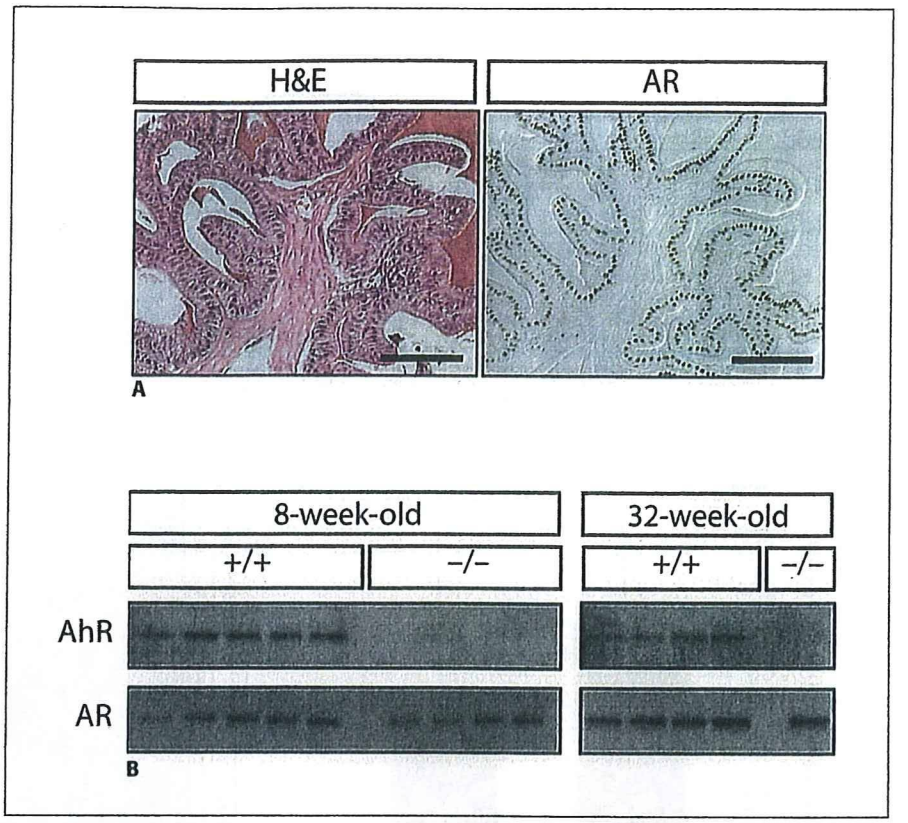

Fig. 2. Unaffected expression of androgen receptor in seminal vesicle of *AhR*(−/−). **A** Expression of AR in the seminal vesicle. Cryosections (10 μm thick) were prepared from seminal vesicles of 10-week-old *AhR*(+/+) and stained with hematoxylin and eosin (H&E) or anti-AR antibody (AR). Scale bars = 200 μm. **B** Expression of AR in seminal vesicles of 8- and 32-week-old *AhR*(+/+) and *AhR*(−/−) males. Whole tissue extracts (10 μg) prepared from seminal vesicles were subjected to Western blot analyses with antibodies for AhR and AR. Five 8-week-old *AhR*(+/+) seminal vesicles, four 8-week-old *AhR*(−/−) seminal vesicles, four 32-week-old *AhR*(+/+) seminal vesicles, and one 32-week-old *AhR*(−/−) seminal vesicle were used.

Since spermatogenesis is one of the physiological events in the testis and is regulated by testosterone, we also determined whether sperm production is affected in the *AhR*(−/−) testes. We counted epididymal sperm numbers in *AhR*(+/+) and (−/−) mice (fig. 3C). The number was decreased in the *AhR*(−/−) mice to approximately two thirds of the *AhR*(+/+), suggesting that low concentrations of serum testosterone affect spermatogenesis in the *AhR*(−/−) testes. To investigate the process of spermatogenesis, serial sections of the *AhR*(+/+) and *AhR*(−/−) testes were prepared. Morphologically, a substantial number of elongated spermatids were differentiated in the *AhR*(−/−) testes as well as *AhR*(+/+) testes (fig. 3D). Moreover, histological examination of the caudae epididymidis revealed the presence of abundant sperm cells even in the *AhR*(−/−) males. No evidence of any histological abnormality that would cause the reduced number of the sperm was observed in the *AhR*(−/−) testes.

Low Expression of 3βHsd in AhR(−/−) *Testes*

Testosterone is synthesized in testicular Leydig cells, and therefore coincident AhR expression was determined in Leydig cells. Consistent with previous reports [Schultz et al., 2003], anti-AhR immunoreactivity was detected in Leydig cells (fig. 4A). The low serum testosterone concentration suggested the potential involvement of AhR in the development and/or function of Leydig cells. Therefore, we performed immunohistochemical staining with antibody for 3βHsd, a Leydig cell marker [Dupont et al., 1990]. As shown in figure 4A, Leydig cells were present in the testes of both genotypes. We then performed fluorescent immunohistochemical examination followed by cell counting to determine if the number of Leydig cells is decreased in the *AhR*(−/−) testes (fig. 4B). There was no significant difference in Leydig cell number between the testes of *AhR*(+/+) and *AhR*(−/−) (fig. 4C), suggesting that the low level of serum testosterone is not due to a decrease in Leydig cell number but rather reduced ability to produce testosterone. To investigate the possibility of suppression of steroidogenic function of Leydig cells, we compared the expression of 3βHsd in *AhR*(+/+) and *AhR*(−/−) testes by Western blot analyses. Comparable expression of 3βHsd was observed in 10-week-old *AhR*(+/+) and *AhR*(−/−) mice, but the expression was less in 24-, 32-, and 52-week-old *AhR*(−/−) testes compared to age-matched *AhR*(+/+) mice (fig. 5A).

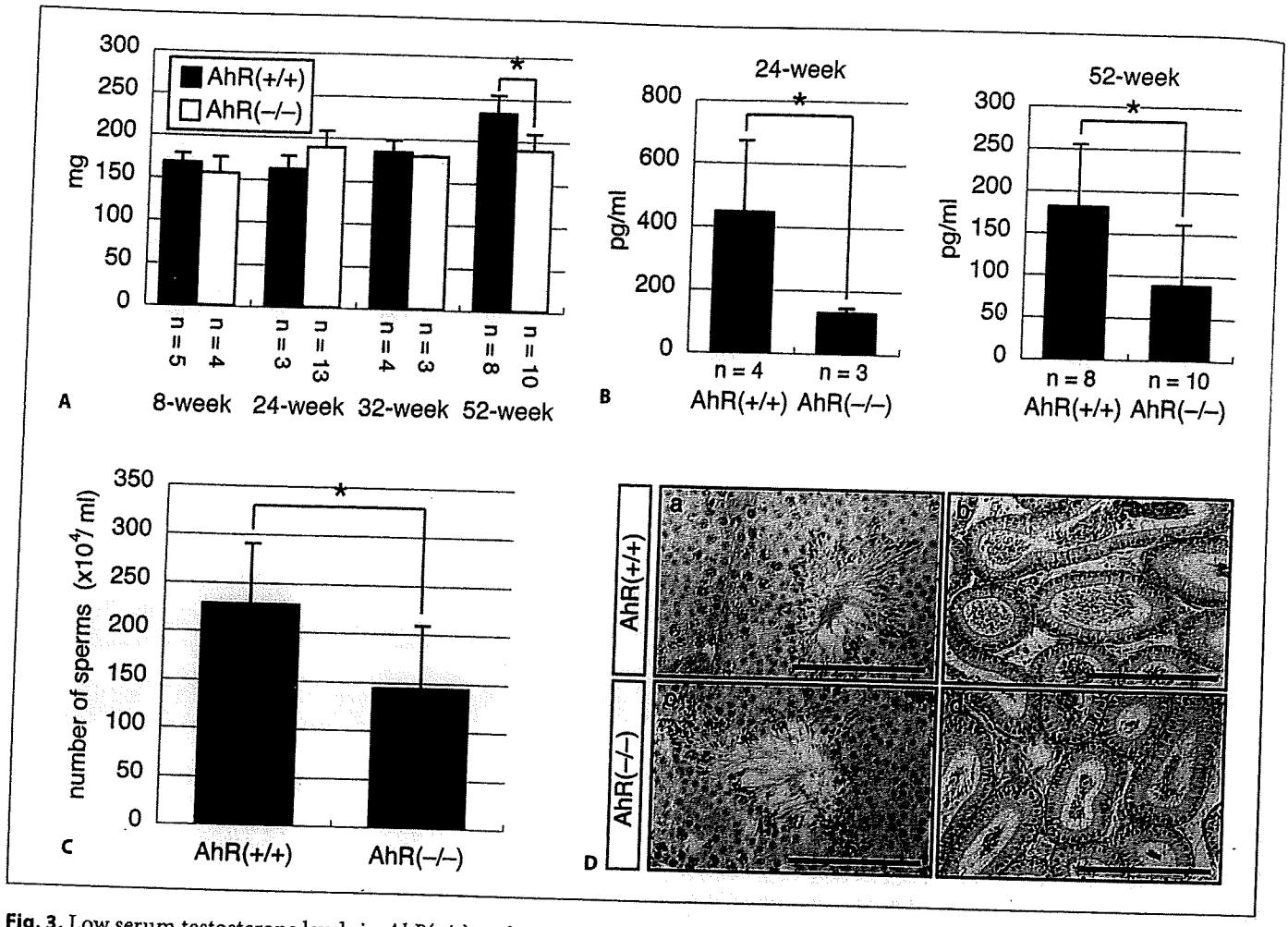

Fig. 3. Low serum testosterone levels in *AhR*(–/–) males. **A** Comparison of testicular weight between *AhR*(+/+) and *AhR*(–/–) mice. The testes isolated from 8-, 24-, 32-, and 52-week-old *AhR*(+/+) and *AhR*(–/–) mice were weighed. Numbers of mice examined are indicated. Values are mean ± SD, * $p < 0.005$. **B** Serum testosterone concentrations in 24- and 52-week-old *AhR*(+/+) and *AhR*(–/–) mice determined by enzymatic immunoassay. Numbers of mice examined are indicated. Values are mean ± SD, * $p < 0.05$. **C** Comparison of epididymal sperm number between 52-week-old *AhR*(+/+) and *AhR*(–/–). Sperm cells were recovered from eight *AhR*(+/+) and ten *AhR*(–/–) 52-week-old mice. * $p < 0.025$. **D** Histological analysis of the testes (**a**, **c**) and caudae epididymidis (**b**, **d**) of *AhR*(+/+) and *AhR*(–/–) mice. Five-micrometer paraffin-embedded sections of the testes and caudae epididymidis from 20-week-old *AhR*(+/+) and *AhR*(–/–) mice were stained with hematoxylin and eosin. Scale bars in **a** and **c** = 100 μm, **b** and **d** = 200 μm.

Our previous study demonstrated that AhR regulates *Cyp19* (aromatase P450) gene expression in the ovary [Baba et al., 2005]. Since aromatase P450 is capable of converting testosterone to estradiol, increased expression of the enzyme would lead to decrease in serum testosterone and thus Cyp19 expression was examined in 24-week-old mice. However, there was no discernible difference in Cyp19 expression between the *AhR*(+/+) and *AhR*(–/–) testes (fig. 5B). Moreover, the expression of StAR (steroidogenic acute regulatory protein), Insl3 (insulin like-3), and P450scc (side chain cleavage) necessary for the endocrine function of Leydig cells was examined by quantitative RT-PCR. As shown in figure 5C, the expression of the *StAR* gene was decreased as well as *3βHsd* in the *AhR*(–/–) testes. In contrast, the expression of *Insl3* and *P450scc* in the *AhR*(–/–) testes was comparable to *AhR*(+/+), indicating that not all the steroidogenic genes are regulated by AhR. Further, we investigated the expression of Ad4BP/SF-1, which is known to regulate the expression of all steroidogenic genes; however, the expression of Ad4BP/SF-1 protein was not affected in the *AhR*(–/–) testes (fig. 5D).

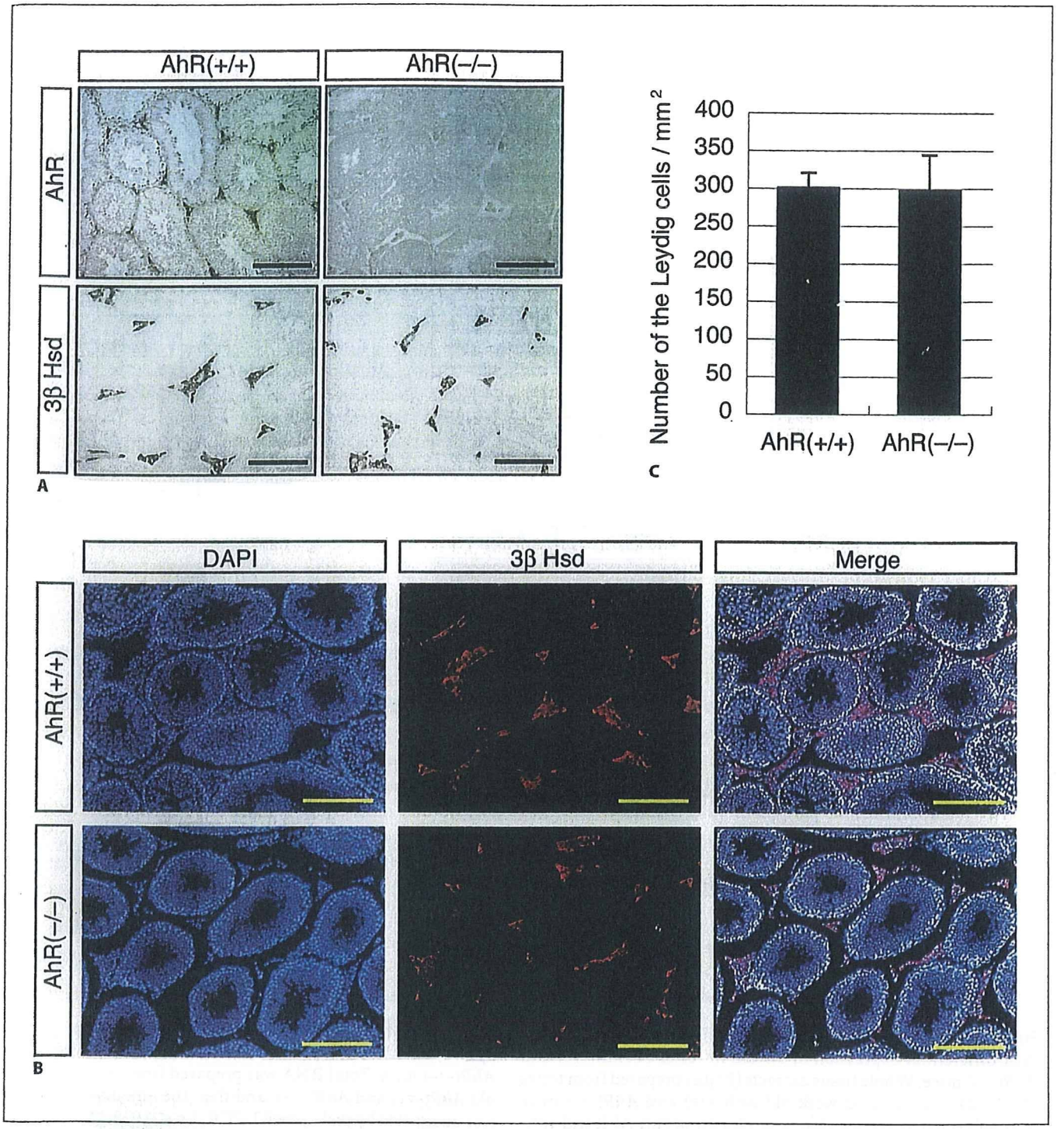

Fig. 4. No difference in number of testicular Leydig cells between *AhR*(+/+) and *AhR*(–/–) mice. **A** Expression of AhR and 3βHsd in Leydig cells. Five-micrometer paraffin sections were prepared from the testes of 24-week-old *AhR*(+/+) and *AhR*(–/–) mice and subjected to immunohistochemical analyses using anti-AhR and anti-3βHsd antibodies. Scale bars = 200 μm. **B** Immunohistochemical staining of testes of 52-week-old *AhR*(+/+) and *AhR*(–/–) mice using anti-3βHsd antibody (red). Nuclei were stained with DAPI (blue). Sections were prepared from eight *AhR*(+/+) and ten *AhR*(–/–) mice. Representative results are shown. Scale bars = 200 μm. **C** Comparison of Leydig cell number between *AhR*(+/+) and *AhR*(–/–). Numbers of 3βHsd-positive cells in testes of eight *AhR*(+/+) and ten *AhR*(–/–). 3βHsd-positive cells were counted in four sections for each animal. Values are average numbers of Leydig cells ± SD per mm².

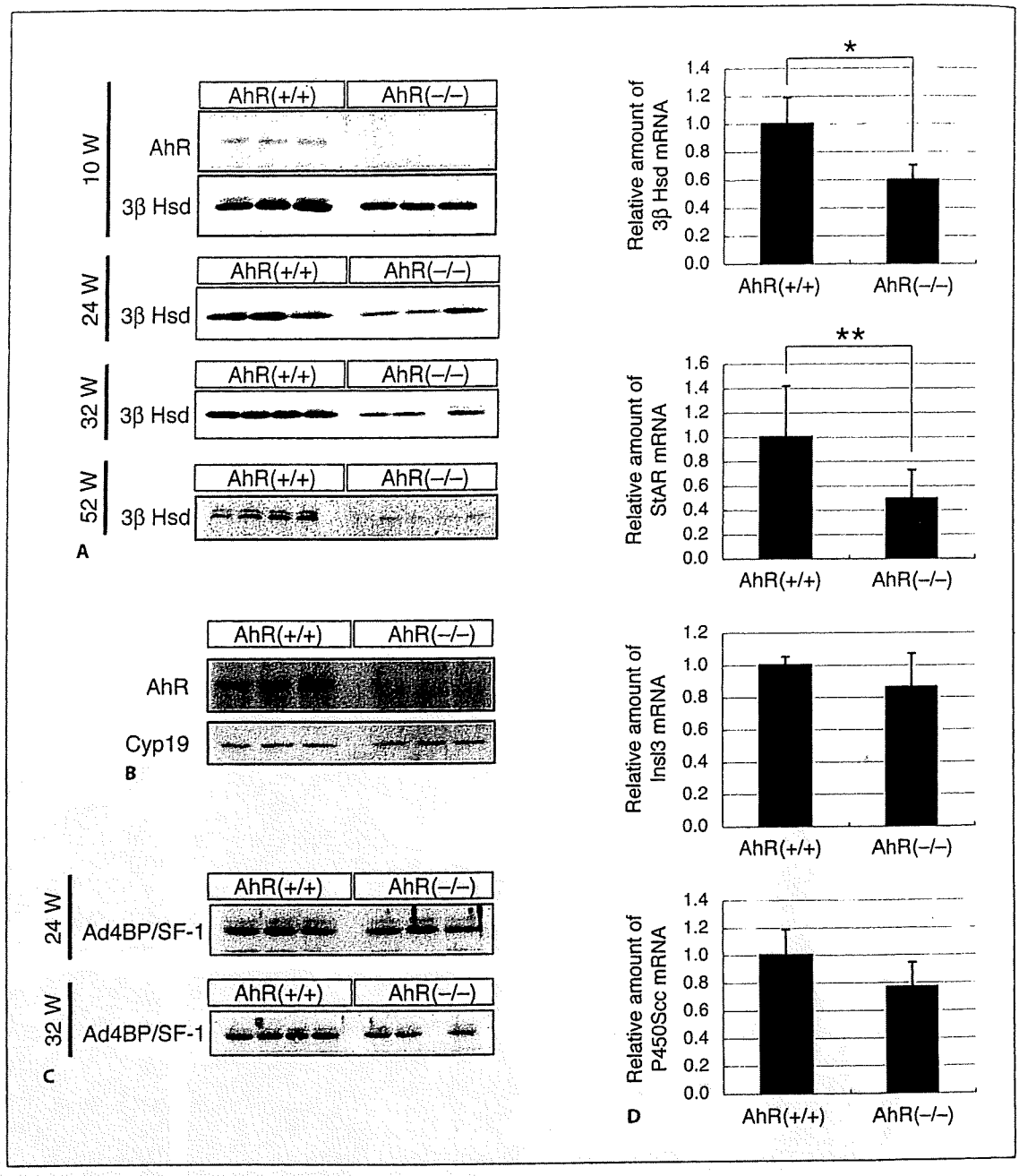

Fig. 5. Low expression of 3βHsd in *AhR*(−/−) testes. **A** Age-dependent differential expression of 3βHsd in testes of *AhR*(+/+) and *AhR*(−/−) mice. Whole tissue extracts (10 μg) prepared from testes of 10-, 24-, 32-, and 52-week-old *AhR*(+/+) and *AhR*(−/−) mice were subjected to Western blot analyses using anti-AhR and anti-3βHsd antibodies. Three or four males were used for each blotting. **B** Expression of Cyp19 in testes of *AhR*(+/+) and *AhR*(−/−) mice. Whole tissue extracts (10 μg) prepared from testes of 24-week-old *AhR*(+/+) and *AhR*(−/−) mice were subjected to Western blot analyses using anti-AhR and anti-Cyp19 antibodies. Three *AhR*(+/+) and three *AhR*(−/−) males were used. **C** Expression of 3βHsd, StAR, Insl3, and P450scc mRNA in testes of *AhR*(+/+) and *AhR*(−/−) mice. Total RNA was prepared from testes of 52-week-old *AhR*(+/+) and *AhR*(−/−), and then the amount of the mRNA was quantified by real-time RT-PCR, * $p < 0.025$, ** $p < 0.1$. **D** Expression of Ad4BP/SF-1 in testes of *AhR*(+/+) and *AhR*(−/−) mice. Whole tissue extracts (10 μg) prepared from testes of 24- and 32-week-old *AhR*(+/+) and *AhR*(−/−) mice were subjected to Western blot analyses using anti-Ad4BP/SF-1 antibodies. Three *AhR*(+/+) and three *AhR*(−/−) males were used.

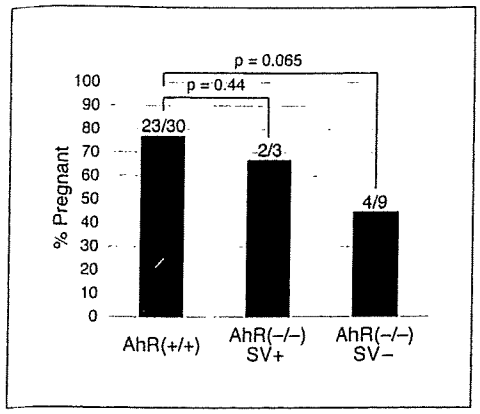

Fig. 6. Low fertility of *AhR*(–/–) males lacking seminal vesicles. Thirty *AhR*(+/+) and three *AhR*(–/–) mice with seminal vesicles, and nine *AhR*(–/–) mice lacking seminal vesicles were mated with *AhR*(+/+) females. Data represent the percentages of successful pregnancies. Numbers on bars represent the number of pregnant females per total number of female mice.

Reduced Fertility of AhR(–/–) *Males*

Lastly, we examined how the reproductive activity is affected in *AhR*(–/–) males. In order to determine reproductive activity, 21- to 33-week-old *AhR*(+/+) and *AhR*(–/–) males were mated with wild-type females. Before mating, the *AhR*(–/–) males were surgically examined to determine whether they still possess the seminal vesicles or not. Three of these males still had their seminal vesicles while nine of them did not. These two groups, together with wild-type males, were then subjected to mating experiments. *AhR*(–/–) males lacking any seminal vesicles showed less reproductive activity than *AhR*(+/+) males and *AhR*(–/–) males harboring the seminal vesicle (fig. 6). The reproductive activity of *AhR*(–/–) males harboring seminal vesicles was not statistically different from that of *AhR*(+/+) males (fig. 6).

During this experiment, the presence of seminal vaginal plugs was checked every morning, and frequently these plugs showed abnormal characteristics with the female mice mated with the *AhR*(–/–) males. Small amounts of white-colored and non-fixed plugs were observed in females mated with males lacking the seminal vesicles (data not shown). Since the vaginal plug is considered to be critical for successful pregnancy, the rate of pregnancy was compared between females with normal and those with abnormal plugs. As expected, successful pregnancies were counted in 4 of the 5 females with normal plugs, while 5 of the 6 females with the abnormal plug had unsuccessful pregnancies.

Discussion

Through the analyses of *AhR*(–/–) males, we demonstrated a novel function for AhR: maintenance of the seminal vesicle. Although the weight of the seminal vesicle was reported previously to be decreased by *AhR* gene disruption [Lin et al., 2002], we showed for the first time a complete regression of the seminal vesicle in *AhR*(–/–) males. At the same time, we noticed that the regression occurs preferentially in aged adult animals. Since the previous study only examined mice that were younger than 90 days old [Lin et al., 2002], it seems perhaps unlikely to encounter any mice lacking the seminal vesicle at that young age. In order to explain the mechanism underlying tissue regression, it was important to examine if apoptosis is increased while cell proliferation is decreased during and just prior to the regression. However, since this regression is considered to occur randomly among individuals, we could not find seminal vesicles in which regression was apparently in progress, suggesting that the process of regression proceeds in a very short period. Because of this regression feature, we neither can predict precisely when the regression starts in each animal, nor determine whether this regression is caused by increased apoptosis or decreased cell proliferation.

Developmentally, the seminal vesicle is derived from the caudal region of the Wolffian duct as a male sex-accessory gland. Likewise, the coagulating gland is derived from the same duct, and thereafter it is fused to the posterior margin of the seminal vesicle. Therefore, the coagulating gland may disappear simultaneously with the seminal vesicle in *AhR*(–/–) males. Functionally, the coagulating gland secretes a substance required for the formation of a vaginal plug to guarantee efficient pregnancies. In this study, we observed a decrease in successful reproductive activity and abnormal vaginal plugs when *AhR*(–/–) males were used in the mating. Therefore, we assumed that the abnormal vaginal plug formation caused by the disappearance of the seminal vesicle together with coagulating gland is a possible reason accounting for the decreased reproductive activity of *AhR*(–/–) males. In addition to vaginal plug formation, sperm number is another factor to guarantee efficient pregnancies. Therefore, we counted the number of epididymal sperms and found that it was reduced in *AhR*(–/–). Although a definite relationship between fertility and sperm number has yet to be determined, this observation raises another possibility: the low sperm count explains the decreased reproductive activity of *AhR*(–/–) males.

The implication of AR in seminal vesicle development was elucidated by *Ar*-knockout mice in which the seminal vesicles failed to develop from the fetal stage [De Gendt et al., 2004]. In addition, castration at adulthood led to regression of the seminal vesicle while administration of dihydrotestosterone rescued such castration-induced regression [Neubauer et al., 1981]. Moreover, administration of androgen antagonists decreased the weight of the seminal vesicle [Vinggaard et al., 2002]. These observations indicated that androgen signaling is indispensable for the maintenance of the adult seminal vesicle as well as for the development of fetal tissue. Considering the importance of androgen signaling in the development and maintenance of the seminal vesicle, we reasoned two possibilities for tissue regression; one is the low expression of AR in the seminal vesicle while the other is reduced testosterone production in testicular Leydig cells. Since AhR is expressed in both seminal vesicles and Leydig cells, any disruption of the *AhR* gene would potentially affect both or either of them. Eventually, examination of the two possibilities strongly suggested that the decreased expression of 3βHsd and StAR in testicular Leydig cells leads to a concomitant decrease in serum testosterone and thus the regression of the seminal vesicle in the *AhR*(–/–) male. Androgen is known to mediate a variety of male functions, and spermatogenesis is one representative event. In fact, sperm production was affected in *AhR*(–/–) males. This differential tissue effect in the decrease of testosterone is possibly due to the sensitivity to testosterone concentration. In fact, administration of androgen antagonist demonstrated that the seminal vesicle is the most sensitive tissue among the male reproductive accessory tissues [Vinggaard et al., 2002].

The expression of AhR in the seminal vesicle implies specific functions of this receptor in the tissue. Although AhR does not regulate AR expression, AhR possibly regulates genes essential for the proliferation of seminal vesicle epithelial cells. In fact, an AhR-defective variant of mouse hepatoma Hepa 1c1c7 cells exhibited a prolonged doubling time caused by G_1 cell-cycle arrest [Ma and Whitlock, 1996]. Embryonic fibroblasts prepared from *AhR*(–/–) tissue grow slower because of accumulation of cells in G_2/M-phase due to an altered expression of G_2/M kinases Cdc2 and Plk [Elizondo et al., 2000]. These observations suggest that AhR promotes cell proliferation. However, opposing functions of AhR in cell cycle regulation have also been demonstrated. For example, in rat 5L hepatoma cells, G_1 arrest was induced by TCDD, an AhR ligand, which resulted in overexpression of CDK2 inhibitor, $P27^{kip1}$ [Kolluri et al., 1999; Sherr and Roberts, 1999]. It was also revealed that AhR forms a protein-protein complex with RB [Ge and Elferink, 1998; Puga et al., 2000; Elferink et al., 2001]. Taken together, it has been well-established that AhR regulates cell proliferation. Thus, in addition to the non-cell-autonomous effects observed in response to decreased testosterone production, the seminal vesicle is potentially regressed cell-autonomously through an abnormal cell cycle regulation in *AhR*(–/–) cells.

Our study demonstrated that the seminal vesicle regressed in the aged *AhR*(–/–) males and that this regression is likely caused by a decrease in testosterone production. In fact, low expression levels of 3βHsd and StAR were found in the *AhR*(–/–) testes. Likewise, our previous study [Baba et al., 2005] demonstrated that AhR activates aromatase *P450* (*Cyp19*) gene transcription in the steroidogenic granulosa cells when the ovaries are at preovulatory phase in the estrous cycle [Lynch et al., 1993]. However, no alteration of Cyp19 expression was observed in the *AhR*(–/–) testes. These results clearly demonstrated that AhR is involved in sex steroid synthesis in both sexes although the affected sites in the steroidogenic process are different between males and females. The mechanism for the sex differences in AhR action remains to be resolved, however, these observations strongly suggested that, similar to the female reproductive activity, AhR has a critical function in the male reproduction as well.

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Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings

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ABSTRACT

The arylhydrocarbon receptor (AhR) mediates sex steroid hormone-related actions in both normal physiology and in dioxin toxicity. In addition to regulation of direct target genes, the ligand-activated AhR associates with estrogen or androgen receptors (ERα or AR) to regulate transcription as a functional unit. Given that endogenous and exogenous AhR-ligands are structurally diverse, it is unclear whether cross-talk regulation of ERα/AR by the activated AhR is an intrinsic function of the AhR or the result of ligand-type-selective differences. To ensure uniform activity of the AhR irrespective of ligand-type-specific differences, we employed CA-AhR, which lacks the ligand-binding domain and has a constitutive activity. We found that CA-AhR, in the absence of a ligand, acted as a transcriptional co-regulator for the unliganded ERα/AR as well as for mutants of ERα/AR lacking a ligand-binding domain. CA-AhR was recruited to estrogen-/androgen-responsive promoters with endogenous ERα/AR. Moreover, CA-AhR had an E3 ubiquitin ligase activity and promoted proteasomal degradation of ERα/AR. Thus, these findings indicate that the cross-talk function of the AhR with sex hormone receptors is an intrinsic function of the AhR.

The arylhydrocarbon receptor (AhR) is a member of the basic helix–loop–helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors. The AhR mediates the toxic effects of dioxins such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) [1–3], in part by modulating estrogen and androgen signaling [4,5]. AhR-deficient mice exhibit both abnormal estrous cycles and defective ovarian follicle maturation in females [6], and developmental defects in the prostate of males [7]. This implies a sex hormone-related innate function of the AhR.

The transcriptional activity of the AhR is primarily regulated by ligand-dependent translocation to the nucleus [3]. Unliganded AhR is sequestered in the cytosol by interacting with the Hsp90-XAP2 (also called AIP or ARA9) chaperon complex through the PAS-B region [1–3]. Ligand binding to the PAS-B region of the AhR induces dissociation of the Hsp90-XAP2 and subsequent translocation of the AhR to the nucleus[1–3]. The AhR then dimerizes with Arnt, recognizes the xenobiotic responsive element (XRE), and recruits co-activators [3]. The AhR induces expression of direct target genes such as the drug metabolizing enzymes CYP1A1 and CYP1A2 [1–3]. Disruption of inhibitory PAS-B function by ligand binding is therefore expected to be sufficient to induce transcriptional activity of the AhR [8]. In fact, a mutant AhR that lacks a PAS-B region (CA-AhR) is constitutively active and exhibits transcriptional activity irrespective of lack of ligand-binding capacity [9].

AhR exhibits other regulatory functions by modulating the function of other transcription factors, including Rb/E2F [10], NF-κB [11], and the estrogen (ERα and ERβ) [12–16] and androgen (AR) [16] receptors. These cross-talk pathways are important components that mediate the functions of endogenous and exogenous AhR-ligands. As for the estrogen-related adverse effects of dioxins, ligand-activated AhR/Arnt associates with ERα and ERβ through the N-terminal A/B region within the ERs [12–16]. By means of this association, the liganded AhR potentiates the transactivation function of 17β-estradiol (E_2)-unbound ERα and represses E_2-bound ERα-mediated transcription upon the estrogen-responsive element (ERE) [12]. Reciprocally, E_2-bound ERα associates with XRE-bound AhR to either potentiate [13] or repress [14] AhR-mediated transcription. Reflecting this functional cross-talk, Arnt also acts as a co-regulator for ERα [17]. In addition, it has been recently shown that the liganded AhR promotes ubiquitination and proteasomal degradation of ERα and the AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [16]. Thus, complexes of the AhR with ERs or

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AR appear to regulate transcription as functional units by multiple mechanisms.

Several diverse types of AhR-ligands have been reported to date, including endogenous [tryptamine, indole-3-acetic acid, and indirubin], exogeneous [TCDD, and benzo(a)pyrene], and synthetic [3-methylcholanthrene (3MC), and β-naphthoflavone (βNF)] ligands [18]. Since these compounds are structurally diverse, and the physiological ligand(s) for the AhR has not been defined, it is unclear whether modulation of ERα and AR function by the liganded AhR may be attributed to an intrinsic function of the AhR.

To ensure uniform activity of the AhR in our experiments, irrespective of ligand-type-specific differences, we employed CA-AhR which lacks binding capacities for the Hsp90-XAP2 as well as for ligands [8]. CA-AhR has constitutive transcriptional activity in the absence of ligands [8], and CA-AhR transgenic mice exhibit dioxin-exposure-related phenotypes in various tissue such as the liver and stomach [9]. These characteristics render this mutant particularly suitable for studying the intrinsic function of the AhR irrespective of ligand-type-specific differences. In this study, we demonstrated that CA-AhR acts as a transcriptional co-regulator for the unliganded ERα/AR, even in the absence of AhR-ligands. Moreover, CA-AhR promoted proteasomal degradation of both ERα and the AR. Therefore, these findings indicate that cross-talk with the sex hormone receptors is an intrinsic function of the AhR that is not subject to ligand-type-selective differences.

Materials and methods

Plasmids. Expression plasmids for the AhR, Arnt, constitutively active AhR, ERα, ERαΔD/E/F, AR, and ARΔE/F were previously described [12]. Luciferase reporter plasmids [estrogen-responsive element (ERE)-Luciferase, androgen-responsive element (ARE)-Luciferase, and xenobiotics-responsive element (XRE)-Luciferase] were described previously [12].

Cells, transfection, and luciferase assay. Human breast cancer-derived MCF-7 cells, human endometrial cancer-derived Ishikawa cells, human prostate cancer-derived LNCaP cells, and human renal cancer-derived 293T cells were cultured as previously described [16]. Cells were cultured in phenol-red-free DMEM containing 0.2% charcoal-stripped FBS and transfected with the receptor expression vectors and the luciferase reporter plasmids [16]. Cells were then treated with E_2 (10 nM), DHT (10 nM), 3-methylcholanthrene (3MC) (1 μM), β-naphthoflavone (β-NF) (1 μM), or MG132 (10 μM), for 24 h (Luciferase assays) or for 3 h (Western blotting).

For the Luciferase assays [19], cells at 40–50% confluence were transfected with the indicated plasmids [0.25 μg reporter plasmids, 0.025 μg ERα, 0.1 μg ERαΔD/E/F, AhR/Arnt (+, 0.05 μg; ++, 0.1 μg; +++, 0.2 μg), 0.1 μg AR, 0.1 μg ARΔE/F] using Lipofectamine reagent (Gibco–BRL). Luciferase activity was determined with the Luciferase assay system (Promega). As a reference plasmid to normalize transfection efficiency, 2.5 ng pRL-CMV plasmid (Promega) was co-transfected. All values represent averages ±SD of at least three independent experiments.

In vitro ubiquitination assay. The *in vitro* ubiquitination assay was performed as previously described, with several modifications [16]. The CA-AhR immunocomplex was purified using anti-FLAG antibody from MCF-7 cells transfected with FLAG-HA-CA-AhR together with HA-DDB1 and myc-TBL3. The immunocomplex was incubated with recombinant ubiquitin and reaction buffer [50 mM Tris–HCl (pH 7.4), 5 mM MgCl, 2 mM NaF, 2 mM ATP and ATP-regenerating system, 0.6 mM DTT, and 12 μg ubiquitin (Calbiochem), 60 ng E1(Calbiochem), 0.3 μg E2 mixture set (Calbiochem)]. The self-ubiquitination of CA-AhR was detected by Western blotting using an anti-HA antibody.

ChIP assays. ChIP assays were performed essentially as previously described, with several modifications [12]. MCF-7 or LNCaP cells were transfected with 5 μg FLAG-CA-AhR in a 100 mm dish. After 36 h, the cells were treated with a transcription inhibitor α-amanitin (2.5 μM) for 2 h, released by a washing twice with PBS for 10 min and medium change. After the medium change, the cells were cross-linked at the indicated time (min).

More detailed methods are supplied as Supplementary information.

Results and discussion

CA-AhR activates the transcriptional function of unliganded ERα and AR

First, we tested if CA-AhR was indeed constitutively active in a xenobiotic-responsive element (XRE)-driven reporter assay in endometrial tumor-derived Ishikawa cells. CA-AhR activated XRE-mediated transcription in the absence of AhR-ligand as efficiently as 3MC-bound wild-type AhR (Supplementary Fig. 1).

Under this experimental condition, the effects of CA-AhR on ERα- and AR-mediated transcription were examined with the reporter assays. A reporter plasmid containing either a consensus estrogen-responsive element (ERE) or a consensus androgen-responsive element (ARE) was co-transfected with different amounts of either wild-type AhR or CA-AhR into Ishikawa cells (for ERE assays) or kidney-derived 293T cells (for ARE assays). When the wild-type AhR was transfected, transfection-dosage-dependent activation of the ERE- or ARE-Luciferase activity in the presence of, but not in the absence of, AhR-ligands [3MC and βNF], was observed (Fig. 1A and B). In that reporter assay, we found that CA-AhR significantly activated the ERE- or ARE-Luciferase activity in a transfection dosage-dependent manner in the absence of AhR-ligands (Fig. 1A and B). The activation function of CA-AhR for ERα-/AR-mediated transcription was comparable with that of the ligand-bound wild-type AhR, as expression levels of these AhR derivatives were not significantly different (Fig. 1C). We obtained similar results on the observed CA-AhR modulation of ERα function in mammary tumor-derived MCF-7 cells (data not shown).

CA-AhR activates ERα and AR mutants which lack C-terminal ligand-binding domains

To further demonstrate direct modulation of ERα-/AR-mediated transcription by the AhR, we employed ERα and AR mutants which lack C-terminal ligand-binding domains (LBDs; also known as E/F regions) [12]. Nuclear receptors exhibit two activation functions (AFs), AF-1 and AF-2 [20]. The AF-1 transactivation function in the A/B domain is ligand-independent, while the AF-2 function in the LBD is induced in a ligand-dependent manner through conformational change [20]. The LBD-deficient ERα and AR derivatives [ERαΔD/E/F and ARΔE/F] have DNA-binding activity but retain only AF-1 function [12].

As shown in lane 2 of Fig. 2A and B, transfected ERαΔD/E/F and AR ΔE/F mutants exhibited AF-1 activity, which was unaffected by the presence of E_2 or DHT, respectively (Fig. 2A and B, lane 12). Nonetheless, CA-AhR activated ERαΔD/E/F- and AR ΔE/F-mediated transcription in a transfection dosage-dependent manner in the absence of ERαAR ligands (lanes 9–11). The wild-type AhR, however, activated ERαΔD/E/F- and AR ΔE/F-mediated transcription only in the presence of 3MC (lanes 6–8). Importantly, since neither CA-AhR nor the LBD mutant of ERαAR has a functional ligand-binding domain, it is highly unlikely that the modulation of ERαΔD/E/F and AR ΔE/F by CA-AhR can be attributed to overlapping ligand-responsibility.

CA-AhR is recruited to estrogen- and androgen-responsive promoters in the absence of ligands

To explore the function of CA-AhR in the endogenous chromatin context, we tested whether CA-AhR was functional in endogenous estrogen-/androgen-responsive promoters by a chromatin immunoprecipitation (ChIP) assay. For this purpose, we used MCF-7 cells and LNCaP cells. Endogenous ERα and AR functionally bind to estrogen-responsive *c-fos* promoters in MCF-7 cells and androgen-responsive *PSA* promoters in LNCaP cells, respectively [12,21]. The wild-type AhR, upon ligand treatment, is recruited to the XRE-containing promoters at 30–60 min, and dissociates from the promoters after 60 min. The ligand-activated AhR is recruited to and dissociates from estrogen-responsive promoters together with ERα on a similar time course [12]. In order to synchronize the transcriptional cycle of CA-AhR in the absence of ligands, we

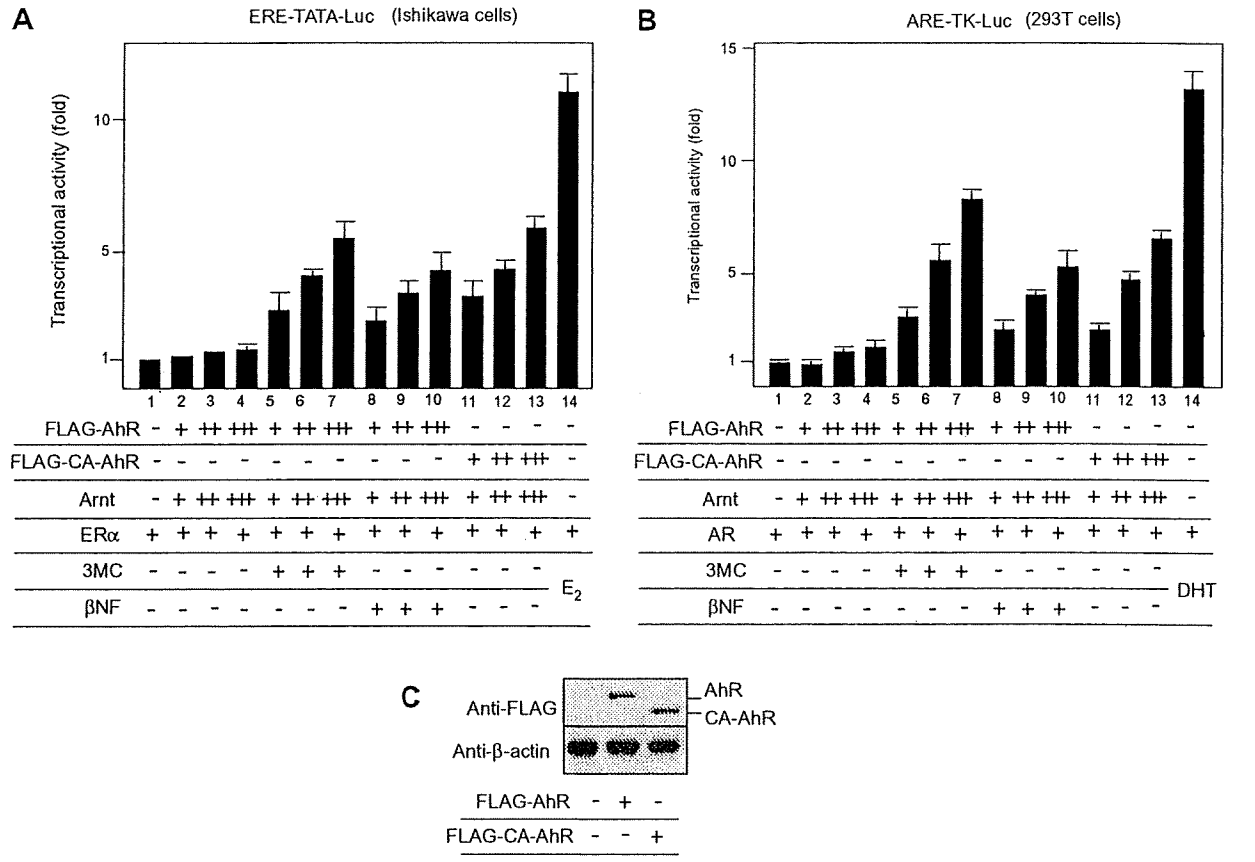

Fig. 1. The constitutively active AhR activates transcription through the unliganded ERα and AR in the absence of AhR-ligands. Ishikawa cells (A) or 293T cells (B) were transfected with the reporter plasmid bearing ERE (A) or ARE (B) together with the indicated expression plasmids (AhR/Arnt; +, 0.05 μg; ++, 0.1 μg; +++, 0.2 μg), in the presence or absence of the indicated ligands (3MC, 1 μM; βNF, 1 μM), and a Luciferase assay performed. Data are means ± SD of three independent experiments. (C) 293T cells were transfected with the indicated expression vectors (0.5 μg for FLAG-AhR and FLAG-CA-AhR), and the lysates subjected to Western blotting.

used the α-amanitin-release method, which is widely used in similar time-course ChIP experiments [22]. The MCF-7 cells and LNCaP cells transfected with CA-AhR were treated with α-amanitin, a transcription inhibitor, for two hours, and were then released by washing and a medium change.

Upon release from α-amanitin, CA-AhR was recruited to the *c-fos* promoter at 30–60 min in MCF-7 cells. Interestingly, endogenous ERα was co-recruited with CA-AhR to the *c-fos* promoter following a similar time-course in CA-AhR-transfected cells. No significant recruitment of ERα was observed 60 min after α-amanitin release in non-transfected cells (Fig. 3A). Similarly, CA-AhR was recruited to the *PSA* promoter at 60 min in LNCaP cells, and transfection of CA-AhR induced co-recruitment of endogenous AR on the *PSA* promoter on a similar time-course as CA-AhR (Fig. 3B). These results suggest that CA-AhR associates with endogenous ERα or AR on estrogen- or androgen-responsive promoters, respectively, as a transcriptional co-regulator.

CA-AhR has ubiquitin ligase activity and promotes proteasomal degradation of ERα in the absence of ligands

Finally, we tested if CA-AhR acted as an E3 ubiquitin ligase in the absence of a ligand. The ligand-activated AhR assembles a $CUL4B^{AhR}$ complex consisting of CUL4B/DDB1/Rbx1/TBL3/AhR/Arnt [16]. It recognizes ERα and the AR and promotes their ubiquitin–proteasome-mediated degradation [16]. Therefore, in this study the ubiquitin ligase activity of CA-AhR was verified. The CA-AhR immunocomplex, which included the components of $CUL4B^{AhR}$, was prepared, and the *in vitro* ubiquitination assay performed. The CA-AhR complex has an E3 ubiquitin ligase activity that is E1/E2-enzyme-dependent *in vitro*, as revealed by its self-ubiquitination activity (Fig. 4A).

The effects of CA-AhR on the degradation of ERα and the AR were then examined. ERα was co-transfected with either the wild-type AhR or CA-AhR into MCF-7 cells. After incubation with ligands and/or a proteasomal inhibitor MG132 for 6 h, cell lysates were prepared and the protein levels of ERα measured with Western blotting. To avoid detection of endogenous ERα protein in the non-transfected cells, we transfected FLAG-tagged ERα and detected ERα with a FLAG antibody. As shown in Fig. 4B, CA-AhR promoted degradation of ERα in the absence of the AhR-ligand in a transfection dosage-dependent manner (Fig. 4B, lanes 4 and 5). In contrast, wild-type AhR promoted degradation of ERα only in the presence of the AhR-ligand (lanes 2 and 3). A proteasomal inhibitor MG132 abolished the promotion of degradation, confirming a ubiquitin–proteasome pathway mediated mechanism (lane 6). Proteasomal degradation of the wild-type AhR itself was promoted by the AhR-ligand (lanes 2 and 3), consistent with previous reports [1–3].

Consistently, when the AR was co-transfected with either the wild-type AhR or CA-AhR into 293T cells, we found that CA-AhR promoted proteasomal degradation of the AR in the absence of AhR-ligand (Fig. 4C). The wild-type AhR promoted degradation of the AR only in the presence of a ligand (data not shown). These re-

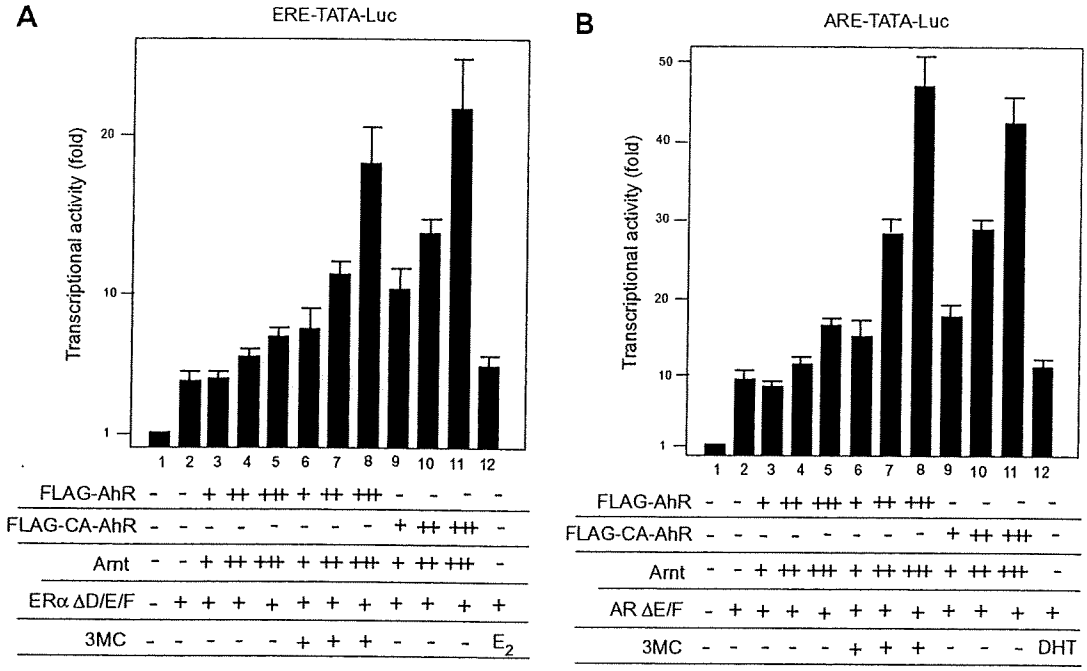

Fig. 2. CA-AhR activates mutants of ERα and AR which lack ligand-binding domains. Ishikawa cells (A) or 293T cells (B) were transfected with the indicated plasmids, and the Luciferase assay was performed as in Fig. 1. Data are means ± SD of three independent experiments. The data show that CA-AhR modulates the transcriptional activity of ERαΔD/E/F and AR ΔE/F in the absence of ligands.

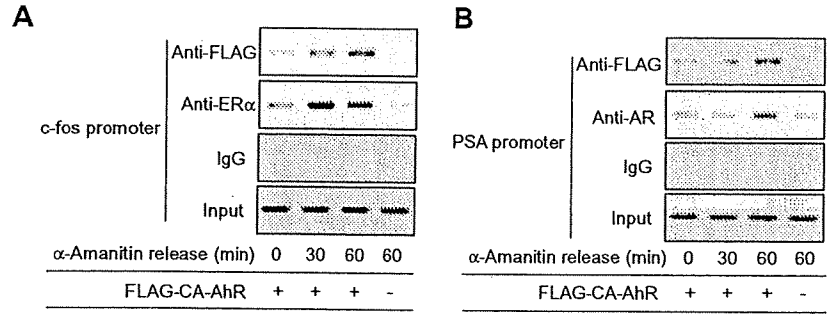

Fig. 3. Promoter occupancy of CA-AhR together with ERα or the AR in the absence of AhR-ligands. MCF-7 cells (A) or LNCaP cells (B), in which endogenous ERα (A) or the AR (B) was functional, were transfected with FLAG-tagged AhR or CA-AhR as indicated. The cells were treated with 10 μM α-amanitin for 2 h, and then subjected to the ChIP assay at the indicated time (min) after release from α-amanitin by a medium change. Samples were immunoprecipitated with the indicated antibodies, and promoter DNAs were amplified by PCR as indicated.

sults suggest that the activated AhR directly modulates stability of ERα and AR proteins by its ubiquitin ligase activity, irrespective of the ligand.

AhR as a transcriptional co-regulator for ERα/AR

Accumulating evidence suggests some of the actions of the AhR are mediated through cross-talk pathways with other transcription factors, including Rb [10], NF-κB [11,23], and nuclear receptors [5,12–16]. We and other groups have previously reported that the ligand-activated AhR directly associates with ERα and the AR to regulate transcription [12–16]. Moreover, AhR-dependent degradation of ERα has been independently reported [15,16]. To provide additional evidence for these AhR-ERα/AR cross-talk pathways, we have shown that CA-AhR, which lacks the ligand-binding domain and has a constitutive activity [8,9], modulates the functions of ERα and the AR in the absence of AhR-ligands. This suggests that activation of the AhR, either by ligand binding or by deletion of an inhibitory domain, directly induces a regulatory AhR-ERα/AR cross-talk pathway. Moreover, we have shown that the ubiquitin ligase activity of CA-AhR is also intact in the absence of a ligand. These results suggest that modulation of ERα and the AR is an intrinsic function of the AhR.

Importantly, we have shown that the mutants of ERα and AR which lack the C-terminal ligand-binding domains are also functionally regulated by CA-AhR. This directly excludes the possibility of cross-binding of AhR-ligands to ERα and the AR. Consistent with this, it was recently demonstrated that the AhR-ligand 3MC does not directly bind to ERα [24]. Together, these results indicate that the active form of the AhR, but not the ligand itself, is required for AhR-ERα/AR cross-talk. Thus, the present data support the existence of a 'direct cross-talk' pathway in which the AhR directly associates with ERα/AR and regulates their function as a transcriptional co-regulator. In the nuclear receptor superfamily of tran-

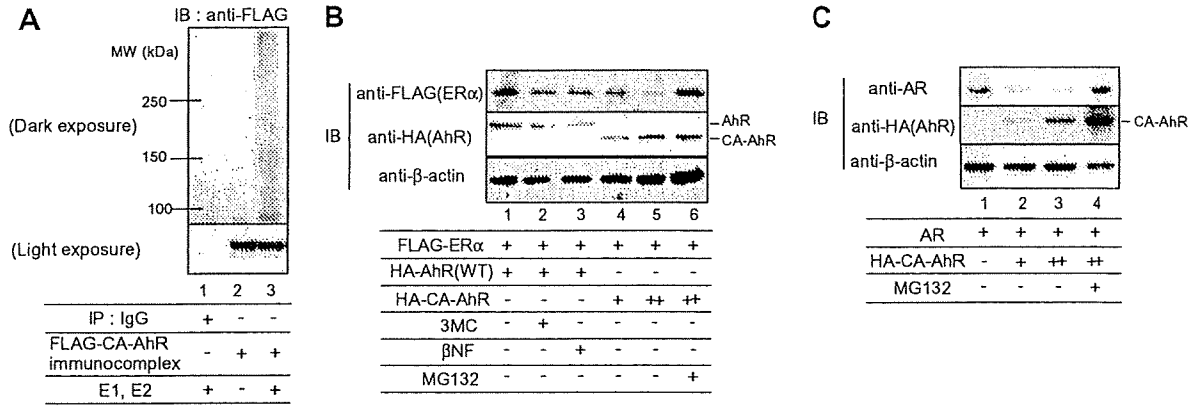

Fig. 4. CA-AhR promotes proteasomal degradation of ERα and the AR. (A) A FLAG-CA-AhR immunocomplex and IgG immunocomplex, prepared from MCF-7 cells, were subjected to the *in vitro* ubiquitination assay. The self-ubiquitination of CA-AhR was detected by Western blotting as indicated. (B,C) MCF-7 cells (B) or 293T cells (C) were transfected with the indicated plasmids [0.25 μg FLAG-ERα, 0.25 μg AR, 0.5 μg HA-AhR, HA-CA-AhR (+, 0.5 μg; ++, 1.0 μg) in 6-well dish]. Twenty-four hours after transfection, the cells were incubated with the indicated ligands or vehicle, then lysed and subjected to Western blotting with the indicated antibodies.

scription factors, ligand-type-selective differences in receptor conformational change affect the interaction with specific co-regulators [21,25]. Given that AhR-ligands are structurally diverse, ligand-specific differences in the modulation of AhR conformation may result in differential interaction with ERα/AR, CUL4B, and co-activators such as p300. This may lead to differential regulation of cross-talk pathways.

In the cross-talk pathways, AhR, like other co-regulators [26], both positively and negatively regulates other transcription factors. For instance, AhR associates with Rb/E2F1 and cooperatively regulates transcription, both positively [27,28] and negatively [10]. Similarly, AhR and NF-κB pathways converge in either a cooperative [11] or inhibitory [23] manner, depending on the cellular conditions. Our presented data indicate that the AhR assembles both a transcriptional co-regulator complex and a ubiquitin ligase complex. These complexes may explain the bi-phasic functions of the AhR-ligand in the regulation of other transcription factors, and are likely related to the physiological function of the AhR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.054.

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Inducibility of cytochrome P450 1A1 and chemical carcinogenesis by benzo[*a*]pyrene in AhR repressor-deficient mice

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Abstract

AhR repressor (AhRR) is an AhR-related bHLH-PAS transcription factor. It is known to repress AhR transcription activity in a competitive manner. To examine AhRR functions in mice, we produced AhRR-deficient mice by gene knockout. *AhRR(−/−)* mice were born in normal Mendelian proportions, grew well, and were fertile. *AhR(−/−)* mice exhibited higher levels of *Cyp1a1* (Cytochrome P450 1A1) mRNA induction in the skin, stomach and spleen than wild-type mice, while expression of *Cyp1a1* mRNA was not significantly altered in the liver, lung, heart or other tissues, suggesting that "super-induction" of *Cyp1a1* mRNA expression in *AhRR(−/−)* mice occurs in a tissue specific manner. *AhRR(−/−)* mice displayed a delayed response to skin carcinogenesis caused by benzo[*a*]pyrene. Since CYP1A1 is involved in the metabolic activation and detoxification of chemical carcinogens, these results suggest that overexpression of CYP1A1 shifts the balance of the metabolic activities in the skin of *AhRR(−/−)* mice in favor of the detoxification of carcinogens.

Keywords: AhR receptor; Gene targeting; Chemical carcinogenesis; CYP1A1; AhR; Transcription; Metabolic activation; Polyaromatic hydrocarbon; Super-induction; Transcription repression

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the bHLH (basic helix-loop-helix)-PAS (Per-Arnt-Sim homology) superfamily [1–3]. Normally, AhR exists in the cytoplasm in association with the HSP90 complex. Upon binding with its ligands, such as 3MC (3-methylcholanthrene) and TCDD (2′,3′,7′,8′-tetrachlorodibenzo-*p*-dioxin), AhR translocates to the nucleus, where it heterodimerizes with Arnt (AhR nuclear translocator, another member of the bHLH-PAS superfamily) to induce the expression of a battery of drug-metabolizing enzymes including CYP1A1, 1B1 and 1A2 [1–3]. In addition, recently, the target genes of AhR have been expanded to those involved in cell cycle regulation, apoptosis, endocrine regulation and the immune system [4,5]. Among them, AhRR is unique, because it represses the transcriptional activity of AhR and thus forms a negative feedback regulatory loop in the xenobiotic signal tranduction pathway [6,7]. AhRR (AhR repressor) which was originally identified in mice, has also been reported in many animal species including human [8], rat [9] and fish [10]. In cell culture, AhRR inhibits AhR transcription activity by competing with AhR for heterodimer formation with Arnt; the AhRR/Arnt heterodimer then competes with AhR/Arnt heterodimer for binding to xeno-

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biotic response element (XRE) sequences [6]. Little is known, however, about the functional role of AhRR in the AhR signaling pathway in living animals.

To investigate the functional roles of AhRR in the AhR signaling system *in vivo*, we generated *AhRR(−/−)* mice by homologous recombination. *AhRR(−/−)* mice were born in normal Mendelian propotions, grew well, and were fertile. We found that *AhRR(−/−)* mice were relatively resistant to skin carcinogenesis induced by benzo[*a*]pyrene (B[*a*]P), compared with the wild type (WT). Skin fibroblast cells derived from *AhRR(−/−)* mice showed a remarkably higher level of *Cyp1a1* mRNA induction in response to B[*a*]P than WT counterparts. This "super-induction" of *Cyp1a1* mRNA was not observed in all the tissues examined of *AhRR(−/−)* mice, indicating that AhRR works as repressor of AhR only in specific tissues.

Materials and methods

Generation of AhRR-deficient mice. We disrupted the AhRR gene in mouse embryonic stem cells as described [11]. A targeting vector was constructed by replacing a part of the 2nd exon and the 2nd intron of the *AhRR* gene with the *NLS-LacZ-neo*^{r} gene cassette as shown in Fig. 1A. The HSV-TK gene was used for negative selection. The linearized targeting vector was electroporated into E14 ES cells, and the cells were subjected to double selection with G418 (0.3 mg/ml) and gancyclovir (2 μM). Double-resistant ES clones were then screened by PCR using a pair of oligonucleotide primers corresponding to the neomycin resistance gene (TV-neo; 5′-TCA GAG CAG CCG ATT GTC TGT TGT GCC CAG TCA T-3′) and *AhRR* gene (AhRR TV-PCR2-2; 5′-AGA CCT GAG AGG TCT AGA CTT GGA TGC TAC-3′) depicted in Fig. 1A as arrowheads. To confirm the homologous recombination, ES clone genomic DNA was digested with PstI or BamHI restriction enzymes for DNA blot analysis using 5′ or 3′ external probes. Positive ES clones were injected into blastocoel cavities of 3.5-day postcoitum (dpc) blastocysts derived from C57BL/6 mice. The injected blastocysts were surgically transplanted into the uteri of pseudo-pregnant ICR recipient mice at 2.5 dpc. Germ-line transmission of the *AhRR* defective allele was screened by PCR to obtain two independently targeted founder mice, and heterozygous F1 mice were intercrossed to obtain *AhRR(−/−)* mice. Tail DNAs of the pups were extracted and subjected to PCR for the presence of the mutated *AhRR* allele using the TV-neo and AhRR TV-PCR2-2 primers. To distinguish easily the mutated *AhRR* alleles from WT by PCR, the following oligonucleotides were used as PCR primers: AhRR KO-5′ (5′-GAA ACT GTA GCC CTG GAT ACT TCT G-3′), AhRR KO-3′ (5′-ATC ATT GCT CTG AGC ATC CAC TAG G-3′) and TV neo primer. The AhRR KO-5′ and 3′ primer pair amplifies only the *AhRR* wild-type allele (190 bp), while that of the AhRR KO-3′ and TV neo primers amplifies only the mutated one (527 bp).

PCR-RFLP analysis. Because the established *AhRR* mutant mice contain both C57BL/6 and 129Sv *AhR* alleles, PCR-restriction fragment length polymorphism (RFLP) analysis was performed to exclude the 129Sv *AhR* allele, as described [8,12]. Briefly, tail genomic DNAs were amplified by PCR with a primers OL72 (5′-GGT TCG AAT TTC CAG GAT GG-3′) and OL111 (5′-CCA CCC CAG GTA CAT GAT GGA ACC-3′). PCR fragments were digested with *Eco47*III restriction enzyme and electrophoresed on an 8.0% acrylamide gel. The C57BL/6 *AhR* allele yields 142 and 76 bp fragments, while the 129Sv *AhR* allele yields a 218 bp fragment. Mice homozygous for the C57BL/6 *AhR* allele were used for further analyses.

Chemical treatment and tumor induction. B[*a*]P and 3MC were obtained from Wako Junyaku Co. (Osaka). To analyze *Cyp1a1* induction in mouse tissues, corn oil (vehicle control) or 3MC dissolved in corn oil (4 mg/ml) was intraperitoneally injected into mice (80 mg/kg body weight), and the mice were sacrificed 24 or 48 h after injection. Tissues were collected from the mice and subjected to RNA extraction for RT-PCR analysis, as described [6]. For tumor induction experiments, *AhRR(−/−)* mice were backcrossed with wild-type C57BL/6 mice for at least 7 generations, and subcutaneously injected with 0.2 ml of B[*a*]P in corn oil (10 mg/ml) twice, a week apart, as described [13]. All mice of 8 weeks of age were examined for development of tumors at least once a week for 30 weeks until death. The tumor sizes were recorded throughout the experimental period. Tumor-bearing mice were counted and presented as percentage of the total. Tumors were dissected, fixed in formalin and embedded in paraffin. Sections at 3-μm thickness were stained with hematoxylin and eosin as described previously [14].

Skin fibroblast cell culture preparation. WT and *AhRR(−/−)* skin fibroblast cultures were prepared from the skin of at least six neonatal mice, respectively. Skin was removed from newborn mice, and then minced into small pieces, followed by digestion with 1% collagenase (SIGMA) in DMEM for 1 h at 37 °C. The digests were then rinsed once with PBS, then maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37 °C in 5% CO_2 until skin fibroblast cells covered the entire culture dish plate. The cells were replated at 2.0×10^6 cells per 10 cm diameter dish for further experiments and passage.

Cell treatments and RT-PCR. Skin fibroblast cell cultures were incubated in the absence (DMSO) or presence of 1 μM B[*a*]P (DMSO solution) as described in figure legends. Total RNA was extracted from the cells with TRIsure RNA extraction reagent and reverse-transcribed into cDNA by using SuperScript II RTase. Quantitative gene expression analysis was

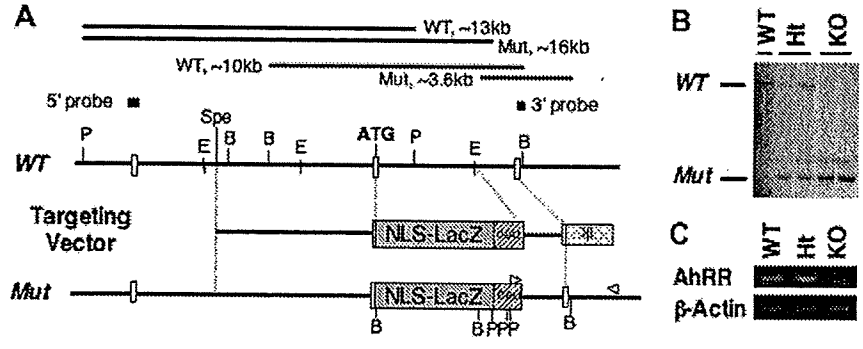

Fig. 1. Targeted disruption of the mouse *AhRR* gene. (A) Schematic representation of the targeting vector, *AhRR*-WT and Mut alleles. Cleavage sites for the restriction enzymes are indicated by E (EcoRI), B (BamHI), Sp (SpeI) and P (PstI). The locations of the 5′ and 3′ probes used for the DNA blot analysis are indicated at the top. Two arrowheads indicate the position of primers used to identify homologous recombinant clones. (B) DNA blot of mouse genome using the 3′ probe. Genomic DNA (10 μg) was digested with BamHI; digested products were then electrophoresed and hybridized. (C) Mice were treated with 3MC for 24 h, and then total RNA from spleen was subjected to RT-PCR analysis.

performed by using Platinum SYBRGreen qPCR premix in an ABI7300 qPCR analyzer. PCR primers for CYP1A1: 5′-GGACATTTGAGAA GGGCCAC-3′ and 5′-CGTCCAGCTTCCTGTCCTGA-3′; for CYP1B1: 5′-GGATGTGCCTGCCACTATTAC-3′, 5′-CCTGAACATCCGGGTA TCTG-3′; for AhRR; 5′-CCTGTCCCGGGATCAAAGATG-3′ and 5′-CTCACCACCAGAGCGAAGCCATTGA-3′; for β-actin: 5′-GTGAAA AGATGACCCAGATCATG-3′ and 5′-GTGGTACGACCAGAGGCA TAC-3′.

β-Galactosidase staining. Tissues were fixed overnight in PBS containing 4% paraformaldehyde, dehydrated with ethanol, embedded in paraffin and sectioned at 3-μm thickness. The sections were dewaxed and stained. β-galactosidase staining was carried out as described [15].

Results

Targeted disruption of mouse AhRR gene

To investigate the functional role of AhRR *in vivo*, we generated an *AhRR* knockout mouse by gene targeting technology as described [15]. The *NLS-LacZ* sequence was fused in a reading frame with the 8th amino acid of the *AhRR* gene so that the inserted *NLS-LacZ* gene could mimic the mode of *AhRR* gene expression (Fig. 1A). Since the resulting protein product lacks most of the coding region of the bHLH domain, which is essential for dimerization and DNA binding, we expected that the knockout mice would lack AhRR function. E14 embryonic stem (ES) cells were electroporated with the linearized targeting vector and subjected to positive-negative selection. Of the 360 clones screened by PCR, nine clones had undergone homologous recombination at the *AhRR* locus, as subsequently confirmed by DNA blot (Fig. 1B). The mutant clones were proliferated and microinjected into C57BL/6 recipient blastocysts to generate chimeric mice, and the male chimeras were crossed with C57BL/6 females. Ultimately, two independent mutant ES cells were successfully transmitted to offspring.

Generation of homozygous AhRR mutant mice

AhRR(+/−) mutant mice were viable and fertile, and were intercrossed for analysis of the phenotypes of *AhRR(−/−)* homozygosity. Offspring of all three genotypes were born at a normal Mendelian proportion in both mixed and C57BL/6 backgrounds (Table 1).

To assess complete inactivation of the gene, the absence of *AhRR* mRNA was confirmed by RT-PCR. Intraperitoneal injection of 3MC induced *AhRR* mRNA expression in the heart of wild-type and *AhRR(+/−)* mice, whereas *AhRR* mRNA was not found in the *AhRR(−/−)* (Fig. 1C). Taken together with the results of the DNA blots (Fig. 1B), these results confirmed the specific disruption of the *AhRR* gene.

Table 1
Genotypes of offspring obtained by double heterozygous mating

Background[a]	WT	Ht	KO	*n*
Mix	63 (20.3%)	166 (53.4%)	82 (26.4%)	311
B6	30 (30.6%)	49 (50.0%)	19 (19.4%)	98

[a] Mating was performed with heterozygous mice in C57BL/6 and 129SV background (Mix) or mice backcrossed to C57BL/6 background 7 generations (B6).

The general behaviors including feeding, growth and mating of AhRR*(−/−)* mice were apparently normal, and the mutants lived a normal lifespan (data not shown). Gross anatomy did not reveal any anomaly in AhR*(−/−)* mice.

Gene expression in AhRR(−/−) mice

To investigate the repressor function of AhRR *in vivo*, 3MC was intraperitoneally injected into *AhRR(−/−)* and WT mice. After 24 h of treatment, expression levels of *Cyp1a1* mRNA, one of the well-known AhR target gene products, were measured in various tissues along with the levels of *AhRR* mRNA (Fig. 2A). As previously observed [6], in WT mice, *AhRR* mRNA was highly induced by 3MC in heart, lung, and spleen, and weakly in liver, kidney, thymus intestine, brain and stomach. On the other hand, *Cyp1a1* mRNA was highly induced in lung, liver and heart in WT mice, while this high induction was not much affected in the same tissues of *AhRR(−/−)* mice. In spleen and stomach, induction level of *Cyp1a1* mRNA was higher in *AhRR(−/−)* mice than in WT. The higher induction of *Cyp1a1* mRNA in *AhRR(−/−)* than in WT mice was not observed in all the tissues examined, i.e., induction was tissue-dependent.

We performed a time-course study of *Cyp1a1* mRNA expression in the 3MC-injected mouse spleen (Fig. 2B). In WT mice, *Cyp1a1* mRNA was gradually increased and reached a plateau at 48 h after the 3MC injection. In contrast, in *AhRR(−/−)* mice, *Cyp1a1* mRNA continued to increase to a higher level than in WT mice throughout the course of the experiment. These results support the idea that AhRR represses AhR activity in WT mice.

In addition to spleen and stomach, 3MC treatment also induced the expression of *Cyp1a1* mRNA in the skin of *AhRR(−/−)* mice to a higher level than in WT skin (Fig. 2C).

Because both *AhR* [12] and *AhRR* disrupted genes were inserted in a reading frame of the respective genes with NLS-*LacZ*, we were able to examine the expression patterns of AhR and AhRR in the skin by β-galactosidase staining. AhRR expression was restricted to the dermal fibroblasts (Fig. 3A and C), while AhR was expressed in both fibroblasts and epidermal cells (Fig. 3B and D). To investigate the expression of AhRR and CYP1A1 in the dermal fibroblasts in detail, we isolated dermal fibroblasts from *AhRR(−/−)* and WT mice, and cultured them for treatment with B[*a*]P. *AhRR* mRNA was clearly induced in response to B[*a*]P in WT fibroblasts (Fig. 3E), whereas no expression of AhRR was observed in *AhRR(−/−)* cells. On the other hand, *AhRR(−/−)* fibroblast cells induced *Cyp1a1* mRNA in response to B[*a*]P to a level much higher

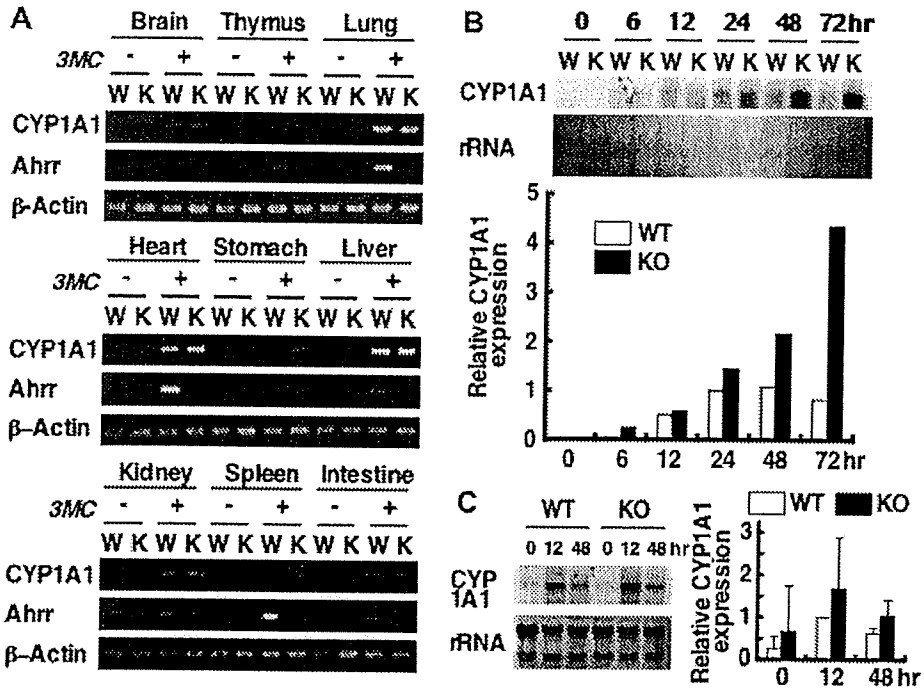

Fig. 2. Inducibility of *AhRR* and *Cyp1a1* mRNA in various tissues. (A) Expression of *Cyp1a1* and *AhRR* mRNA in various tissues of wild type (W) and *AhRR(−/−)* (K) mice. The mice were intraperitoneally injected with 3MC (80 mg/kg body weight). After 24 h, *Cyp1a1* and *AhRR* mRNA expression levels were examined by RT-PCR. Expression levels were normalized on the basis of β-actin expression. (B) A time course of *Cyp1a1* mRNA expression in spleen of wild-type (W) and *AhRR(−/−)* (K) mice after 3MC treatment. Wild-type or *AhRR(−/−)* mice were treated with 3MC; at indicated times after the treatment, RNA was extracted from spleen of the 3MC-treated mice and *Cyp1a1* mRNA expression was examined by RNA blot. *Cyp1a1* mRNA levels are presented relative to the wild-type value at 24 h. (C) Induction of *Cyp1a1* mRNA in skin of wild-type (WT) and *AhRR(−/−)* (KO) mice. 3MC was intraperitoneally injected into wild-type or *AhRR(−/−)* mice. RNA was extracted from the skin of the treated mice and used for determination of *Cyp1a1* mRNA by RNA blot. Average values of the four mice for each group are presented relative to the wild type at 12 h, with standard deviation.

than the wild type, and the induction continued to increase throughout the 24 h experiment. In contrast, wild-type cells slightly increased *Cyp1a1* mRNA but had decreased its expression by 24 h after the treatment. This "super-induction" of CYP1A1 in *AhRR(−/−)* skin fibroblast cells clearly suggest that AhRR works as a negative regulator of CYP1A1 in skin fibroblasts.

Skin carcinogenesis induced by B[a]P in AhRR KO mice

It is known that AhR mediates B[*a*]P carcinogenicity in the skin through expression of CYP1A1 [14]. We wished to investigate the carcinogenicity of B[*a*]P in the skin of *AhRR(−/−)* mice. Both *AhRR(−/−)* and WT mice were injected with B[*a*]P subcutaneously twice, a week apart, and the generation of skin carcinomas was observed thereafter (Fig. 4A). In WT mice, the first subcutaneous tumor was observed 12 weeks after the first treatment of B[*a*]P, and all the mice bore skin tumors 25 weeks after the treatment. On the other hand, the incidence of skin tumors in *AhRR(−/−)* mice was significantly delayed, ~5 weeks behind WT mice. Since CYP1A1 is known to be involved in both metabolic activation and detoxification of chemical carcinogens [16], these results suggest that overexpression of CYP1A1 shifts the balance of metabolic activity of *AhRR(−/−)* skin fibroblasts in favor of detoxification. Histological analysis of the tumors revealed that they were mostly fibrosarcomas, with a minor population of rhabdomyosarcomas and squamous cell carcinomas (data not shown), consistent with a previous report [14]. WT mice showed a slightly higher mortality than *AhRR(−/−)*, but without statistical significance (Fig. 4B).

Discussion

Previously, we reported that AhRR functions as a repressor of the AhR activity, based on transient DNA transfection experiments using cultured cell lines. AhRR represses the transactivation activity of AhR by competing with AhR for heterodimer formation with Arnt; the Arnt-AhRR heterodimer then competes for binding to XRE sequences [6]. To investigate the physiological roles of AhRR *in vivo*, we generated *AhRR(−/−)* mice by homologous gene recombination. The homozygous *AhRR(−/−)* mice were born at normal Mendelian ratios in genetic cross experiments using heterozygous AhRR mutant female and male mice. Mutants grew well and were fertile, indicating that AhRR is dispensable for mouse development and homeostasis. When given 3MC intraperitoneally as an inducer, *AhRR(−/−)* mice exhibited a higher level of CYP1A1 induction in the spleen, stomach and skin than WT mice. In contrast, CYP1A1 induction was not signifi-

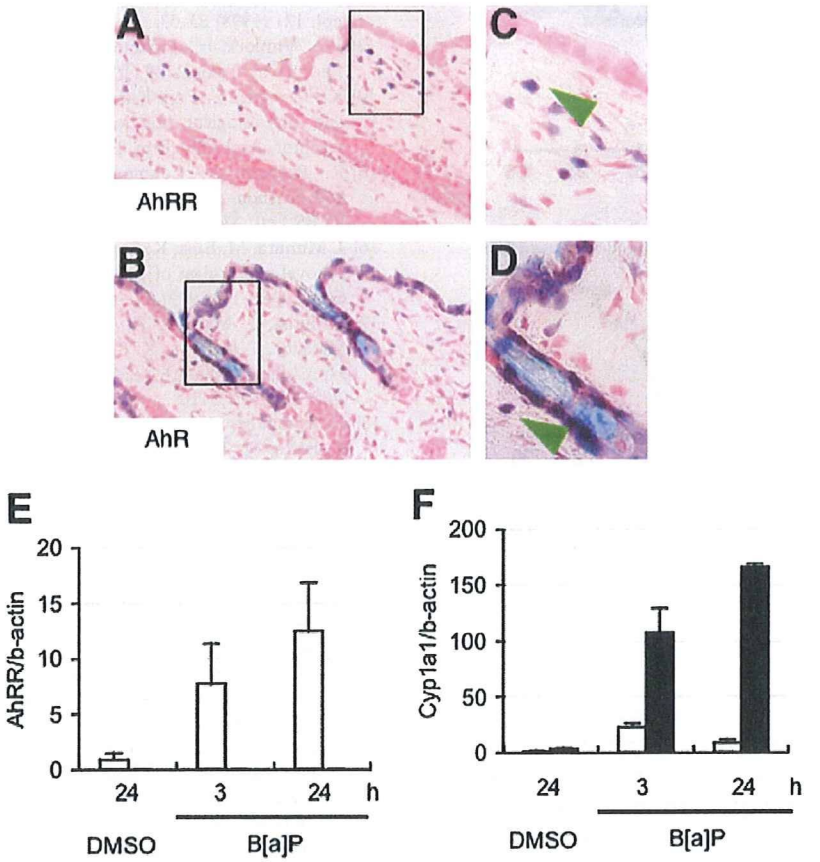

Fig. 3. Cyp1a1 and AhRR expressions in the skin and the skin fibroblast cells of wild-type and *AhRR(−/−)* mice. (A–D) AhRR and AhR expression in the skin. Skin sections prepared from *AhRR(−/−)* (A,C) or *AhR(+/−)* mice (B,D) were subjected to β-galactosidase staining for detection of AhRR or AhR expressing cells. Arrowheads indicate the blue (positive) signal in skin fibroblasts. (E) Expression of *AhRR* mRNA in the isolated skin fibroblast cells of wild-type mice after B[*a*]P treatment. Skin fibroblast cells were treated with vehicle (DMSO) or 1 mM B[*a*]P for 3 and 24 h. Total RNA was extracted from the treated cells and used for quantitation of AhRR by RT-PCR. (F) Expression of *Cyp1a1* mRNA in isolated skin fibroblast cells of wild-type and *AhRR(−/−)* mice after B[*a*]P treatment. Skin fibroblast cells were prepared from wild-type (open bars) and *AhRR(−/−)* mice (closed bars) as described above. The cultured cells were treated with B[*a*]P, and analyzed for *Cyp1a1* mRNA expression by the RT-PCR.

cantly affected in other tissues such as heart and lung, despite high inducibility of *AhRR* mRNA in these tissues of WT mice. Although the reason for this tissue-specific variation of the inducibility of CYP1A1 remains to be investigated, we speculate that protein levels of AhRR may vary from tissue to tissue, probably due either to stability of the protein or translational control. Recently, we have observed that the AhRR protein can undergo modifications, such as ubiquitination and sumoylation has been found to occur (our unpublished observations), and these alterations may be associated with the tissue-specific variation in the inducibility of *Cyp1a1* mRNA expression in *AhRR(−/−)* mice. Investigations of the detailed tissue-specific expression profiles of the AhRR protein are now underway. A study of expression of *LacZ*, which is knocked-in to the *AhR* and *AhRR* loci, revealed that AhR and AhRR are coexpressed in the skin fibroblasts under uninduced conditions. In the heterozygous *AhRR(+/−)* mice skin sections, we could not detect any ß-galactosidase staining, which was observed only in the skin of *AhRR(−/−)* mice (Fig. 3 and data not shown). This is probably because AhRR, which was expressed in *AhRR(+/−)* mice, repressed AhR activity; therefore, AhR-regulated *AhRR* expression was repressed below a detectable level in *AhRR(+/−)* mice. The lack of AhRR expression resulted in the enhancement of AhR activity, leading to the LacZ expression from the *LacZ*-knocked-in *AhRR* gene, in support of the notion that AhRR represses the AhR activity in the skin fibroblasts. These results are confirmed by the experiments using isolated skin fibroblast cells. *AhRR* mRNA was enhanced in WT cells in response to B[*a*]P, together with a slight, but significant enhanced expression of CYP1A1. On the other hand, induction of *Cyp1a1* mRNA was observed in *AhRR(−/−)* skin fibroblasts with significantly higher levels than WT.

AhR mediates carcinogenesis caused by chemical carcinogens through expression of CYP1A1 [14,17]. *AhR(−/−)* mice are resistant to chemical carcinogenesis caused by B[*a*]P [14], because they have essentially no expression of CYP1A1. In this report, *AhRR(−/−)* mice were found to be relatively resistant to chemical carcinogenesis induced by B[*a*]P, as compared with WT mice. Since CYP1A1 is

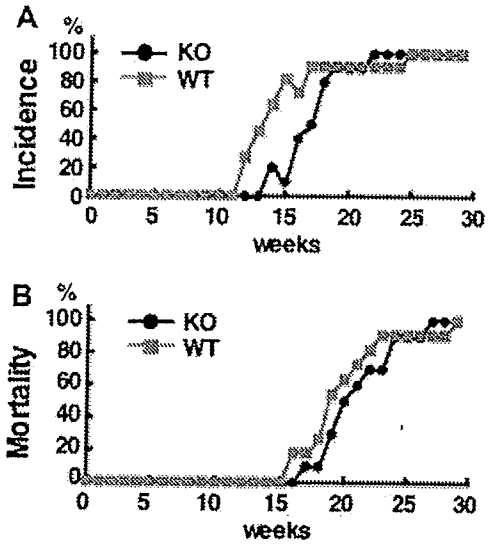

Fig. 4. B[*a*]P-induced tumor incidence and mortality of *AhRR(−/−)* mice. (A) WT (black; $n = 11$) and *AhRR*-KO (magenta; $n = 10$) mice were injected B[*a*]P subcutaneously twice. Tumor formation (A) and mortality (B) were observed as described in Materials and methods and shown as a percentage of the total.

known to be involved in both activation and detoxification of chemical carcinogens [16], "super-induction" of CYP1A1 in *AhRR(−/−)* mice is considered to shift the balance in favor of detoxification by accelerating the rate of drug metabolism to lower the carcinogenic intermediates of B[*a*]P.

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Molecular mechanism of transcriptional repression of AhR repressor involving ANKRA2, HDAC4, and HDAC5

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Abstract

The Aryl hydrocarbon receptor repressor (AhRR) has been proposed to inhibit Aryl hydrocarbon receptor (AhR) activity by competing with AhR for forming a heterodimer with AhR nuclear translocator (Arnt) and subsequently binding to the xenobiotic responsive elements (XRE). However, the precise mechanism of AhRR inhibitory activity remains unknown. Analysis of the inhibitory activity of AhRR on the expression of a TK promoter-driven reporter has localized a core repressor domain in the sequence of amino acid residue 555–701. The inhibitory activity of AhRR is sensitive to a histone deacetylase (HDAC) inhibitor, trichostatin A. By using the yeast two-hybrid screening method with the C-terminal sequence of AhRR as bait, we identified a binding partner, Ankyrin-repeat protein2 (ANKRA2), a protein known to interact with HDAC4 and HDAC5. RNA interference experiments using ANKRA2 and AhRR siRNAs indicate that ANKRA2 is important for transcriptional repression by AhRR. We have found that under normal conditions, *CYP1A1* gene is kept silent in MEF cells by AhRR/Arnt heterodimer, which binds to the XRE sequence in its promoter and recruits ANKRA2, HDAC4, and HDAC5 as co-repressors.

Keywords: Aryl hydrocarbon receptor (AhR); AhR repressor (AhRR); ANKRA2; HDAC4; HDAC5; CYP1A1; RFXANK

The Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to a superfamily with basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) structural motifs and functions as an intracellular mediator of xenobiotic signaling pathways [1]. Normally, AhR exists within the cytoplasm in association with a complex of HSP90, XAP2, and p23. Upon binding a ligand such as tetrachlorodibenzo-*p*-dioxin (TCDD), the AhR complex translocates into the nucleus and forms a heterodimer with the structurally related AhR nuclear translocator (Arnt) [1]. Thereupon, the AhR/Arnt heterodimer binds to XRE (xenobiotic responsive elements) sequences in the promoter regions of the target genes encoding drug-metabolizing enzymes, such as *CYP1A1* and *CYP1B1* to enhance their expressions [2]. The AhR signaling pathway mediates not only the adaptive response required for the detoxification of xenobiotics, but also a variety of xenobiotics-induced toxicological effects such as tumor promotion, teratogenesis, and endocrine disruption [3–7]. In addition, AhR is also known to mediate multiple physiologic processes such as female reproduction [8,9].

During the course of our study on the AhR transcription factor, we found a novel bHLH-PAS family protein with a high similarity to AhR in the N-terminal bHLH-PAS A domain. In contrast, its C-terminal region shares a minimal degree of similarity to that of AhR and lacks the obvious PAS B domain of the ligand-binding site in AhR [10]. In transient DNA transfection assays, we found

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