiation bringing about the upregulation of Lhcgr.

We next aimed to clarify, whether the different effects of PMSG and FSH on Ahr mRNA and protein might be due to the short half-life of FSH or if the specific activity of LH is required for Ahr upregulation. For this, we injected mice 4 times every 12 h with FSH alone or in combination with LH. Western blot analysis

indicated that FSH alone was again not able to influence Ahr protein levels. However, we observed a slight but significant increase in *Ahr* mRNA expression. Simultaneous injection of FSH and LH, in turn, resulted in significant upregulation of both Ahr protein and mRNA indicating that both FSH and LH activities are needed to influence Ahr in GCs (I, Figure 1 d–f). The results are concordant with the fact that indeed both gonadotropins are known to be essential for the maturation of ovarian GCs. It has been determined that FSH-treatment without LH results in reduced number of large follicles and thus the follicular maturation is disturbed (Ruman et al. 2005). By analysing *Fshr*, *Cyp19a1* and *Lhcgr* expression, we observed that these hallmark follicular maturation genes are upregulated only when mice received both FSH and LH (I, Supplementary Figure S1 c). The role of LH in influencing Ahr can additionally be attributed to its ability to affect protein levels post-transcriptionally e.g. by miRNAs (Bahrami et al. 2017; Khan et al. 2015). Additionally, miRNAs have been shown to control Ahr protein level (Huang et al. 2011; Liu et al. 2018a; Nakano et al. 2016).

4.1.2. PMSG-dependent expression dynamics of Ahr and follicle maturation marker genes

To understand the mechanism of how Ahr is regulated during follicular maturation in GCs, we sought to determine the temporal pattern of *Ahr* expression. Mice were injected with PMSG and granulosa cells harvested every 12 h up to 48 h time point. Western blot analysis indicated that Ahr protein levels start to increase 24 h post-injection and continue to rise until 48 h after injection. We observed a similar pattern in mRNA expression, though significant differences were apparent 36 h post-injection (I, Figure 2 a–c).

It has been described previously that Ahr regulates the expression of *Fshr* and *Cyp19a1* (Baba et al. 2005; Barnett et al. 2007a). We next determined the temporal expression patterns of the aforementioned GC differentiation markers and *Lhcgr* to validate our experimental setting of PMSG administration. More importantly, we aimed to gain insight into the possible interplay between these genes and Ahr. We observed a significant increase in *Fshr* expression 24 h post-injection, coinciding with the expression of *Ahr*. The expression of *Cyp19a1* and *Lhcgr*, however, started to increase 12 h post-injection, earlier than Ahr (I, Figure 2 d–f). Considering that Ahr has been reported to regulate *Cyp19a1*, this seems contradicting. However, one should keep in mind that this superovulation scheme, using relatively high doses of PMSG, might not exactly mimic the events taking place in normally cycling adult mice and thus other proteins can fulfil the function of Ahr. Supporting this, it has been shown that high amounts of PMSG and hCG can rescue the ovarian phenotype common to *Ahr*-KO mice, as the ovarian weight, number of *corpora lutea* and ovulated ova were comparable to

that of wild-type mice (Barnett et al. 2007a). Another possibility is that the regulation of *Cyp19a1* by Ahr is dependent on the degree of GC differentiation. Although initially induced by FSH signalling, the continued expression of *Cyp19a1* may indeed depend on Ahr as the follicular maturation proceeds. Additionally, we cannot exclude the possibility that Ahr regulates *Cyp19a1* on a basal level, since both genes are expressed in immature follicles. The relatively early upregulation of *Lhcgr* compared to Ahr, however, corroborates our notion that it might be involved in the control of Ahr protein levels as determined above (I, Figure 1).

4.1.3. PMSG-dependent upregulation of Ahr occurs in large antral follicles and is caused by attenuation of PKA signalling

Until now, there was little data about the spatial expression of Ahr in murine ovaries. Thus, our goal was to determine where Ahr is expressed in the ovary and to clarify in which GCs the upregulation of Ahr occurs. Mice were primed with PMSG or injected with vehicle. After 48 h, ovaries were excised and cryosections subjected to immunofluorescence with an Ahr-specific antibody. We found that PMSG treatment led to maturation of follicles, exemplified by the presence of large antral follicles. Importantly, the upregulation of Ahr took place exclusively in large antral follicles, more precisely in mural GCs, whereas the cumulus GCs and smaller follicles showed little or no Ahr expression (I, Figure 3). Our results are supported by a previously published study showing elevated *Ahr* mRNA expression in mural GCs of large antral follicles (Wigglesworth et al. 2015). Moreover, the same study showed higher *Lhcgr* expression in mural GCs additionally supporting our idea about its role in *Ahr* regulation.

FSH initiates the maturation of ovarian follicles. It is known to activate adenylate cyclase resulting in higher cAMP formation and subsequent PKA activation (Ratoosh et al. 1987). PKA, in turn, phosphorylates Creb, making it a good surrogate to evaluate PKA activity (McNulty et al. 1994; Puri et al. 2016). In order to clarify if PKA might regulate Ahr, mice received PMSG and GCs were extracted 24 h later, at the time when *Ahr* expression starts to increase. The GCs were further cultured for 4 h in the presence of Forskolin, another known activator of PKA signalling, or vehicle alone. Western blot analysis revealed a robust downregulation of p-Creb protein levels in vehicle-treated cells. This was somewhat expected, considering the absence of external stimuli (e.g. FSH) present *in vivo*. Forskolin, in turn, increased the amount of p-Creb in cultured GCs. In terms of Ahr, the lack of external stimuli had a positive effect on Ahr protein levels. Forskolin treatment, however, influenced Ahr negatively (I, Figure 4 a–c). This negative correlation led us to the notion that PKA pathway suppresses *Ahr* expression. Accordingly, FSH-dependent repression of *Ahr*

expression *in vitro* has been demonstrated previously in rats (Bussmann & Barañao 2006). Additionally, we measured the expression of *Fshr*, *Cyp19a1* and *Lhcgr* and observed a rapid downregulation of these genes following *in vitro* culture, whereas Forskolin counteracted this effect indicating that *in vitro* culture indeed lacked PKA activation (I, Supplementary Figure S3).

To assure further, that PKA signalling regulates *Ahr*, we next used the PKA inhibitor H89. GCs were cultured with or without Forskolin in the presence or absence of H89 for 4 h. We observed a significant Forskolin-dependent down-regulation of *Ahr* expression. H89, however, abolished this effect efficiently, allowing us to assume that PKA indeed downregulates the expression of *Ahr* (I, Figure 4 d). Additional time course experiments revealed that *Ahr* expression continues to increase at least up to 24 h in culture. Forskolin, in turn, repressed *Ahr* in all corresponding time points tested (I, Supplementary Figure 4 a). In order to determine whether PKA controls *Ahr* expression *in vivo* we assessed the temporal pattern of p-Creb during 48 h of PMSG and vehicle treatment as previously with Ahr (I, Figure 2 a–c). We observed that p-Creb is present in vehicle-treated mice in every time point tested, indicating basal PKA activity and its possible constitutive repressive effect on *Ahr* expression. Importantly, two Creb response elements have been identified in the mouse *Ahr* promoter (Schmidt et al. 1993). PMSG-treatment in turn resulted in downregulation of p-Creb protein levels. More precisely, densitometry analysis revealed that p-Creb level is significantly lower 24 h after PMSG injection, at the time when *Ahr* expression starts to increase (I, Figure 4 e and f). This is in accordance with Maizels et al. who showed a reduction of p-Creb levels at the 24 h time point in rat GCs (Maizels et al. 2001). Although they observed decreased p-Creb protein levels also 48 h after PMSG treatment, our results did not reveal a significant difference in 36 and 48 h time points compared to vehicle treatment. More importantly, we observed high variability in p-Creb levels, which may be caused by varying numbers of maturing follicles in different mice. Collectively, these data indicate that *Ahr* expression in ovarian GCs during follicular maturation is controlled by PKA activity as shown by both *in vitro* as well as *in vivo* experiments.

4.1.4. PMSG regulates chromatin accessibility at *Ahr* promoter

To further characterise the regulation mechanisms of *Ahr* during follicular maturation, we next aimed to elucidate, how *Ahr* expression is regulated at the mRNA level. Gene expression can be influenced by both transcription rate and mRNA degradation. We first measured the levels of heteronuclear RNA (hnRNA), which is a surrogate method to evaluate transcription rate, in GCs of mice treated with PMSG or vehicle alone at different time points. By using specific primers complementary to the exon-intron junction and intron, we

detected the nascent unspliced *Ahr* hnRNA. Further analysis revealed that treatment of mice with PMSG increases the transcription rate of *Ahr* gene and this is comparable to mRNA and protein expression observed earlier (I, Figure 5 a). Additionally, we examined whether PMSG might also affect *Ahr* mRNA stability. For this, GCs from PMSG-primed mice were cultured *in vitro* in the presence of PMSG or vehicle for 2 and 4 h. We found that PMSG led to increased expression of *Ahr* compared to vehicle treatment. When GCs were treated with the transcription inhibitor actinomycin D (ActD), we observed a robust decline in *Ahr* mRNA detection. However, there was no difference between PMSG or vehicle treatment (I, Figure 5 b). This led us to the conclusion that PMSG indeed influences *Ahr* transcription rate and not mRNA stability in maturing ovarian GCs.

It is known that regulatory elements responsible for gene expression can be situated far from the transcriptional start site. However, previous studies characterising *Ahr* promoter have defined a region necessary for its constitutive basal activity. Additionally, it has been shown that *Ahr* promoter contains several response elements for gonadotropin-dependent modulators (Fitzgerald et al. 1998; Garrison & Denison 2000). Thus, we aimed to determine if PMSG influences *Ahr* expression by modulating its promoter activity. GCs were transfected with a 1792 bp *Ahr* promoter-reporter vector, treated with PMSG or vehicle and luciferase activity measured 48 h later. We were able to detect the activation of the reporter construct compared to empty vector in vehicle-treated cells. PMSG, however, had no effect on luciferase activity (I, Figure 5 c). This indicated that the regulatory elements responsible for *Ahr* regulation by PMSG might be situated further upstream. Another possible explanation might be the context of chromatin, which is not precisely mimicked by exogenous vectors.

There are studies presenting epigenetic control of gene expression in the ovary and previous publications demonstrating epigenetic regulation of *Ahr* expression (DeManno et al. 1999; Garrison et al. 2000; Salvador et al. 2001; Zhang et al. 1996)**.** This led us to investigate whether PMSG-dependent upregulation of *Ahr* expression might be controlled by changes in chromatin structure – i.e. relaxation of chromatin – thus enabling accessibility to factors necessary for transcription. For this, we used CHART-PCR (chromatin accessibility by real-time PCR), which allows evaluation of chromatin condensation using nuclease digestion and detection of undigested DNA by specific primers. We concentrated on *Ahr* promoter region –176 to –77 bp upstream of transcriptional start site (TSS), as the proximity of this region to the TSS makes it essential for the binding of transcription machinery. *Cyp19a1* and *Pax7* were used as positive and negative controls, respectively. Analysis of PMSG vs vehicle-treated GCs revealed that chromatin at *Cyp19a1* promoter is open following PMSG treatment, consistent with its mRNA expression. There were no significant changes in case of *Pax7*, a control gene not expressed in GCs (I, Supplementary Figure S5). Analysis of *Ahr*

promoter revealed a similar pattern to *Cyp19a1*, indicating that the expression of *Ahr* like *Cyp19a1* is indeed regulated by chromatin remodelling at proximal promoter (I, Figure 5 d). There are studies describing direct phosphorylation of histones by FSH-PKA signalling, which resulted in decondensation of chromatin (DeManno et al. 1999; Salvador et al. 2001). Contrarily to this, others have provided evidence that PKA activity is required to maintain chromatin condensation and thus silencing of gene expression (Collas et al. 1999). Although it is reasonable that both processes are simultaneous, the latter seems to be responsible for regulation of *Ahr* in the ovarian GCs.

Taken together, we have provided evidence that PMSG induces *Ahr* expression in large antral follicles during maturation of granulosa cells. This effect is elicited by reduction in PKA signalling and involves decondensation of chromatin at *Ahr* promoter (Figure 6).

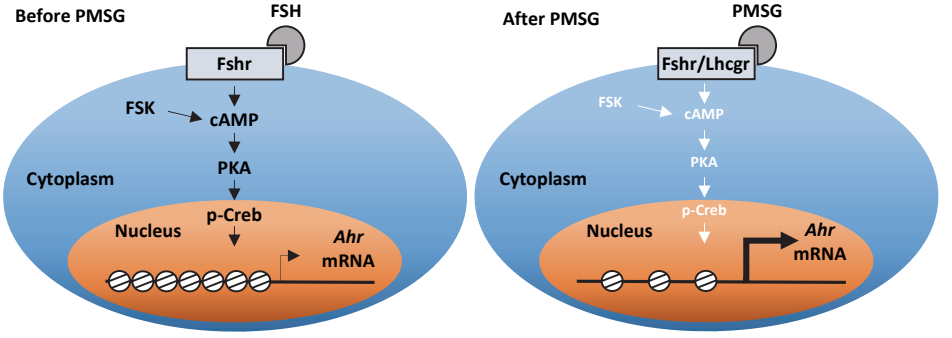

Figure 6. Schematic representation PMSG-dependent upregulation of *Ahr* expression. *Ahr* expression is repressed in GCs of immature ovarian follicles. Following PMSG treatment, PKA signalling is transiently silenced (pictured in white), enabling upregulation of *Ahr* via opening of chromatin at its promoter.

4.2. Lhcgr signalling represses *Ahr* expression in preovulatory granulosa cells by chromatin remodelling (II)

Previously published data indicate that following LH surge *Ahr* is downregulated in preovulatory stage of the rat reproductive cycle *in vivo* (Chaffin et al. 2000). We sought to determine, whether this effect can be observed also in mouse granulosa cells. Moreover, we aimed to characterise the molecular mechanisms involved in *Ahr* downregulation.

4.2.1. Ahr protein and mRNA expression are repressed by hCG

To determine the expression of *Ahr* in preovulatory GCs we used a classical superovulation scheme. This includes administration of PMSG to immature female mice, injection of hCG (LH analogue) or vehicle 48 h later followed by excision of ovaries after 12 h and harvesting of GCs. Western blot analysis revealed that hCG-treatment results in a robust downregulation of Ahr protein levels compared to vehicle-treated cells. Additionally, gene expression analysis revealed that *Ahr* mRNA is upregulated following PMSG-treatment as determined in Ref I but hCG injection renders *Ahr* mRNA to the levels of GCs from immature mice. We also determined *Ahr* expression in whole ovaries and observed a negative though statistically insignificant effect by hCG, which may be due to the cells other than GCs in the ovary that lack Lhcgr and thus do not respond to this stimulus (II, Figure 1 A and B). These results are in agreement with previous *in vivo* observations in rats, where downregulation of *Ahr* was evident prior to ovulation (Chaffin et al. 2000).

Additional experiments revealed that *Ahr* expression continued to increase if mice received vehicle after 48 h of PMSG treatment. Importantly, we observed a similar pattern, including hCG-dependent downregulation, when the vehicle and hCG treatments were performed *in vitro*, ascribing the downregulation of *Ahr* specifically to Lhcgr signalling, rather than any possible secondary effects taking place *in vivo*. It was our next objective to determine the temporal pattern of *Ahr* in response to vehicle/hCG treatment. We again noted that *Ahr* expression continues to increase following vehicle treatment. However, downregulation of *Ahr* was evident 8 h and 12 h post hCG injection when compared to cells from mice that received PMSG for 48 h. More importantly, the hCG-dependent reduction of *Ahr* mRNA opposing the upregulation of vehicle treatment was evident already 4 h after injection (II, Figure 1 C and D). Hence, the down-regulation of *Ahr* expression is rapid and under control of Lhcgr signalling.

A previous publication has provided evidence that hCG and LH actions, although both binding the same receptor Lhcgr, result in somewhat different intracellular signalling pathways quantitatively and qualitatively (Casarini et al. 2012). Concerning this, we also assessed the effect of LH on *Ahr* expression and observed similar outcomes compared to hCG (unpublished data). Therefore, we concluded that Lhcgr signalling, irrespective of its activator, reduces *Ahr* expression both *in vivo* and *in vitro* and this effect occurs in a rather rapid manner.

4.2.2. Downregulation of *Ahr* expression is dependent on PKA signalling and does not involve *de novo* protein synthesis

It is known that Lhcgr signalling activates adenylate cyclase (AC) resulting in elevation of cAMP levels, which in turn results in PKA activation. PKA has been shown to modulate, among others, ERK1/2 (Extracellular Signal-Regulated Kinase 1/2) and modify chromatin proteins (DeManno et al. 1999; Puri et al. 2016). In order to shed light on the possible mechanisms involved in hCG-dependent downregulation of *Ahr*, GCs from PMSG-primed mice were cultured *in vitro* in various conditions. First, as seen previously, we observed a significant downregulation of *Ahr* mRNA when cell culture media was supplemented with hCG. Forskolin, which is another activator of adenylate cyclase, showed similar albeit more profound repressive effects on *Ahr* expression, consistent with previous publication presenting cAMP-dependent downregulation of *Ahr* (Fitzgerald et al. 1996). Although we paid no attention to this, it may be caused by its direct action on AC rather than intermediate Lhcgr signalling by hCG. The activation of AC and subsequent cAMP formation led us to hypothesise that PKA pathway may be responsible for regulating *Ahr*. To test this, additional experiments using the PKA inhibitor H89 revealed the importance of PKA as the downregulation of *Ahr* was abolished. As PKA can also modulate ERK1/2 activity, we next aimed to test whether ERK1/2 participates in *Ahr* downregulation. Inhibition of ERK1/2 by its inhibitor U0126, however, had no effect on hCG/Forskolin (FSK)-dependent modulation of *Ahr* expression (II, Figure 2 A). Thus, *Ahr* seems to be under direct control of PKA.

Since the onset of *Ahr* downregulation appeared rather fast (II, Figure 1 D), we next aimed to determine, whether this occurs via direct signalling or requires *de novo* protein synthesis. For this, GCs from PMSG-injected mice were cultured *in vitro* in the presence of vehicle or cycloheximide (CHX), a known inhibitor of new protein synthesis. After 1 h pre-treatment, either hCG, FSK or vehicle were added to the culture medium. Analysis of *Ahr* mRNA expression indicated that CHX treatment alone resulted in robust induction of gene expression. Although the mechanisms remain unknown, it is possible that CHX treatment resulted in elimination of inhibitory proteins tightly controlling *Ahr* expression. Concomitant treatment of GCs with FSK and hCG, however, counteracted this effect emphasising that repression of *Ahr* expression is independent of new protein synthesis and may occur via signal transduction between proteins (II, Figure 2 B). Although we did not investigate this in detail, it is reasonable to argue that p-Creb, a direct phosphorylation target of PKA, may be responsible for this in a similar way as identified in Ref I.

Collectively, these results demonstrate that PKA pathway is involved in the downregulation of *Ahr* expression in preovulatory GCs. Modulation of ERK1/2 activity, in turn, revealed that it does not participate in the repression of *Ahr*,

referring that PKA influences *Ahr* directly rather than through other downstream pathways. Moreover, we determined that CHX boosted *Ahr* expression underscoring the repression of *Ahr* by *de novo* protein synthesis at basal level. FSK/hCG counteracted the effects of CHX and therefore exerted their effect on *Ahr* independent of new protein synthesis.

4.2.3. PKA represses *Ahr* expression by transcriptional silencing

To characterise how PKA exerts its effect on *Ahr* expression we next aimed to elucidate, whether this occurs through reduced transcription rate or increased mRNA degradation – the two fundamental processes regulating mRNA abundance in cells. The results revealed that both hnRNA, indicative of transcription rate, and mRNA levels are reduced in a similar pattern following hCG treatment of PMSG-primed mice. This indicates that hCG indeed downregulates *Ahr* transcription. Data on posttranscriptional regulation of *Ahr* is scarce. For example, regulation of *Ahr* by miR-124 has been determined (Huang et al. 2011). Additionally, a recent study demonstrated A-to-I RNA editing with subsequent miR-378-dependent reduction of *Ahr* mRNA (Nakano et al. 2016). To corroborate our findings indicating reduced transcription rate rather than post-transcriptional control of *Ahr*, we aimed to evaluate whether hCG might have an effect on *Ahr* mRNA stability. For this, GCs from mice injected with PMSG were cultured *in vitro* in the presence of hCG or vehicle followed by addition of the transcription inhibitor actinomycin D (ActD). Analysis of the results revealed that there were no differences between hCG or vehicle treatment when *de novo* RNA synthesis was inhibited, eliminating the possibility of *Ahr* regulation by e.g. miRNAs (II, Figure 3 B and C). This led us to conclude that hCG does not influence *Ahr* mRNA stability and exerts its effect primarily through reduction of transcription.

4.2.4. Chromatin remodelling at proximal promoter is involved in hCG-dependent downregulation of *Ahr* expression

Previous studies have described the promoter of *Ahr* and identified the region and elements necessary for its expression (Fitzgerald et al. 1996; Garrison & Denison 2000). In order to clarify, whether hCG might exert its effect on *Ahr* expression by some trans-acting factors, we used a reporter gene construct containing *Ahr* promoter as described in Ref I. GCs from PMSG-primed mice were transiently transfected with the reporter vector followed by hCG treatment. By analysing the results, we found that the reporter gene was indeed functional, as induction of luciferase expression was observed. When GCs were treated with hCG, however, we did not see any changes in promoter activity (II, Figure 3 D). Additionally,

we analysed the endogenous *Ahr* expression and noted downregulation as seen in our previous experiments (unpublished data). Considering that exogenous vectors do not precisely mimic the endogenous chromatin structure, we hypothesized that hCG may exert its effect on *Ahr* expression through modifications at the level of chromatin.

Histone acetylation and deacetylation, among others, are processes that are responsible for open and closed chromatin structures, respectively. The use of histone deacetylase (HDAC) inhibitor enables to investigate, whether gene expression is downregulated via closing of chromatin. By using the HDAC inhibitor trichostatin A (TSA), we next aimed to elucidate the mechanisms behind hCG-dependent downregulation of *Ahr*. GCs from PMSG-primed mice were cultured *in vitro* in the presence of TSA followed by addition of hCG/FSK. By quantifying *Ahr* mRNA we observed that hCG as well as FSK again repressed *Ahr* expression. Addition of TSA, however, reversed this effect indicating that chromatin remodelling may be involved in the regulation of *Ahr* expression. This finding is in accordance with previous studies, where inhibition of HDAC activity by TSA and n-butyrate counteracted *Ahr* repression (Garrison et al. 2000; Zhang et al. 1996).

One possibility to assess the state of chromatin is using CHART-PCR. In our experiments we focused on the promoter region –176 to –77 bp relative to TSS and the first intron far downstream (II, Figure 4 A). PMSG-primed mice were injected with hCG or vehicle and harvested GCs were subjected to CHART-PCR. We found that hCG did not influence the state of chromatin in the first intron as the amount of recovered DNA was the same as in vehicle treated GCs. This indicates that chromatin at the first intron is constantly open. Analysis of the promoter region, however, clearly demonstrated that hCG causes chromatin condensation as the recovered DNA was more abundant compared to vehicle treatment (II Figure 4 B). Thus, we concluded that following hCG surge, *Ahr* is downregulated by condensation of promoter chromatin. Indeed, comparable results have been obtained previously, where *Cyp19a1* expression was reported to be controlled by similar mechanisms post-hCG stimulation (Nimz et al. 2010).

Collectively, we demonstrated that Lhcgr signalling downregulates *Ahr* expression in preovulatory granulosa cells both *in vitro* and *in vivo*. This involves activation of PKA, but not ERK1/2 pathways, with subsequent reduction in *Ahr* transcription rate but not mRNA stability. Finally, we observed that the decrease in *Ahr* mRNA abundance was due to closing of chromatin at *Ahr* promoter thereby disabling the access of transcription factors necessary for gene expression (Figure 7).

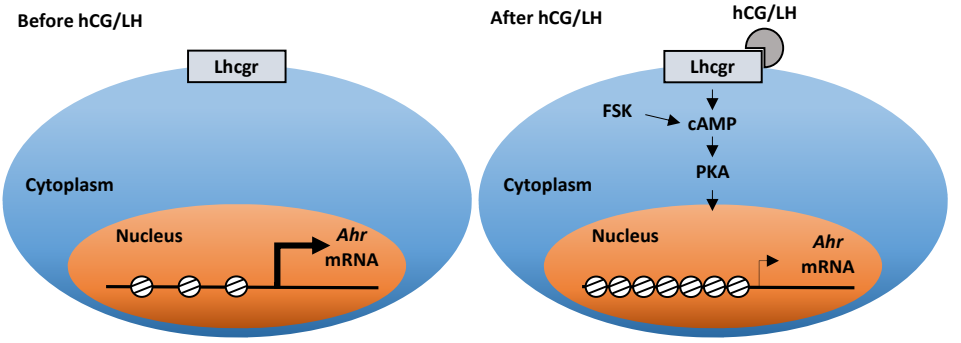

Figure 7. Schematic representation of hCG/LH-dependent *Ahr* repression. LH/hCG stimulation results in PKA-dependent downregulation of *Ahr* expression via closing of chromatin at *Ahr* promoter.

4.3. Ahr activates *Fshr* promoter via direct interaction with E-Box binding site (III)

Previous experiments exploring the role of Ahr in female reproductive system have observed that the environmental contaminant and most potent Ahr ligand TCDD has negative effects on *Fshr* expression (Hirakawa 2000). On the contrary, experiments with *Ahr* knockout mice indicate that Ahr influences *Fshr* expression positively, as lack of Ahr resulted in reduced *Fshr* mRNA levels. Additionally, binding of Ahr on *Fshr* promoter was identified without exogenous ligands by chromatin immunoprecipitation (Barnett et al. 2007a). Therefore, we aimed to shed light on the interplay between Ahr and *Fshr* and elucidate the possible mechanisms behind this.

4.3.1. Ahr and Arnt overexpression enhances *Fshr* reporter gene induction

To clarify the mechanisms by which Ahr exerts its effect on *Fshr* expression we used a constitutively active Ahr (CA-AHR) expression vector. CA-AHR lacks ligand binding ability thereby resulting in constitutive transcriptional activity (McGuire et al. 2001). This allowed us to study the endogenous role of Ahr i.e. without exogenous ligands such as TCDD, which has been shown to inhibit *Fshr* expression. Additionally we were able to avoid any possible Ahr-independent adverse effects the Ahr ligands could exert on granulosa cells. First, we transiently transfected the murine granulosa cell line KK-1 with CA-AHR and ARNT expression vectors together with the luciferase reporter gene containing a 1553bp

Fshr promoter. We found that CA-AHR indeed was functional and able to induce the promoter activity of *Fshr* (III, Figure 1 A).

The consensus motif XRE bound by Ahr/Arnt dimer consists of the sequence 5'-TNGCGTG-3', where TNGC and GTG are recognised by Ahr and Arnt, respectively. We next searched for transcription factor binding sites in the *Fshr* proximal promoter and identified a putative XRE site – TTGCCTG – differing from the consensus sequence by one nucleotide (underlined). Additionally, we identified AP-1 and E-box motifs. Previous studies have determined that Arnt homodimers are capable to bind E-box motifs and regulate gene expression (Arpiainen et al. 2007; Huffman et al. 2001). Thus, we next aimed to clarify whether Arnt homodimers rather than Ahr/Arnt complex might regulate *Fshr* promoter activity. For this, KK-1 cells were co-transfected with *Fshr* reporter vector and CA-AHR or Arnt expression vectors alone or their combination. We found that Arnt alone had no effect on *Fshr* promoter activity. Ectopic expression of CA-AHR modestly but statistically significantly activated *Fshr* promoter suggesting the possibility that endogenous Arnt may be the limiting factor in supporting the action of CA-AHR. Indeed, when CA-AHR and Arnt were co-transfected, we observed a robust induction of *Fshr* promoter reporter gene (III, Figure 1 B).

These results provide evidence that the constructed CA-AHR was functional i.e localised in the nucleus and induced target gene expression. Moreover, CA-AHR was capable to activate *Fshr* promoter and this was further enhanced with Arnt co-transfection. This indicates that Ahr/Arnt heterodimers, rather than Arnt homodimers, as shown previously, drive *Fshr* expression (Arpiainen et al. 2007). The modest induction of *Fshr* reporter gene transfected with CA-AHR alone is probably due to endogenous Arnt expressed in these cells, although dimerization of Ahr with other proteins cannot be excluded. For example dimerization of Ahr with KLF6 has been reported (Wilson et al. 2013). However, the extensive induction of *Fshr* promoter activity by CA-AHR and Arnt co-transfection indicates that indeed the Ahr/Arnt heterodimer is responsible for modulating *Fshr* expression. Considering this, all subsequent transfection experiments always included Arnt expression vector.

4.3.2. Ahr regulates *Fshr* activity through an E-box motif

It has been reported that the region necessary for *Fshr* promoter activity lies between –555 to –99 bp upstream of translational start site (Levallet et al. 2001). Considering this, we next aimed to specify the region essential for Ahr-dependent regulation. In addition to the 1553bp promoter reporter used in our previous experiments, we generated a series of reporter vectors harbouring 562bp, 209bp and 99bp of *Fshr* promoter. These constructs, together with CA-AHR and Arnt,

were transiently transfected into KK-1 and primary granulosa cells isolated from PMSG-primed mice. We observed that CA-AHR was able to induce the reporter genes containing 1553bp, 562bp or 209bp *Fshr* promoter constructs. However, the 99bp construct was unresponsive to CA-AHR (III, Figure 2). Thus, we identified that the region where Ahr exerts its effect on *Fshr* promoter lies within –209 to –99 bp upstream of translational start site. This is in accordance with a previous study, where binding of Ahr on the *Fshr* promoter in the same region was identified by chromatin immunoprecipitation (Barnett et al. 2007a).

Barnett and colleagues stated that the *Fshr* proximal promoter contains Ahr response elements (Barnett et al. 2007a). As discussed above, our transcription factor binding site search resulted in only two motifs in the aforementioned region – AP-1 and E-box – similar to that found previously (Levallet et al. 2001). Additionally, a putative XRE site was present, differing only by one nucleotide – GCCTG (III, Figure 3 A). We next aimed to clarify, which of these regulatory elements may be important for *Fshr* promoter activation by Ahr. For this, mutations were introduced into each of the three sites in the 209bp reporter construct. The mutated vectors were then transfected into KK-1 cells as well as GCs together with CA-AHR and Arnt expression vectors. Further analysis of luciferase expression revealed that mutations in the putative XRE or AP-1 sites had no effect on CA-AHR-dependent *Fshr* promoter activity. The disruption of E-box, however, rendered the luciferase levels comparable to transfections without CA-AHR (III, Figure 3 B).

We identified the minimal region of *Fshr* promoter responsive to Ahr-dependent activation. To date, several varying binding sites for Ahr have been identified. Thus, it would not be surprising to discover that Ahr could interact with DNA distinct from its classical XRE sequence. Our results indicate that the putative XRE site indeed is irrelevant in Ahr-dependent activation of *Fshr* promoter as shown by site-directed mutagenesis. More importantly, Ahr seems to exert its effect via binding to E-box element. Strengthening this notion, a previous report has suggested interactions of Ahr and E-box element on the promoter of *IL17* in Th17 cells, thereby activating its expression (Cui et al. 2011).

4.3.3. Ahr interacts with the E-box motif on *Fshr* promoter

The E-box motif is a binding site for various transcription factors. Among others, c-Myc/Max dimers are known to bind to E-box (Blackwell et al. 1993). Moreover, Usf1 has been identified to bind the E-box and regulate mouse *Fshr* expression (Hermann et al. 2008). To further characterise the interplay between Ahr and *Fshr* promoter, we next used DNA affinity precipitation assay (DAPA) to clarify whether Ahr binds E-box directly or may this be facilitated by other proteins e.g. Usf1. DAPA relies on the precipitation of nuclear protein and labelled DNA probe complexes, thus we first needed to determine the localisation

of Ahr in KK-1 and GCs. Cell fractionation and subsequent Western blot analysis revealed that in KK-1 cells Ahr is in an inactive form located in the cytoplasm and the nuclear fraction lacked Ahr. When cells were treated with TCDD, the highest affinity ligand, we observed a robust nuclear accumulation of Ahr. GC fractionation indicated that Ahr is mainly located in the cytoplasm. However, nuclear Ahr was also detected, indicating that GCs might produce endogenous Ahr ligands. Accordingly, the tryptophan metabolite kynurenine has been reported to be present in GCs (Dahiya et al. 2019). Interestingly, TCDD treatment resulted only in modest nuclear import of Ahr (III, Figure 4 A). Considering this, KK-1 cells were pre-treated with TCDD in our next experiments. GCs from PMSG-primed mice, in turn, were used without prior treatment to avoid possible side-effects of TCDD.

For DAPA experiments, we used biotinylated oligonucleotides harbouring the identified wild type or mutated transcription factor binding sites and flanking regions. AP-1 and E-box motifs were overlapping, thus on the same probe. First, DAPA experiments were performed with KK-1 cells. Nuclear lysates were incubated with biotinylated probes containing the E-box/AP-1 and putative XRE sites of *Fshr* promoter. Probes harbouring mutated binding sites served as controls. Analysis of precipitated proteins by Western blot revealed that both Ahr and Usf1 bound the wild type E-box/AP-1 probe, whereas no binding was detected when E-box was mutated. Disruption of the AP-1 sequence did not abolish the binding of Ahr or Usf1 to the probe. We did not detect any Ahr or Usf1 protein when the putative XRE or its mutated probes were used indicating that this indeed is not a functional binding site for Ahr. To determine if Ahr binds to E-box via interactions with Usf1 or *vice versa*, unlabelled competitor probes with Usf1 and XRE consensus sequence were used in 10-fold excess. The use of competitor containing the consensus XRE sequence abolished precipitation of Ahr with E-box probe. However, we still detected Usf1 indicating that Usf1 does not require Ahr for binding to E-box. Usf1 competitor abrogated the binding of Usf1 to the E-box probe, whereas it did not affect the binding of Ahr, indicating that Usf1 does not facilitate the binding of Ahr to the E-box probe containing *Fshr* promoter sequence (III, Figure 4 B). Similar experiments were performed with GCs. Again, we observed that both Ahr and Usf1 bound the intact E-box/AP-1 probe. The use of mutated E-box probe abrogated Ahr and Usf1 detection, whereas AP-1 mutation resulted in no changes. Additionally, Usf1 competitor again displaced Usf1 but had no effect on the binding of Ahr to the wild type E-box probe (III, Figure 4 C). These results are in line with our previous experiments using mutated *Fshr* promoter constructs, where disruption of E-box sequence abolished the Ahr-dependent activation of the reporter gene.

At first, the interplay between Ahr and Usf1 on binding to E-box seems somewhat contradicting. Especially when using the Usf1 competitor, that contains the core E-box sequence CACGTG. This, however, can be explained by different

flanking nucleotides in the E-box probe vs Usf1 competitor, where A-to-G and T-to-C substitutions were introduced at +1 and +3 positions downstream of CACGTG, respectively. Indeed, previous publications have underscored the importance of these nucleotides in binding of Ahr to DNA (Matikainen et al. 2001; Shen & Whitlock 1992). In addition, the importance of flanking nucleotides has been emphasised in the binding of Arnt homodimers to the E-box element (Swanson & Yang 1999). Considering that Arnt without Ahr had no effect on *Fshr* promoter activity, we concluded that Ahr/Arnt heterodimer seems to interact with the E-box motif directly rather than via Usf1.

4.3.4. Ahr DNA binding is necessary for transactivation of *Fshr* promoter

Two previously published studies have described that specific mutations in *Ahr* sequence render it unable to bind DNA (Bunger et al. 2008; Levine et al. 2000). More precisely, insertion of glycine and serine between arginine-39 and aspartic acid-40 (AHR_DBD) and a substitution of alanine-78 to aspartic acid (AHR_A78D) rendered Ahr incapable to bind DNA. By exploiting this, we aimed to determine whether Ahr interacts with the E-box motif on *Fshr* promoter directly or via other proteins. We constructed two mutant CA-AHR expression vectors (CA-AHR_A78D and CA-AHR_DBD), transfected them into KK-1 cells and performed DAPA experiments. Following Western blot, we again observed that CA-AHR was binding the E-box probe, this did not require Usf1 as shown by Usf1 competitor, and mutation of the E-box motif abolished the binding of both proteins. When KK-1 cells were transfected with CA-AHR_A78D or CA-AHR_DBD, no binding to the E-box probe was detected (III, Figure 5 A). This, however, did not influence the binding of Usf1 to the intact E-box probe indicating that Ahr and Usf1 interact independently with the E-box motif.

Further reporter gene experiments using CA-AHR, CA-AHR_A78D and CA-AHR_DBD were undertaken to ensure our notion that the DNA binding ability of Ahr is indeed obligatory for *Fshr* promoter activation. For this, GCs and KK-1 cells were transiently transfected with the respective expression vector and *Fshr* reporter gene. Analysis of luciferase expression revealed that CA-AHR again induced *Fshr* promoter activity in both cell types. When CA-AHR_A78D or CA-AHR_DBD were ectopically expressed, we observed a considerable reduction in *Fshr* promoter activity compared to CA-AHR, demonstrating that DNA binding of Ahr is essential for the induction of *Fshr* promoter (III, Figure 5 B). In GCs but not KK-1 cells, we observed a modest increase in *Fshr* promoter activation by mutant Ahr compared to empty vector transfection. This, however, can be explained by the activity of endogenous Ahr, as observed in fractionation experiments (I, Figure 4 A). Additionally, we cannot completely

exclude the option that CA-AHR_A78D or CA-AHR_DBD may have disturbed binding potencies with other proteins necessary for *Fshr* transcription. However, the results obtained from DAPA experiments with Usf1 competitors still strongly indicate that the binding and thus transactivation of *Fshr* promoter rely on the direct interaction between Ahr and E-box motif.

A previous publication has emphasised that TCDD – the most potent ligand of Ahr – negatively regulates *Fshr* mRNA expression in cultured rat GC (Hirakawa 2000). Barnett and colleagues and our data herein, however, ascribe an opposite function to Ahr in regard of mouse *Fshr* expression (Barnett et al. 2007b,a). Thus, we next aimed to clarify, whether this is due to species-specificity or due to different activation of Ahr i.e. endogenous/constitutive activity vs exogenous ligand (TCDD). For this, GCs from PMSG primed mice were transfected with *Fshr* promoter reporter constructs and wild type Ahr expression vector or respective controls. Analysis of luciferase expression revealed that TCDD alone significantly although to a small extent activates *Fshr* promoter (III, Figure 6). This seems to be in line with our experiments showing TCDD-dependent accumulation of endogenous Ahr in GCs (III, Figure 4 A). Small changes in reporter gene activity were observed when Ahr was overexpressed without TCDD treatment, indicating activation of Ahr by an endogenous ligand. However, when GCs were transfected with Ahr and received additionally TCDD, we observed a robust transactivation of *Fshr* promoter. Considering that ectopic overexpression of Ahr and subsequent activation by TCDD probably results in ubiquitous nuclear accumulation of Ahr, this was not surprising. Moreover, the TCDD-responsiveness of *Fshr* promoter is reinforced by the fact that it harbours A and T at positions +1 and +3 downstream E-box motif, respectively. These nucleotides have been shown to be essential for TCDD-induced target gene activation (Matikainen et al. 2001).

Our results from reporter gene experiments differ from previously published data in rats. There is a slight possibility that these contradicting results stem from the differences in model organisms. However, the reason might also lie within different experimental protocols, as Hirakawa observed TCDD-dependent down-regulation of *Fshr* expression *in vitro* in GCs derived from diethylstilbestrol (DES)-treated immature rats (Hirakawa 2000). As FSH/LH activities are needed to upregulate Ahr in GCs (I, Figure 1), it is reasonable to argue that DES-treatment does not increase Ahr protein levels. Supporting this, oestradiol did not influence *Ahr* expression in rats (Chaffin et al. 2000). Thus, it is possible that the reduction in *Fshr* expression results from TCDD-induced silencing of Ahr signalling via Tiparp and/or Ahrr, rather than direct inhibitory action of Ahr. In our experiments with PMSG-primed mice, the Ahr levels are relatively high and potentially superior to these inhibitory signals. Our findings are supported by Barnett and colleagues, who also ascribed a positive role for Ahr in regulating Fshr expression (Barnett et al. 2007a).

Conclusively, published data indicate that Ahr has an important role in granulosa cells, as knockout mice have disordered oestrous cyclicity, reduced expression of *Fshr*, *Cyp19a1* and decreased oestradiol levels (Baba et al. 2005; Barnett et al. 2007a,b). We, herein, provide evidence that Ahr regulates *Fshr* expression positively via direct interaction with the E-box motif (Figure 8). Although the effects various environmental pollutants, like TCDD, exert on reproduction may seem somewhat contradicting, future studies will shed light in this matter, as toxicological studies are still ongoing.

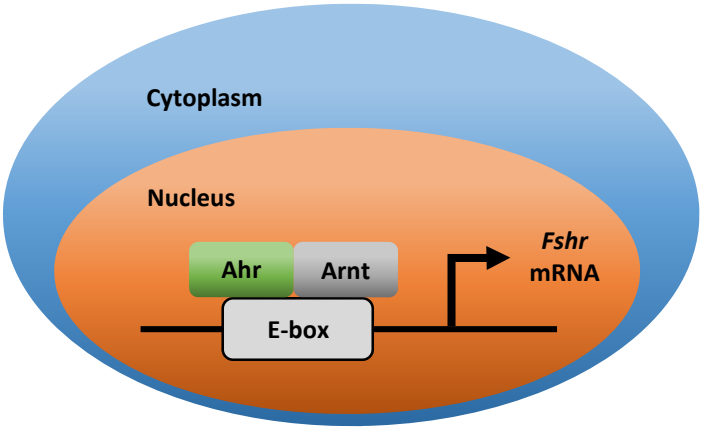

Figure 8. Regulation of *Fshr* expression by Ahr. Ahr and Arnt directly interact with an E-box motif located in the promoter region of *Fshr*.

SUMMARY

Initially, the aryl hydrocarbon receptor was studied in regard of its role in toxicology. Additionally, the *Ahr* gene was characterised, including its promoter and regulatory elements therein. Today, the majority of research investigating Ahr focuses on elucidating its role in normal physiology as well as disease, identifying its novel ligands and exploiting them to counteract health disorders. However, little attention is paid on how Ahr itself is regulated. Thus, the research presented in the current thesis aimed to delineate the mechanisms that regulate Ahr expression. Moreover, we intended to clarify how Ahr regulates its target gene devoid of the consensus response element in the regulatory region. For this, we used ovarian granulosa cells from mice, as fluctuations in Ahr levels and regulation of *Fshr* have been reported in these cells previously.

In the first part of the thesis, we ascertained that Ahr is upregulated as follicular maturation of granulosa cells proceeds and this requires both FSH and LH activity. Moreover, Ahr protein levels increased in mural rather than cumulus GCs of large antral follicles. The temporal pattern of Ahr expression indicated that its upregulation coincides with *Fshr*, a gene reported to be regulated by Ahr. Cyp19a1, another Ahr target gene, however, was induced prior to changes in Ahr expression. Additional experiments revealed that PKA pathway inhibits *Ahr* expression. This was determined *in vitro*, as the use of PKA inhibitor recovered Ahr expression, and *in vivo*, where *Ahr* induction was observed when p-Creb levels decreased. We provide evidence that Ahr is upregulated via increased transcription rate and not by changes in mRNA stability. This in turn appeared to be the result of the opening of chromatin at the Ahr promoter as revealed by CHART-PCR.

In the second part of this thesis we determined that the upregulation of Ahr expression during follicular maturation is reversed in the preovulatory phase following LH surge. By using the LH analogue hCG, we observed a rapid decrease in Ahr expression at both mRNA and protein levels *in vitro* and *in vivo*. This effect was elicited directly by PKA, as *de novo* protein synthesis was not required and PKA inhibition abolished the downregulation of Ahr. Investigation into the possible mechanisms revealed that Ahr decrease is the result of reduced transcription rate and did not involve changes in mRNA stability. Additional experiments using the HDAC inhibitor exposed the importance of epigenetic modifications in the regulation of *Ahr*. In accordance with this, we provide evidence that Lhcgr signalling results in chromatin remodelling i.e. reduced accessibility at Ahr promoter but not in the first intron, as shown by CHART-PCR experiments.

The third part of the current thesis reveals that Ahr transactivates *Fshr* expression in GCs by direct binding to DNA at the E-box motif located in the

proximal promoter region. We first constructed a CA-AHR expression vector and verified its functionality. Deletion constructs of *Fshr* promoter reporter gene indicated that Ahr exerts its effect on Fshr promoter activity in the region –209 to –99 bp relative to translational start site. Mutations of the transcription factor binding sites in this region underscored the importance of E-box motif but not AP-1 or a putative XRE. Further DAPA experiments indicated that Ahr binds E-box directly rather than via USF1. This was later confirmed with CA-AHR mutants incapable of DNA binding in both DAPA experiments as well as reporter gene assay where *Fshr* promoter activation was abolished. Finally, we established that TCDD-activated Ahr stimulates *Fshr* promoter induction, rather than inhibits its expression as observed previously.

The upregulation of Ahr expression has been reported in cancer. Considering that the majority of recent studies regarding Ahr focus on the modulation of its activity, the results herein provide another means in disrupting its signalling, i.e. activation of PKA pathway, and thus adverse outcomes in disease. Additionally, the establishment of the E-box regulatory element as an Ahr binding site enables to identify novel Ahr target genes, thereby expanding our understanding of this controversial protein.

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SUMMARY IN ESTONIAN

Arüülsüsivesinike retseptor hiire munasarja granuloosarakkudes

On teada, et paljud keskkonnas levinud saasteained, sealhulgas dioksiinid (nt TCDD) mõjutavad oluliselt inimese tervist, põhjustades muuhulgas vähki ja häireid immuunsüsteemis ning reproduktsioonis. Seetõttu on pikka aega uuritud nende ksenobiootiliste kemikaalide toimemehhanisme ning leitud, et mitmed neist avaldavad mõju läbi arüülsüsivesinike retseptori (AHR). AHR on transkriptsioonifaktor, mis ligandi poolt aktiveerituna liigub raku tuuma ning aktiveerib mitmete märklaudgeenide transkriptsiooni. Nende hulka kuuluvad ka ksenobiootikume lagundavate ensüümide geenid. Kui algselt arvati, et AHR-i peamiseks funktsiooniks on nende saasteainete metabolism, siis hilisemad uuringud on tõestanud, et AHR-il on ka oluline endogeenne ehk ksenobiootikumidest sõltumatu roll. Lisaks on tänaseks tuvastatud hulgaliselt AHR-i agoniste ning antagoniste, mida sünteesitakse organismis või omastatakse toiduga. Seejuures on oluline märkida, et erinevad ligandid võivad mõjutada geenide ekspressiooni erinevalt ning samuti võib see olla sõltuv rakutüübist ja mudelorganismist. Kuigi eelkõige on AHR-iga seotud uurimistööde põhifookuseks olnud AHR-i aktiivsuse muutumine, on teada, et ka selle ekspressioon võib varieeruda. Näiteks on tuvastatud kõrge AHR-i tase mitmetes vähitüüpides. Seega on AHR-i ekspressiooni moduleerimisel potentsiaalne terapeutiline väärtus.

Mitmed keskkonnakemikaalid, mis avaldavad oma toimet läbi AHR-i, kutsuvad esile häireid reproduktiivsüsteemis. Teisest küljest on Ahr-il ka oluline endogeenne funktsioon, indutseerides *aromataasi* (*Cyp19a1*) ja *folliikuleid stimuleeriva hormooni retseptori* (*Fshr*) ekspressiooni granuloosarakkudes. Samuti on teada, et *Ahr*-i ekspressiooni dünaamika muutub emase roti munasarjas reproduktiivtsükli jooksul, viidates *Ahr*-i regulatsiooni olulisusele. Sellest tulenevalt oli käesoleva doktoritöö eesmärgiks selgitada, millised mehhanismid mõjutavad *Ahr*-i ekspressiooni hiire munasarja granuloosarakkude küpsemisel enne ja pärast ovulatsiooni stimuleerimist. Lisaks kirjeldati, milliste interaktsioonide kaudu mõjutab Ahr *Fshr*-i promootori aktiivsust.

Käesoleva doktoritöö esimene osa keskendus Ahr-i ekspressiooni regulatsioonile granuloosarakkudes folliikulite küpsemise käigus. Esmalt tehti kindlaks, et sarnaselt rotis täheldatule, suureneb Ahr-i ekspressioon granuloosarakkude diferentseerumise käigus ning see on sõltuv nii folliikuleid stimuleeriva hormooni (FSH) kui ka luteiniseeriva hormooni (LH) signaliseerimisest. Kõrge Ahr-i tase oli omane eelkõige suurte antraalsete folliikulite granuloosarakkudele. Ahr-i ekspressiooni dünaamikat vaadeldes selgus, et see on ajaliselt võrreldav *Fshr*-i ekspressiooni suurenemisega, mis on kooskõlas viimase regulatsiooniga Ahr-i poolt. See-eest teise Ahr-i märklaudgeeni – *Cyp19a1* – ekspressioon indutseeriti ajaliselt varem. Järgnevalt selgitati, milline signaalirada võiks mõjutada *Ahr*-i ekspressiooni. Kasutades proteiinkinaas A (PKA) inhibiitorit *in vitro*, selgus, et PKA represseerib *Ahr*-i geeni avaldumist. Antud tulemust kinnitasid *in vivo* katsed, kus täheldati PKA märklaudvalgu fosforüleeritud vormi vähenemist

samaaegse *Ahr*-i induktsiooniga. Lisaks tehti kindlaks, et *Ahr*-i ekspressiooni suurenemine on tingitud mRNA sünteesi aktiivsuse suurenemisest, kuid mitte muutunud mRNA stabiilsusest. Viimaks selgitati, et antud efekt on põhjustatud *Ahr*-i promootorala kromatiini avanemisest.

Doktoritöö teises osas kontsentreeruti Ahr-i regulatsioonimehhanismidele granuloosarakkudes pärast LH analoogi hCG (inimese koorini gonadotropiin) manustamist hiirtele. Selgus, et hCG mõjul väheneb *Ahr*-i ekspressioon kiirelt nii mRNA kui valgu tasemel. See oli tingitud otseselt PKA signaliseerimisest, mida tõendasid *de novo* valgusünteesi ja PKA inhibiitori katsed. Lisaks määratleti, et Ahr-i ekspressiooni vähenemine on põhjustatud mRNA sünteesi aktiivsuse langusest ja mitte mRNA stabiilsuse kahanemisest. Edasised katsed näitasid, et antud efekt on omakorda tingitud kromatiini struktuuri ümberkorraldustest. Nimelt leiti, et hCG mõjul sulgub kromatiin *Ahr*-i promootoralal, kuid mitte geenisiseselt.

Antud doktoritöö kolmandas osas keskenduti Ahr-i ja *Fshri*-i promootorala interaktsioonide kirjeldamisele. Esmalt konstrueeriti konstitutiivselt aktiivse Ahr-i (CA-AHR) ekspressioonivektor ning valideeriti selle funktsionaalsus. Järgnevalt määratleti reportergeeni analüüsil, et Ahr-sõltuvaks Fshr-i promootori aktivatsiooniks on oluline piirkond, mis paikneb vahemikus –209 kuni –99 aluspaari translatsiooni alguspunktist ülesvoolu. Antud piirkonnas paiknevaid transkriptsioonifaktorite seondumiskohti muteerides selgus, et Ahr-i toime avaldub läbi E-box motiivi. Seda kinnitasid ka DNA afiinsus-sadestamise meetodil saadud tulemused, mis osundasid, et Ahr seondub E-box motiivile pigem otseselt. Kinnitamaks, kas Ahr interakteerub otseselt DNA-ga või siiski mõne tuvastamata valgu vahendusel, konstrueeriti järgnevalt vektorid, millelt ekspresseeritakse Ahr-i variante, mis pole võimelised DNA-le seonduma. Edasistest katsetest selgus, et muteeritud Ahr valgud polnud võimelised *Fshr*-i promootorile seonduma ning seda aktiveerima. Seega järeldati, et Ahr seondub E-box motiivil otseselt DNA-ga. Viimaseks uuriti TCDD mõju *Fshr*-i promootori aktiivsusele. Vastupidiselt varasemalt publitseeritule, kus täheldati Fshr-i ekspressiooni langust TCDD mõjul, ilmnes, et TCDD indutseerib Ahr-sõltuvalt Fshr-i promootori aktivatsiooni.

Käesoleva doktoritöö uurimistulemuste põhjal võib üldistavalt väita, et PKA signaliseerimine represseerib Ahr-i ekspressiooni hiire munasarja granuloosarakkudes. Arvestades, et kõrget AHR-i valgu taset on täheldatud mitmetes vähitüüpides, on sellel leiul ka potentsiaalne rakenduslik väljund meditsiinis, kus PKA aktivatsiooni muutmine võiks olla üheks terapeutiliseks sihtmärgiks. Lisaks aitab antud doktoritöö käigus selgunud teadmine, et Ahr on võimeline interakteeruma E-box motiiviga, tuvastada uusi Ahr-i märklaudgeene ning seeläbi laiendada arusaama selle valgu funktsioonidest organismis.

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Farewell, my "professional childhood"!

PUBLICATIONS

CURRICULUM VITAE

Name: Indrek Teino
Date of birth: 08. December 1985
Contact: Chair of Cell Biology, Institute of Molecular and Cell Biology, University of Tartu, Riia Street 23, 51010, Tartu, Estonia
E-mail: indrek.teino@gmail.com

Education and professional employment:

2018–	University of Tartu, Institute of Molecular and Cell Biology, national coordinator of ERA-NET project HEPAMUT (Mutated neo-antigens in hepatocellular carcinoma)
2011–	University of Tartu, PhD studies (Molecular and Cell Biology); laboratory assistant
2009–2011	University of Tartu, MSc in Natural Sciences (Gene Technology)
2004–2009	University of Tartu, BSc in Natural Sciences (Gene Technology)
2001–2004	Hugo Treffner Gymnasium

Scientific work:

- The main research is focused on aryl hydrocarbon receptor in mouse ovarian granulosa cells and human embryonic stem cells.
- Supervised three BSc and one MSc dissertations, co-supervised two BSc and two MSc dissertations

Special courses:

2019 International Federation for Adipose Therapeutics and Science annual meeting, France, Marseille
2018 International Society for Stem Cell Research annual meeting, Australia, Melbourne
2018 AHR Meeting 2018, France, Paris
2017 International Society for Stem Cell Research annual meeting, USA, Boston
2016 International Society for Stem Cell Research annual meeting, USA, San Francisco
2015 International Society for Stem Cell Research annual meeting, Sweden, Stockholm
2014 International Conference on Gonadotropins & Receptors, France, Tours
2014 International Society for Stem Cell Research annual meeting, Canada, Vancouver
2014 Practical course “Laboratory Animal Science I”, Estonia, Tartu

Fellowships:

2018 Doctoral School of Biomedicine and Biotechnology, grant for foreign visits
2018 Kristjan Jaak grant for foreign visits, Archimedes Foundation
2018 Olev and Talvi Maimets scholarship, University of Tartu Foundation
2017 Kristjan Jaak grant for foreign visits, Archimedes Foundation
2016 Kristjan Jaak grant for foreign visits, Archimedes Foundation
2016 Olev and Talvi Maimets scholarship, University of Tartu Foundation
2015 Doctoral School of Biomedicine and Biotechnology, grant for foreign visits
2014 Doctoral School of Biomedicine and Biotechnology, grant for foreign visits
2014 Kristjan Jaak grant for foreign visits, Archimedes Foundation
2013 Olev and Talvi Maimets scholarship, University of Tartu Foundation

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2. Ervin, E. H., Pook, M., **Teino, I**., Kasuk, V., Trei, A., Pooga, M., & Maimets, T. (2019). Targeted gene silencing in human embryonic stem cells using cell-penetrating peptide PepFect. Stem Cell Res. Ther. 10(1):43
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4. Pook, M., **Teino, I**., Kallas, A., Maimets, T., Ingerpuu, S., & Jaks, V. (2015). Changes in Laminin Expression Pattern during Early Differentiation of Human Embryonic Stem Cells. PLoS ONE. 10(9): e0138346
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ELULOOKIRJELDUS

Nimi: Indrek Teino
Sünniaeg: 08. detsember 1985
Aadress: Rakubioloogia õppetool, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool, Riia 23, 51010, Tartu, Eesti
E-post: indrek.teino@gmail.com

Haridus- ja teenistuskäik:

2018– Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, ERA-NET projekti HEPAMUT (Muteeritud neoantigeenid hepatotsellulaarses kartsinoomis) koordinaator
2011– Tartu Ülikool, doktoriõpe (molekulaar- ja rakubioloogia); Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, laborant
2009–2011 Tartu Ülikool, loodusteaduste MSc (geenitehnoloogia)
2004–2009 Tartu Ülikool, loodusteaduste BSc (geenitehnoloogia)
2001–2004 Hugo Treffneri Gümnaasium

Teadustegevus:

- Peamiseks uurimisteemaks on arüülsüsivesinike retseptor hiire munasarja granuloosarakkudes ja inimese embrüonaalsetes tüvirakkudes.
- Kolme bakalaureuse- ja ühe magistritöö juhendaja ning kaasjuhendaja kahes bakalaureuse- ja kahes magistritöös.

Erialane enesetäiendus:

2019 International Federation for Adipose Therapeutics and Science aastakonverents, Prantsusmaa, Marseille
2018 AHR Meeting 2018, Prantsusmaa, Pariis
2018 International Society for Stem Cell Research aastakonverents, Austraalia, Melbourne
2017 International Society for Stem Cell Research aastakonverents, USA, Boston
2016 International Society for Stem Cell Research aastakonverents, USA, San Francisco
2015 International Society for Stem Cell Research aastakonverents, Rootsi, Stockholm
2014 International Conference on Gonadotropins & Receptors, Prantsusmaa, Tours
2014 International Society for Stem Cell Research aastakonverents, Kanada, Vancouver
2014 Koolitus “Katseloomateadus I”, Eesti, Tartu

Stipendiumid:

2018 Biomeditsiini ja biotehnoloogia doktorikool, välislähetuse toetus
2018 Kristjan Jaagu välislähetuse stipendium, SA Archimedes
2018 Olev ja Talvi Maimetsa stipendium
2017 Kristjan Jaagu välislähetuse stipendium, SA Archimedes
2016 Kristjan Jaagu välislähetuse stipendium, SA Archimedes
2016 Olev ja Talvi Maimetsa stipendium
2015 Biomeditsiini ja biotehnoloogia doktorikool, välislähetuse toetus
2014 Biomeditsiini ja biotehnoloogia doktorikool, välislähetuse toetus
2014 Kristjan Jaagu välislähetuse stipendium, SA Archimedes
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