Review

Molecular mechanisms of the physiological functions of the aryl hydrocarbon (dioxin) receptor, a multifunctional regulator that senses and responds to environmental stimuli

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Abstract: The aryl hydrocarbon receptor (AhR) was originally identified as a ligand-activated transcription factor that is involved in the induction of xenobiotic-metabolizing Cytochrome P4501A1 (CYP1A1). For several decades, AhR has been studied in relation to toxicology and pharmacology. With recent discoveries on novel AhR functions, AhR research has expanded into multiple aspects of physiology, such as reproduction, innate immunity and tumor suppression. In this review, we summarize and discuss recent progress in mechanistic and functional studies on AhR with particular emphasis on physiological processes.

Keywords: bHLH-PAS transcription factor, innate immunity, reproduction, tumor suppression, ubiquitylation, environmental stimuli

Introduction: A brief history of AhR research

Studies on the aryl hydrocarbon receptor (AhR, also known as dioxin receptor) began about 50 years ago when drug-metabolizing enzymes were found to be induced by polycyclic aromatic hydrocarbons (PAHs), such as 3-methylchoranthrene (3MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Around 1960, three research groups independently reported that drug-metabolizing enzymes were induced in rat livers.[1)–4)] Subsequently, in 1962, Omura and Sato identified a microsomal component that gave a unique absorption spectrum with a peak at 450 nm when bound to CO in the presence of NADPH, as a hemoprotein and designated this newly discovered hemoprotein Cytochrome P450[5)] and Cooper *et al.* revealed that this hemoprotein was a drug-metabolizing enzyme using a photo-reactivation method in which the CO-inhibited drug-metabolizing activity was reversed by treatment with flash light.[6)]

In 1976, Poland *et al.* identified a cytoplasmic factor that bound TCDD with high affinity and called this novel protein AhR.[7)] Based on both the behavior of AhR in induced cells and the genetics of the inducibility of the drug-metabolizing activity, they proposed that this cytoplasmic factor was involved in inducing the drug-metabolizing P450.[8)] In the early 1980s, cDNA clones of phenobarbital-inducible and 3MC-inducible P450s, and subsequently, their genomic clones were isolated by the then-newly-developed molecular cloning technology.[9),10)] Biochemical and molecular biological approaches used a reporter gene, in which the chloramphenicol acetyltransferase structural gene was under the control of the rat *P450c* (*CYP1A1*) promoter, to identify regulatory sequences within the promoter that induce the reporter gene in response to 3MC and designated these sequences XRE (Xenobiotic Responsive Element, subsequently named DRE: Drug Responsive Element, or AhRE).[11)] Gel mobility shift assays (GMSA) revealed that an XRE-binding factor translocates from the cytoplasm into the nucleus upon TCDD treatment. In addition, this XRE-binding factor contained TCDD as a ligand, suggesting that the XRE-binding factor was either AhR or contained AhR as a component.[12)] Subsequently, studies on the mechanisms that regulate mouse *Cyp1A1* gene expression revealed a similar

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array of enhancer sequences in the promoter, which were designated DRE.[13]

Using the XRE-binding property of AhR and a partial N-terminal 20 amino acid sequence determined by Bradfield *et al.* of the purified mouse AhR fragment, which was photo-labeled with a TCDD derivative,[14] we isolated a cDNA clone of AhR from a cDNA library of the mouse cell line Hepa-1. Using this clone, we were able to determine the amino acid sequence of AhR and show that AhR has a bHLH motif and a PAS[15] domain (an amino acid sequence that is conserved among the Per, Arnt and Sim proteins). Our findings were subsequently confirmed by similar data from Bradfield *et al.*[16] The isolation of the AhR cDNA clone furthered our understanding of the molecular mechanisms that regulate the transcription of AhR target genes in response to xenobiotics including TCDD, and the toxicological effects of TCDD that are mediated by AhR.[17,18]

In 1995, Gonzalez *et al.* first described AhR-deficient mice that were generated by homologous recombination,[19] and two other research groups including ours subsequently reported the generation of these mice.[20,21] Experiments using these mutant mice clearly demonstrated that AhR mediates many pharmacological and toxicological effects, including the induction of drug-metabolizing CYP1A1, teratogenesis, immune suppression, tumor promotion, and liver damage caused by TCDD, as the AhR-deficient mice were resistant to these effects.[21]–[23] Until the turn of the century, most if not all of the AhR research work had focused on elucidating the molecular mechanisms by which AhR mediated the TCDD-induced pharmacological and toxicological effects, which are detrimental to most living organisms.[17,18]

Throughout these investigations, together with the fact that AhR is highly evolutionarily conserved across a variety of animal species,[24] it has been suggested that AhR is involved in xenobiotic-independent and physiological functions.[25]–[27] Furthermore, because AhR and Arnt (AhR nuclear translocator) are expressed during early mouse embryogenesis,[28] it has been proposed that AhR also plays a role in development. In addition, AhR has recently been reported to be involved in cell proliferation, apoptosis, adipose differentiation, tumor suppressor function, immune cell differentiation and reproductive function.[29]–[31] Consistent with these physiological roles of AhR, it has been reported that AhR can be activated in response to cell density, even in the absence of obvious exogenous ligands,[25,32] and in the presence of a variety of natural chemicals, such as lipoxin A4, prostaglandin G2 (PGG2), bilirubin and tryptophan derivatives including FICZ (6-formylindolo-[3,2-*b*]carbazole) and indirubin.[33] Moreover, the characterization of AhR homologues in invertebrates has indicated that these proteins are also involved in development, despite the fact that they cannot apparently bind xenobiotics.[34] Elucidation of the endogenous functions of AhR will provide deeper insight into the toxicological and adverse effects caused by xenobiotics, because these deleterious effects can be understood as a consequence of the unscheduled activation of AhR. In this short review, we will summarize and discuss our current knowledge on the regulatory mechanisms of AhR and its physiological functions in tumor suppression, reproduction, and immunity.

Activation of AhR

It is well established that AhR exists in a latent state in a complex with HSP90, immunophilin-like XAP2 (also named ARA9 or AIP), and p23 in the cytoplasm[17] (Fig. 1A). HSP90 binding is essential to retain AhR in the cytoplasm and this interaction is thought to mask the nuclear localization signal (NLS) of AhR. Overexpression of XAP2 increases the amount of AhR in the cytosol. Furthermore, the LxxLL motif (x is any amino acid) in the middle of the AhR molecule, which was found to mediate protein-protein interactions between transcriptional cofactors and nuclear receptors, is also involved in the cytoplasmic retention of AhR through protein-protein interactions.[25,35]–[37] The roles of the three components (HSP90, XAP2 and p23) in the HSP90 complex are described in detail in a recent review.[38] Upon ligand binding, HSP90-bound AhR translocates into the nucleus and then dissociates from the HSP90 complex in the face of and binds to Arnt.[17] In *in vitro* experiments, HSP90 is released from the ligand-bound AhR when incubated with nuclear extracts from mouse Hepa-1 cells containing Arnt, but not from Arnt-deficient mutant cells, suggesting that Arnt stimulates the ligand-induced displacement of HSP90 from AhR.[39]

It is well established that many synthetic PAHs and halogenated PAHs (HAHs) activate the AhR signal pathway. Although absolute planarity is not required for receptor binding, many agonists are planar compounds, and coplanarity is one of the most influential factors that affect the affinity of AhR for
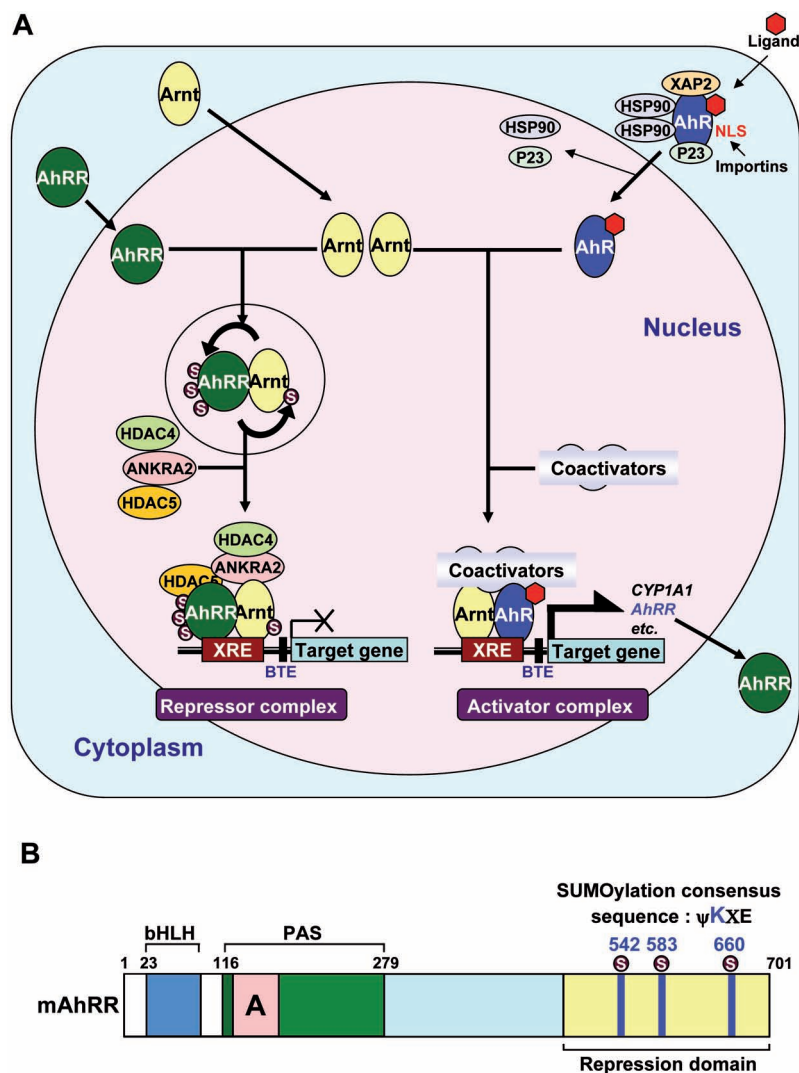
Fig. 1. A, A schematic model for the transcriptional regulation of the AhR/Arnt activator complex and the AhRR/Arnt repressor complex. Unmodified Arnt forms a heterodimer with AhR and recruits coactivators, such as CBP/p300, to form the transcriptional activator complex. Meanwhile, Arnt forms a heterodimer with AhRR, which significantly enhances the SUMOylation of both proteins. SUMOylated AhRR recruits corepressors ANKRA2, HDAC4, and HDAC5 to form the transcriptional repressor complex. B, A schematic representation of the full-length 701-amino acid mouse AhRR. The characterized domains represented are the basic helix-loop-helix (bHLH), Per-Arnt-Sim (PAS), and repression domain. Three putative SUMOylation sites are located within the repression domain, with the target lysine residues indicated.
XRE: Xenobiotic Responsive Element; BTE: Basic Transcription Element; a GC box sequence

its ligand.[40] A recent review assessed and discussed these xenobiotic ligands.[40,41] In association with the increasing interest in the intrinsic functions of AhR, natural or endogenous AhR ligands have been drawing much attention. These compounds contain tryptophan derivatives such as FICZ, IAA (indole-3-acetic acid), tryptamin, indirubin, ICZ (indolo[3,2-b]carbazole) and DIM (3,3'-diindolylmethane), prostaglandins such as PGG2 and lipoxin A4, and heme metabolites including bilirubin and biliruverdin.[33,40,41] However, it is quite difficult to determine which of these natural ligands are physiological cognate ligands or casual ones.

In contrast, several recent studies have reported ligand-independent AhR activation. When cells such as Hepa-1 and 10T1/2 fibroblasts are cultured in sus-

pension, AhR translocates into the nucleus and induces CYP1A1 and 1B1 mRNA synthesis, even in the absence of an exogenous ligand.[42),43)] In addition, prevention of cell-cell interactions in the keratinocyte cell line, HaCaT, by growing the cells at a low cell density or in Ca^{2+}-deficient conditions induces the nuclear accumulation of AhR, resulting in the activation of a reporter gene driven by XRE sequences.[32)] It has been recently reported that addition of second messenger cAMP, an endogenous mediator of hormone and neurotransmitter signaling, to the culture medium activates and translocates AhR into the nucleus, leading to its nuclear accumulation.[44)] The nuclear accumulation of AhR has been reported to be caused by phosphorylation of Ser 68 within the nuclear export sequence (NES). An analysis of specific kinase inhibitors suggests that this phosphorylation is catalyzed by p38MAPK.[32)] An increasing number of reports have indicated that phosphorylation regulates the functions of AhR in physiological signaling pathways, such as cell proliferation and xenobiotic signal transduction.[45)]

Omeprazole is known to induce CYP1A1 in an AhR-dependent manner without directly binding to AhR.[46),47)] It is reported that tyrphosphins AG17 and AG879 tyrosine kinase inhibitors, selectively inhibited omeprazole-mediated induction of CYP1A1, while no inhibitory effect was observed on the TCDD-dependent induction of CYP1A1. Further, a Tyr320Phe mutation in AhR abolished omeprazole-dependent AhR signaling, but minimally affected TCDD-induced activation of CYP1A1 expression. These results suggest that Tyr320 is a phosphorylation site in AhR that is activated by omeprazole in a ligand-independent manner through a signal transduction pathway that involves protein tyrosine kinases.[48)] Therefore, this signal pathway is independent of the AhR pathway that is induced by high affinity ligand binding. Although the detailed mechanisms are still unknown, it is highly likely that AhR is activated by phosphorylation in a ligand-independent manner.

Transcriptional activation by AhR

In the nucleus, the translocated AhR dissociates from the HSP90 complex and then heterodimerizes with Arnt, another bHLH-PAS protein, and the newly formed AhR/Arnt heterodimer binds XRE sequences in the promoters of target genes[17)] (Fig. 1A). The mechanisms of AhR transcriptional enhance-

ment are best characterized for the *CYP1A1* gene.[49)] In the approximately 2 kb sequence upstream of the *CYP1A1* gene, a cluster of XREs (5'-TNGCGTG-3') functions as an enhancer[11),13)] and a basic transcription element (BTE) of a GC box sequence located immediately upstream of the gene is also required for high CYP1A1 expression levels[50)] (Fig. 1A). SP1 binds the BTE, while the AhR/Arnt heterodimer binds the XRE sequence.[50),51)] The binding of these two transcription factors to their cognate enhancer sequences is synergistic; the binding of one of the transcription factors to its cognate sequence facilitates the binding of the other to its cognate sequence. It is also interesting that the sequences of the XRE and GC box are frequently arranged in the promoters of AhR-target genes.[52)]

It has been reported that binding of the AhR/Arnt heterodimer to XREs induces chromatin remodeling, resulting in DNase hyper-sensitivity within 300 bp upstream of the transcription start site of the target genes.[49),53)] Furthermore, studies have shown that when it binds to the XRE sequence, the AhR/Arnt heterodimer recruits CBP/p300, SRC1, NCoA2/GRIP1/TIF2, p/CIP and other coactivators including RIP140, components of ATP-dependent chromatin remodeling complexes such as BRG-1, p-TEFβ and RNA elongation factors.[54),55)] In addition, the TRAP/DRIP/ARC/Mediator complex is recruited to the *CYP1A1* promoter to activate target gene expression in response to xenobiotic stress.[54),56)] Although multiple components are reported to form the transcription complex on the AhR/Arnt heterodimer, the interactions and order of the complex formation have only begun to be partially elucidated, and the current state of these studies has been recently reviewed in detail.[38)]

Negative regulation of AhR activity

AhR signaling can be downregulated by at least two independent mechanisms, proteasomal degradation of AhR and competitive inhibition of AhR by the AhR repressor (AhRR), which is induced by activated AhR (Fig 2). It has been reported that the AhR protein is rapidly depleted *in vitro*, following exposure to AhR ligands, most likely after target gene activation.[57)–59)] This AhR degradation has been observed in rat smooth muscle A7, murine Hepa-1 and human HepG2 cells and was blocked by treating with the proteasome inhibitor MG-132. Since this degradation was inhibited by leptomycin B (LMB), a nuclear export inhibitor, it is likely that AhR degradation
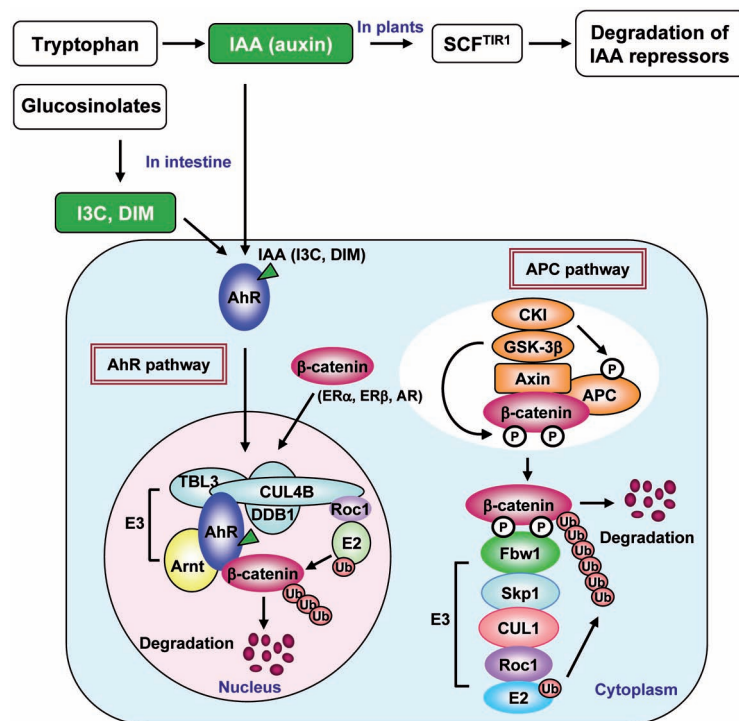
Fig. 2. A schematic model of the AhR- and APC-dependent β-catenin degradation pathways. Ligand-activated AhR translocates into the nucleus where it forms a ubiquitylation complex containing CUL4B and Arnt, whereas the APC-dependent-pathway functions in the cytoplasm. This function of AhR is activated by both xenobiotics and natural AhR ligands, such as indole derivatives (IAA, I3C, DIM. etc.) that are converted from dietary tryptophan and glucosinolates by intestinal microbes.

occurs in the cytoplasm.[57],[58] However, AhR degradation was also shown to occur in the nucleus, and this degradation did not require nuclear export. The ubiquitin ligase protein, C-terminal hsp70-interacting protein (CHIP), has also been implicated in the nuclear degradation of AhR.[59]

Recently, we have reported that when AhR is ligand-activated and translocates into nucleus, it forms an E3 ubiquitin ligase complex with CUL4B (cullin4B), DDB1 (damaged-DNA-binding 1), TBL3 (transducin β–like 3) and Rbx1 (ring box protein 1)/ Roc1 and catalyzes the ubiquitylation of sex steroid hormone receptors, ERα, ERβ, AR (androgen receptor) and AhR itself.[60] Very recently, we have found that this AhR E3 ubiquitin ligase ubiquitylates β-catenin and functions as a tumor suppressor in the colon, as will be described later in detail.[61] In this complex, CUL4B and AhR are thought to serve as the scaffold and substrate recognition component, respectively. The functional relationship between the two ubiquitylation pathways in the nucleus remains to be investigated.

Like other bHLH-PAS signaling pathways such as HIFs,[62] we have revealed that the AhR pathway is also downregulated through feedback inhibition. AhRR was originally found as a TCDD-induced protein and subsequently shown to inhibit AhR transcriptional activity.[63] The *AhRR* promoter has a functional XRE sequence, and gene expression is enhanced upon ligand activation of AhR, resulting in the inhibition of AhR signaling activity.[63],[64] AhRR contains a bHLH-PASA region that is structurally similar with AhR, followed by a C-terminal transcription repression domain (Fig. 1B). AhRR also forms a heterodimer with Arnt[63] (Fig. 1A). Since AhRR has both NLS and NES sequences and does not require an agonist to dimerize with Arnt, newly synthesized AhRR translocates into nucleus and forms a heterodimer with Arnt. This heterodimer binds competitively to the XRE sequence with the AhR/Arnt heterodimer, and subsequently recruits co-repressors such as ANKRA2, HDAC4 and HDAC5.[65] The C-terminal repression domain of AhRR has three SUMOylation sites that are conserved across verte-

brate species (Fig. 1A), and all three sites must be SUMOylated for full suppressive activity.[66] In MEF cells that do not express CYP1A1, AhRR/Arnt was shown to function as a repressor by ChIP (chromatin immunoprecipitation) analysis.[65] It has been recently reported that AhRR functions as a putative tumor suppressor[67] and that susceptibility to endometriosis and male infertility are associated with genetic polymorphisms in the *AhRR* gene.[68]–[70] The two independent mechanisms of downregulating the AhR signaling pathways implicate that there is evolutionary selection against over-activation of AhR.

The E3 ubiquitylation and tumor suppressor functions of AhR

It is well known that AhR ligands negatively or positively regulate the estrogen receptor (ER) activity.[71],[72] The loss of the transactivation potential of ER in the presence of TCDD is reportedly due to a sharp decrease in its ability to bind to the estrogen response element (ERE). AhR ligand-induced degradation and functional repression of sex steroid hormone receptors were attenuated by treating with the proteasome inhibitor, MG-132, resulting in polyubiquitylation of the ER, as determined by polyacrylamide gel electrophoresis.[73] Consistent with this observation, we revealed that polyubiquitylation of ERα and even AhR was promoted by AhR ligands in AhR immunoprecipitated complexes, when ubiquitin E1 and E2 ligases were supplemented *in vitro*.[60] The AhR-containing complexes were isolated from 3MC-treated HeLa cells expressing Flag-tagged AhR by anti-Flag antibody. Glycerol gradient centrifugation of the isolated AhR-containing complexes revealed five major 3MC-dependent complexes. While two of these complexes contained well known coactivators TRAP220/DRIP205/Med220 and p300 as transcription components, one complex was composed of the ubiquitin ligase core components, CUL4B, DDB1 and Rbx (Roc), along with subunits of proteasomal 19S regulatory particle (19SRP), Arnt and TBL3.[60]

When individual recombinant AhR, CUL4B, DDB1, and Rbx1 proteins from Sf9 cells were incubated together, the complex formation was dependent on 3MC, and the complex exhibited ubiquitin ligase activity for the ER *in vitro* when supplemented with E1 and E2 ligases. We revealed that within this AhR ubiquitinylation complex, AhR recognizes and binds both substrates and CUL4B as a scaffold to form the ubiquitinylation complex. ERα, ERβ, and AR are found to be substrates for the CUL4B/AhR complex (Fig. 2). It was previously reported that human ERα (hERα) degradation is accelerated upon E2 ligand binding or phosphorylation of Ser112, whereas a partial agonist, tamoxifen, stabilizes ERα.[74],[75] In contrast, we have shown the 3MC-activated AhR to induce the ubiquitylation and subsequent degradation of tamoxifen-bound ERα and the ERα S118A mutant.[60] Furthermore, AhR was dispensable for E2-dependent ERα degradation. These results indicate that the CUL4B/AhR complex may act independently of the innate protein degradation system(s) for ERα.[76]

Most recently, our result has revealed that the CUL4B/AhR complex ubiquitylates and degrades β-catenin in intestinal epithelial cells and functions as a tumor suppressor in the intestine.[61] AhR-deficient mice were found to frequently develop colonic tumors, mostly in the cecum at approximately 10 weeks of age.[61] It is well known that the APC ubiquitylation system in the colon functions as a tumor suppressor to regulate the β-catenin concentration in the intestine[77] (Fig. 2). Because 3MC- or β-naphthoflavone (βNF)-induced AhR activation was associated with markedly decreased endogenous β-catenin levels in DLD, SW480, or HCT116 cells derived from colon cancers, which are known to stabilize β-catenin because of mutations in APC or the β-catenin gene,[78] it has been suggested that APC- and AhR-containing ubiquitylation complexes function independently. Furthermore, we have revealed that siRNA-mediated knockdown of AhR or CUL4B, but not APC reversed repression of the β-catenin transcription activity on TCF/LEF that was induced by the AhR ligands, 3MC, βNF, and IAA in an *in vitro* reporter gene assay.[61] These results confirm that AhR is involved in previously undescribed ligand-dependent mechanisms of proteasome-mediated β-catenin degradation that are distinct from the canonical APC-dependent pathway.

We investigated the functional association between the APC- and AhR-dependent pathways of β-catenin degradation in intestinal tumor development by generating the mice with compound mutations in the *APC* and *AhR* genes. These $APC^{Min/+} \cdot AhR^{+/-}$ and $APC^{Min/+} \cdot AhR^{-/-}$ mice had greatly accelerated intestinal carcinogenesis compared with the parental mice that had single mutations in the respective genes. These results provide further support that AhR and APC have a cooperative tumor suppression function in intestinal epithelial cells. In connection

with these observations, the compound mutant mice had much higher β-catenin expression in their intestines than the respective parental mutant mice. These results indicate that the AhR ubiquitylation complex functions as tumor suppressor independently of and parallel to the canonical APC-dependent pathway. While the APC system is cytoplasmic, AhR which is activated by ligand-binding is translocated into the nucleus where it forms a ubiquitylation complex. Therefore, these two ubiquitylation complexes are considered to function in different cellular compartments (Fig. 2).

It has been reported that natural AhR ligands derived from indole, such as IAA, I3C (indole-3-carbinol) and DIM, are generated through the conversion of dietary tryptophan and glucosinolates by commensal intestinal microbes and exert chemopreventive effects on colorectal cancers in humans.[79]–[81] Remarkably, $APC^{Min/+}$ or $APC^{Min/+} \cdot AhR^{+/-}$ mice, which develop multiple intestinal tumors on a normal diet, show reduced levels of carcinogenesis in the intestine when fed an I3C- or DIM-containing diet immediately after weaning at 3 or 4 weeks of age. We conclude that these chemopreventive effects of I3C and DIM are mediated by AhR, because no tumor suppressive effect was observed in $AhR^{-/-}$ mice.[61] Since AhR shows a broad ligand specificity, the ability to bind to AhR should provide an effective screening method for the most suitable chemopreventive chemicals against carcinogenesis.

Novel physiological functions of AhR

AhR in reproduction. Previous studies have shown that the lack of *AhR* gene expression in *AhR*-null mice and the unscheduled activation of the AhR signaling pathway by HAHs result in adverse phenotypes in female reproduction organs and lead to impaired reproduction.[82] Together with the fact that AhR is ubiquitously expressed in female genital organs, these results indicate that AhR plays an important physiological role in female reproduction. Female *AhR*-null mice exhibit defects in multiple reproductive aspects, such as conception, litter size and pup survival.[83],[84] These defective functions are reflected in the decreased number of ovulated oocytes and disordered estrous cycle.[84] The estrous cycle and oocyte development in the ovary are regulated by the coordinated actions of the anterior pituitary hormones, LH and FSH, and ovarian steroid hormones including androgen, estrogen and progestin.[82],[85] During

the normal estrous cycle, hormonal signals induce the maturation of granulosa cells surrounding the oocyte, trigger follicle rupture and lead to the subsequent release of the oocyte into the fallopian tube.[82] Our result has revealed that while the number and morphology of immature, preovulatory follicles were similar between wild-type and *AhR*-null females,[82],[84],[86] the numbers of mature follicles and corpora lutea (CL) were remarkably reduced in the super-ovulation process.[84] Several lines of evidence including ours showed that serum LH and FSH levels were similar between *AhR*-null and wild-type mice at all stages of the mouse estrous cycle, but that reduced estradiol synthesis within the ovary caused defective reproduction in *AhR*-null female mice.[84],[86],[87]

It is known that estrogen is synthesized from cholesterol in the ovary and, therefore, that all the enzymes involved in estradiol synthesis are present in the ovary.[88],[89] Specifically, FSH secreted from the anterior pituitary binds to the FSH receptor (FSHR) to stimulate P450 aromatase (CYP19) expression, the rate-limiting enzyme in estrogen synthesis that converts testosterone to estradiol, with a peak of expression in the proestrous stage in granulosa cells.[90] In contrast, other steroidogenic enzymes such as $P450_{SCC}$ (CYP11A), P450 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase (3β-SHD) and 17β-hydroxysteroid dehydrogenase (17β-SHD) are constitutively expressed.[90] We have revealed that although the expression of steroidogenic enzymes other than CYP19 was essentially unchanged between *AhR*-deficient and wild-type mice, CYP19 expression was not enhanced in the proestrous stage in *AhR*-deficient mice, leading to markedly reduced ovarian estradiol concentrations compared to wild-type mice.[84],[91] We have also found that the female reproductive defects in *CYP19* KO and *ER* KO mice are similar to those in *AhR* KO mice, albeit with more severe phenotypes.[84],[92],[93] The phenotypic similarities in defective reproduction between *AhR* KO, *CYP19* KO and *ER* KO mice provide further evidence that *AhR* KO mice have deficits in estrogen production or function.

The AhR and Ad4BP/SF-1 binding sites in the promoter of the *CYP19* gene are conserved in several vertebrates, including many kinds of fish, humans, and mice, suggesting an evolutionarily conserved role for the interactions between these binding sites and their cognate transcription factors in ovarian physiology.[84] Furthermore, reporter gene assay re-

vealed that AhR and Ad4BP synergistically activated the promoter of the *CYP19* gene. The interaction between AhR and Ad4BP transcription factors was confirmed by ChIP analysis.[84] In addition, interestingly, we have found that in a super-ovulation experiment, the expression of both AhRR and CYP19 was similarly regulated in an AhR-dependent manner, while the *CYP19* gene peaked earlier than AhRR, which was upregulated when CYP19 expression began to decline. It is possible that AhR and AhRR regulate the ovarian biological clock by governing the estrous cycle. Several lines of evidence indicate that AhR plays physiological roles in reproduction beyond the estrous cycle.[26],[82] During the peri-implantation period, AhR is abundantly expressed in the uterine vasculature and developing tissues between the embryo and mother.[94] In addition to reduced fertility, *AhR* KO females have difficulty in raising their pups to the weaning age, and these pups have a significantly lower survival rate than pups raised by heterozygous females,[83] a phenotype that may be related to impaired development of the mammary glands.[95]

AhR in immunity.   There is strong evidence that AhR plays significant roles in the immune system.[96] Exposure to TCDD leads to severe thymic involution and profound suppression of both the humoral and cellular immune responses, resulting in increased susceptibility to infection. In addition, *AhR* KO mice are defective in T cell differentiation and are more susceptible to bacterial infection.[97],[98] Most of the cells that participate in immune responses constitutively or inducibly express AhR at substantial levels. Many genes that participate in the immune response, such as all 9 TLRs, IL-1Rs, IL-6, IL-18, etc., have XRE sequences in their promoters, although it is mostly unknown whether AhR is directly involved in their expression.[97],[98]

Although the mechanism has not been investigated in detail, greatly enhanced TNFα and IL-1β production from macrophages rendered the AhR wild-type mice treated with TCDD much more susceptible to LPS-induced septic shock.[99],[100] In contrast, we have observed that the *AhR* KO mice were also hypersensitive to LPS-induced lethal shock compared to wild-type mice.[101],[102] Upon LPS treatment, the serum levels of IL-6, TNFα and IL-1β were significantly higher in *AhR* KO mice than wild-type mice. Consistent with these *in vivo* results, we have revealed that peritoneal macrophages isolated

from *AhR* KO mice induced much higher IL-6 secretion in response to LPS treatment than those from wild-type mice. Furthermore, transfecting the *AhR* KO macrophages with an AhR expression plasmid suppressed this enhanced IL-6 expression upon LPS treatment.[101] These results indicate that AhR suppresses the expression of inflammatory cytokines in response to LPS. Interestingly, we have revealed that peritoneal macrophages markedly enhanced AhR expression in response to LPS treatment.[102] It has been suggested that AhR plays a suppressive role by forming a complex with Stat1 and NF-κB in macrophages, leading to the inhibition of the promoter activity of the *IL-6* gene.[101]

In the case of IL-1β secretion, we have observed that AhR activates the expression of Pai-2,[102] an inhibitor of caspase-1 activation that functions in the process of IL-1β secretion.[103] AhR ligands markedly enhanced Pai-2 expression in wild-type macrophages and transduction of Pai-2 into *AhR* KO macrophages restored suppression of IL-1β secretion.[102] Activated AhR together with NF-κB, but not with Arnt, directly enhanced the expression of *Pai-2* gene by binding to its promoter.[102] It is reported that experiments with the human macrophage cell line U937 showed that TCDD induces the expression of IL-8, Bcl (B-cell chemoattractant) and Baff (B-cell activating factor).[104] These increases in gene expression were associated with the binding of the AhR-Rel complex to a novel NF-κB binding site in the promoters of these genes, and this process did not require Arnt, suggesting a new mechanism of transcriptional regulation that involves TCDD-activated AhR.[104]

There have been several lines of evidence indicating that AhR ligands stimulate naïve T cells (Th0) to differentiate into helper T-cell subsets that suppress or accelerate the immune responses by modulating effector T cell proliferation and cytokine secretion.[97],[98] Previous studies have shown that the profound suppression of an acute graft versus host (GVH) response in TCDD-treated mice was associated with the generation of donor-derived CD4$^+$ CD25$^+$ T_{reg} cells that were dependent upon AhR activation.[105],[106] VAF is a low molecular weight compound that activates AhR. *In vivo* VAG539 treatment increased the frequency of splenic CD4$^+$ T cells expressing CD25 and FoxP3 and induced pancreatic islet allograft tolerance through direct as well as DC-mediated induction of T_{reg} cells.[106] We have reported that another AhR ligand, M50354,

exerts an anti-allergic effect in an AhR-dependent manner both *in vitro* and *in vivo*.[107] TCDD-induced AhR activation was found to induce functional T_{reg} cells that suppressed experimental autoimmune encephalomyelitis (EAE).[108] Interestingly, incubation of Th0 cells under T_{reg} polarizing conditions containing TGFβ$_1$ along with CD3 and CD28 antibodies induced AhR expression. Furthermore, the promoter of the *FoxP3* gene, a transcription factor thought to play a master role in T_{reg} differentiation, contains functional XRE sequences that are responsible for inducing this gene in response to activated AhR. In addition, ChIP analysis revealed that AhR was recruited to the *FoxP3* promoter.[108]

It has been recently reported that AhR plays an essential role in the development of IL-17-producing T helper cells (Th17), a new subset of CD4$^+$ T cells thought to be involved in autoimmunity and the clearance of infectious agents.[109],[110] Th17 cell development is reciprocally related to the generation of T_{reg} cells. While TGFβ$_1$ induces the differentiation of T_{reg} cells, TGFβ$_1$ in combination with IL-6 boosts the differentiation of Th17 cells from naïve T cells under T cell differentiation conditions. The presence of an AhR ligand, FICZ, further enhances Th17 differentiation under Th17-polarizing conditions. AhR expression is remarkably accelerated in induced Th17 cells, with approximately 10-fold higher expression than that in induced T_{reg} cells.[109],[110] Consistent with these observations, we have revealed that Th17 cell differentiation is severely impaired in *AhR* KO Th0 mice, despite normal expression of RORα and RORγ, which are thought to be key players in Th17 cell differentiation.[110] FICZ treatment exacerbates EAE symptoms in wild-type mice compared to *AhR* KO or untreated wild-type mice.[109] We have suggested that Th17 cell differentiation is stimulated by activated AhR through interactions with Stat1 and Stat5, which negatively regulate Th17 cell development.[110] Although the molecular mechanisms by which AhR regulates the helper T cell differentiation process are still unclear, these results indicate that AhR is centrally involved in regulating immune responses by modulating the T_{reg}/Th17 balance through its ligand in specific cytokine milieus. In addition, these data suggest that AhR is a unique target for therapeutic manipulation of immune responses.

Conclusions

The molecular mechanisms of the transcriptional activity of AhR have been elucidated in more detail, with particular emphasis on coactivators, mediators and chromatin remodeling factors. Furthermore, there is increasing evidence that AhR can be activated in a ligand-independent manner, although this mechanism needs to be further studied. The AhR signaling pathway can be precisely regulated by a feedback inhibitory mechanism in which AhRR, whose expression is enhanced by activated AhR, represses the transcription activity of AhR via SUMOylation and the subsequent recruitment of corepressors. In addition to the role of AhR in toxicology and pharmacology, recent evidence indicates that AhR is also involved in reproduction and innate immunity. One of the major contributions of AhR is to protect against invading agents ranging from various small molecule chemicals to microorganisms. With the novel finding that AhR is involved in ubiquitylation, our knowledge on the function of AhR further extends into tumor suppression and the regulation of steroid hormones.

Acknowledgements

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Profile

Yoshiaki Fujii (-Kuriyama) was born in 1938 and after graduating from Faculty of Science and the Master Course of the University of Tokyo, he jointed Sankyo Pharmaceutical Co., Central Research Laboratories in 1965. He received the degree of Doctor of Science from Osaka University in 1970 and moved to Kansai Medical University as lecturer in 1975. Then, he jointed Cancer Institute, Japan Foundation for Cancer Research as associate member in 1977 and subsequently as member. From studies on the xenobiotic metabolism and metabolic activation of chemical carcinogens, he was the first to elucidate the primary structure of drug-metabolizing P450 and paved the way to molecular biological studies on P450 which shows a extremely broad distribution in living organisms from bacteria to plants and animals by clarifying the primary structures of other drug-metabolizing P450s and steroidogenic P450s, and their structure/function relationship. The P450 super-family is now known to consist of more than 6000 molecular species in nature which are involved in vast varieties of functions ranging from drug metabolism, steroidogenesis, synthesis of many lipophilic bioactive compounds including prostaglandins and gibberellin to flower coloring. He was promoted to Professor of Tohoku University in 1987 and continued to work until his retirement as Professor Emeritus in 2002. From his work on the induction mechanisms of drug-metabolizing P450, he identified AhR as a transcription factor working in response to PAH and was the first to solve the primary structure of AhR, leading to elucidation of the induction mechanisms of P450 and its involvement in teratogenesis and carcinogenesis caused by dioxins. With his recent finding that AhR acts as an E3 ubiqutin ligase, he demonstrated that AhR functions as a multiregulatory factor in drug metabolism, innate immunity and colonic tumor suppression. After serving as Guest Professor in University of Tsukuba from 2002 to 2009, he is Guest Professor in Medical Research Institute, Tokyo Medical and Dental University and Visiting Scientist in Institute of Molecular Cellular Biosciences, The University of Tokyo. He was awarded the Princess Takamatsu Cancer Prize in 2006.