showed that the xenobiotic response of CYP2E1 up-regulation mediated by AhR for benzene hematotoxicity was metabolized specifically in the BM (Hirabayashi et al., 2005a). In this article, the detailed changes in blood parameters during the benzene exposure duration and their recovery three days after the cessation of exposure are shown.

2. Materials and methods

2.1. Animals

The establishment of homozygous AhR-KO ($AhR^{-/-}$) mice originating from the 129/SvJ strain has been described elsewhere (Mimura et al., 1997; Yoon et al., 2002). The crossing of heterozygous AhR-KO ($AhR^{+/-}$) males with $AhR^{+/-}$ females generated wild-type ($AhR^{+/+}$), $AhR^{+/-}$, and $AhR^{-/-}$mice. The neonates were genotyped by PCR screening of DNA from the tail. Male AhR-KO ($AhR^{-/-}$) mice and their Wt littermates (12 weeks old) were used in the study as donors. Eight-week-old C57BL/6 male mice from Japan SLC (Shizuoka, Japan) were used as recipients for the repopulation assay and the assay of colony-forming unit in the spleen (CFU-S). All the mice were housed under specific pathogen-free conditions at 24 ± 1 °C and 55 ± 10% relative humidity, under a 12-h light dark cycle. Autoclaved tap water and food pellets were provided *ad libitum*.

2.2. Benzene and benzene exposure

Benzene, CAS. No. 71-43-2, MW 78.11, was purchased from Wako Fine Chemical Company (Osaka, Japan). Experimental mice were intragastrically (*i.g.*) administered with freshly prepared corn oil solutions of benzene (150 mg/kg body weight) once daily for 5 days/week for 2 weeks). The dose used in this study was 150 mg/kg body weight which corresponds to the daily dose for leukemic induction, that is, 300 ppm for 6 h/day (Cronkite et al., 1984; Li et al., 2006). Both doses administered for 5 days/week for 26 weeks induce hematopoietic malignancies at the highest frequency. The aim of this study using this dose is to examine the corresponding toxicity of benzene for inducing hematopoietic malignancies. Note, this dose is over 100-fold higher than the occupational tolerable exposure dose.

2.3. Blood and BM parameters

Peripheral blood was collected from the orbital sinus. Peripheral blood leukocyte (WBC), red blood cell (RBC) and platelet (PLT) counts were determined using a blood cell counter (Sysmex K-4500, Sysmex Co., Kobe, Japan). BM cellularity was evaluated by harvesting BM cells from the femurs of each mouse (Yoon et al., 2001). The animals were sacrificed on days 5, 12, and 15 to evaluate recovery. Then, a 27-gauge needle was inserted into the femoral bone cavity through the proximal and distal edges of the bone shafts, and BM cells were flushed out under pressure by injecting 2 ml of α-MEM. A single-cell suspension was obtained by gently triturating the BM cells through the 27-gauge needle, and cells were counted using the Sysmex K-4500.

2.4. Irradiation

Recipient mice were exposed to a lethal radiation of 800.1 cGy, at a dose rate of 124 cGy/min, using a ^{137}Cs-gamma irradiator (Gammacell 40 Exactor, MDS Nordin Inc., Canada) with a 0.5 mm aluminum–copper filter.

2.5. CFU-S assay

The Till and McCulloch method (Till and McCulloch, 1961) was used for determining the number of colony-forming units in the spleen (CFU-S). Aliquots of BM cell suspensions were used for evaluating the number of CFU-S. The number of BM cells was adjusted to that appropriate for producing non-confluent spleen colonies, and the cells were then transplanted into lethally irradiated mice by injection through the tail vein. Spleens were harvested nine and 13 days after the injection, and fixed in Bouin's solution. Macroscopic spleen colonies were counted under an inverted microscope at a magnification of 5.6×.

2.6. Assays for CFU-GM and CFU-E

Colony formation *in vitro* was assayed in a semisolid methylcellulose culture (Yoon et al., 2001; Hirabayashi et al., 2002a). Briefly, 8×10^4 BM cells suspended in 100 μl of a medium were added to 3.9 ml of a culture medium containing 0.8% methylcellulose, 30% fetal calf serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, with 10 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF) for CFU-GM assay or 1 ng/ml murine interleukin-3 and 2 U/ml erythropoietin for erythroid CFU (CFU-E) assay. One-milliliter aliquots containing 2×10^4 BM cells were plated in triplicate in a 35-mm tissue culture plate, and incubated for six days in a completely humidified incubator at 37 °C with 5% CO_2 in air. Under an inverted microscope, CFU-GM after a six-day culture was counted at a magnification of 40× and CFU-E after a three-day culture at 100×.

2.7. BM repopulation assay

BM repopulation assay (Hirabayashi et al., 1992) was performed similarly to the assay of CFU-S, except that 1×10^6 BM cells were injected into lethally irradiated mice. One month after the transfusion of BM cells, the repopulated mice exposed to benzene.

3. Results and discussion

As previously reported, the AhR-KO mice showed a significantly higher WBC counts than the Wt mice (Fig. 1a). This was also consistent with the high number of myeloid progenitor cells, that is, CFU-S-9 and CFU-S-13, observed in the AhR-KO mice (Fig. 1b). Thus, steady-state hemopoiesis in the Wt mice, on the other hand, is presumed to be suppressed by AhR signaling because of the possible presence of a physiological ligand, which is not readily observed in the AhR-KO mice. In contrast, the numbers of mature progenitor cells in the BM, that is, CFU-E and CFU-GM, decreased (Fig. 1c). This is assumed to be due to a homeostatic negative regulation for the above-mentioned increase in the number of immature myeloid progenitor cells, although direct evidence for a possible feedback was not obtained. These lines of experimental evidences from the AhR-KO mice suggest that the numbers of mature progenitor cells, CFU-E, and CFU-GM in the Wt mice increase.

The assumption that immature progenitor cells in the Wt mice are suppressed by AhR signaling, however, is inconsistent with some reports that certain cell lines showed an enhanced proliferation in the presence of AhR signaling (Ma and Whitlock, 1996; Shimba et al., 2002). In the case of the A549 cell line, cells proliferated by AhR overexpression with the presence of ligands (Shimba et al., 2002). In the case of Hepa 1c1c7, the cell line, cell proliferation was suppressed with deceleration of cell cycling, when antisense AhR was introduced to the cell line (Ma and Whitlock, 1996). Our interpretation of these inconsistent reports is based

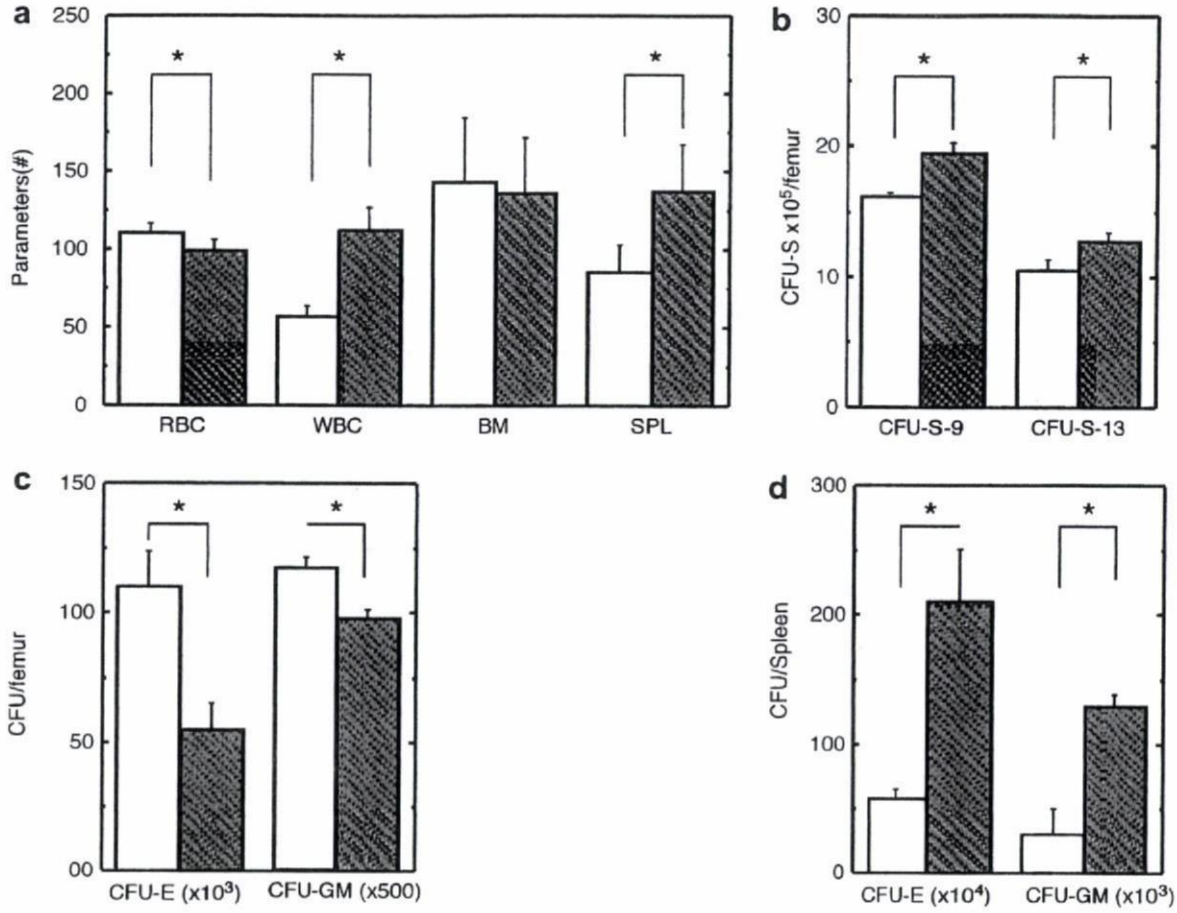

Fig. 1. Comparison of various blood parameters between Wt mice (open columns) and AhR-KO mice (shaded columns). (a) Peripheral blood, bone marrow (BM) and spleen weight. #: vertical axis "Parameters" indicate the counts of peripheral red blood cells (RBCs, $\times 10^8$/ml) and white blood cells (WBCs, $\times 10^6$/ml), BM cellularity ($\times 10^5$/femur), and weight of the spleen (SPL, mg). (b) Number of colony-forming units in spleen (CFU-S, $\times 10^5$/femur) observed on days 9 (CFU-S-9) and 13 (CFU-S-13). (c) Numbers of *in vitro* granulocyte- macrophage CFUs (CFU-GM, ×500/femur) and erythroid CFU (CFU-E, $\times 10^3$/ femur) in femoral BM. (d) Numbers of CFU-GM ($\times 10^3$/spleen) and CFU-E ($\times 10^4$/spleen) in the spleen. *: Significant difference between Wt and AhR-KO mice determined by *t*-test at $p < 0.05$.

on *in vitro* characteristics, that is, the A549 cell line was reported to show an altered p27 expression, and Hepa 1c1c7 cells were supposed to maintain their its survival by AhR signaling. Moreover, a contradictory report was also found in which artificial AhR signaling suppression induced cellular proliferation (Fong et al., 2005). The above-mentioned evidence suggests that the receptor function in the presence or absence of a ligand may differ; therefore, it is of interest to determine whether signaling from AhR in Wt mice that suppressed cell proliferation might be altered by ligand signals.

Taking together the above lines of evidence, the function of AhR signaling is presumed to be expressed solely in immature progenitor cells, which suppresses their proliferation, maintains their quiescence, and thereby, conserves the characteristic features of stem cells, that is, stemness.

Interestingly, in response to such an AhR-null effect, the AhR-KO mice contrarily showed extensive hemopoiesis in the spleen (Fig. 1d), which resulted in a significant increase in spleen weight (Fig. 1a; most right) (Hirabayashi et al., 2003, 2005a).

Because of the above-mentioned difference in BM function between the AhR-KO mice and the Wt mice, in this study, benzene-induced hematotoxicity was evaluated in the Wt mice after subjecting them to a lethal dose of whole-body irradiation followed by repopulation with BM cells that lack AhR. Six weeks after the repopulation, the steady-state hematopoietic parameters for repopulated mice were obtained and are shown in Fig. 2a and b. The results were essentially the same between the mice repopulated with Wt BM cells (open columns) and those repopulated with AhR-KO BM cells (shaded columns) except that there is no anemic tendency, leukocytotic change, nor evidence of splenomegaly in Wt mice as compared with the AhR-KO mice as shown in Fig. 1.

Fig. 3a and b shows the percentages of RBCs (a) and WBCs (b) with respect to the control in the peripheral blood after the repopulation with BM cells. In the Wt mice repopulated with Wt BM cells and those with AhR-KO BM cells (open and closed symbols, respectively), benzene exposure induced a slight but statistically significant decrease in RBC count compared with the sham-exposure except on day 5 in the Wt groups (100% with standard deviation of the mean indicated by horizontal lines: Fig. 3a). The dose used in the present study was sufficiently high, and the decrease in RBC count was readily observed within 2 weeks of exposure.

The decreases in WBC count shown in Fig. 3b are more significant than those in RBC count throughout the exposure period except on day 5 in the AhR-KO group (the data were significantly different between Wt mice ($50.8 \pm 11.2\%$) and AhR-KO mice ($70.6 \pm 17.6\%$; $p = 0.024$)). In contrast to the previous observation (data not shown), mice repopulated with AhR-KO BM cells showed no significant difference in decrease of RBCs or WBCs. This difference in the observation of AhR-KO BM cells between the previous experiment and this study may be due to the difference in the route of benzene exposure, that is, intraperitoneal (*i.p.*) and intragastric (*i.g.*), respectively. Despite BM repopulation with AhR-KO BM cells, benzene exposure by the *i.g.* route in this study may have induced portal hepatic drug metabolism at a much greater extent than that by the *i.p.* exposure route. There was no significant differ-

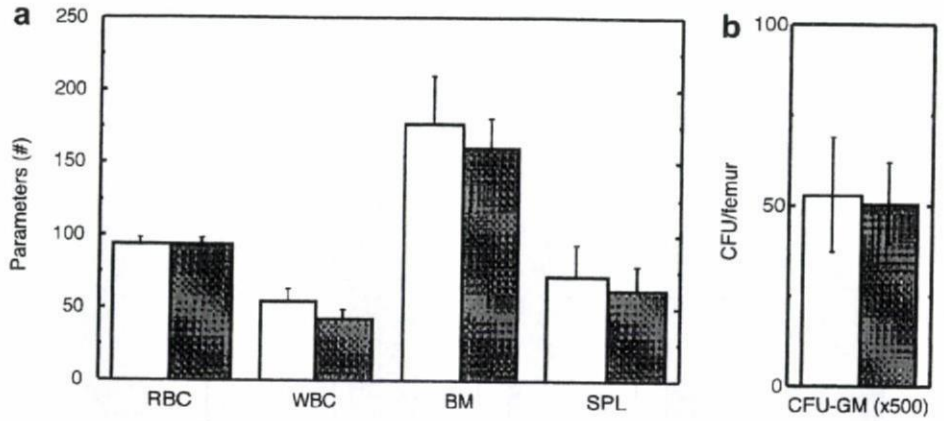

Fig. 2. Comparison of various blood parameters between mice repopulated with Wt BM (open columns) and AhR-KO BM (shaded columns) cells. (a) Peripheral blood, BM and spleen weight. #: vertical axis "Parameters" indicate the counts of peripheral RBCs ($\times 10^8$/ml) and WBCs ($\times 10^6$/ml), BM cellularity ($\times 10^5$/femur), and weight of the spleen (SPL, mg). (b) Numbers of CFU-GM (5×10^2/femur) per femur.

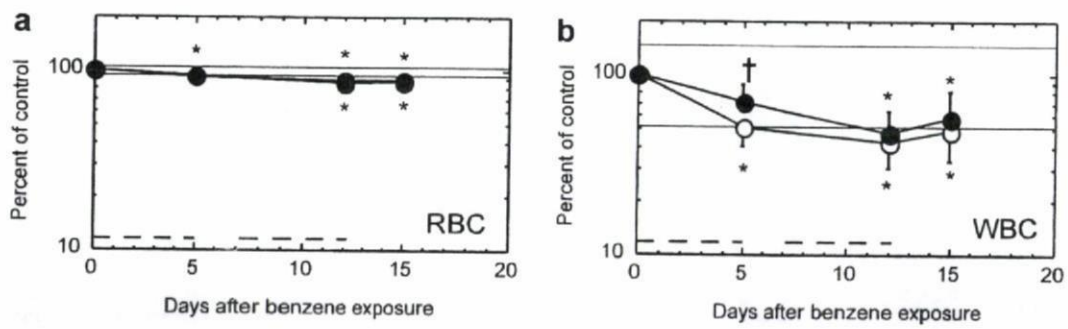

Fig. 3. Changes in percentage numbers of RBC (a) and WBC (b) of mice repopulated with Wt BM (open symbols) and AhR-KO BM (closed symbols) cells during and after benzene exposure, with respect to each sham-exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed line indicates the mean (100%) and the solid lines indicate the standard deviation of the mean (100%) from the sham-exposure control Wt group. The dashed line at the bottom indicates benzene exposure duration. *: Significant difference between sham-exposure group and benzene-exposed group determined by *t*-test at $p < 0.05$. †: Significant difference between Wt mice and AhR-KO mice.

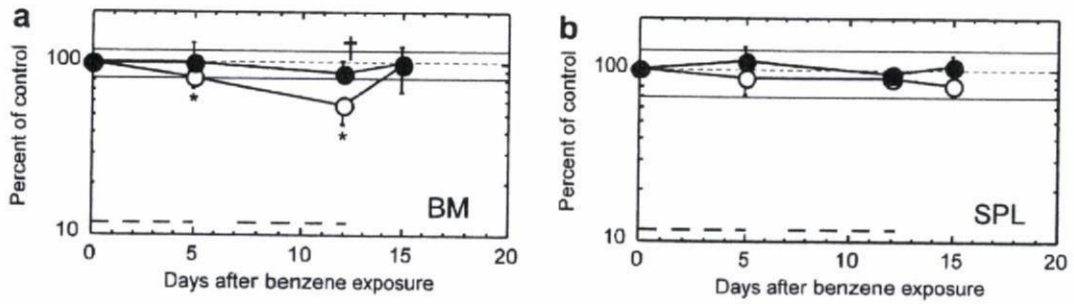

Fig. 4. Changes in percentage number of BM cells (a) and weight of spleen (b) of mice repopulated with Wt BM (open symbols) and AhR-KO BM (closed symbols) cells during and after benzene exposure, with respect to each sham-exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed line indicates the mean (100%) and the solid lines indicate the standard deviation of the mean (100%) from the sham control Wt group. The dashed line at the bottom indicates benzene exposure duration. *: Significant difference between sham-exposure group and benzene-exposed group determined by *t*-test at $p < 0.05$. †: Significant difference between Wt mice and AhR-KO mice.

ence in data between the Wt mice and AhR-KO mice except on day 5 in the number of WBCs.

As shown in Fig. 4a, the decrease in the number of BM cells after benzene exposure is significant in the mice repopulated with Wt BM cells specifically on days 5 and 12 ($82.2 \pm 12.0\%$, $p = 0.035$ and $65.4 \pm 20.3\%$, $p = 0.007$, respectively; number of cells obtained on day 12 was also significantly different between the Wt mice and the AhR-KO mice ($86.7 \pm 14.9\%$; $p = 0.013$)), which returned to the normal range by day 15, that is, three days after cessation of benzene treatment. In contrast to the peripheral blood parameters (Fig. 3a and b), the number of BM cells in the mice repopulated with AhR-KO BM cells did not show any decrease, but the mice showed a clear nullification of benzene-induced decrease in the number of BM cells ($86.7 \pm 14.9\%$; $p = 0.057$). Concerning the weight of the spleen, there are no significant differences among the groups regardless of the duration of benzene treatment and AhR expression (Fig. 4b).

In Fig. 5, the number of CFU-GM in the BM of mice repopulated with Wt BM cells much more significantly decreased on day 12 (open symbols, $37.8 \pm 14.2\%$, $p = 0.019$; the number was also significantly different between Wt mice and AhR-KO mice ($82.0 \pm 7.0\%$; $p = 0.0008$)), which quickly returned to the normal range by day

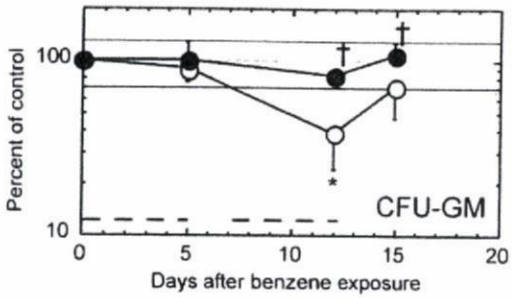

Fig. 5. Changes in percentage number of CFU-GM of mice repopulated with Wt BM (open symbols) and AhR-KO BM (closed symbols) cells during and after benzene exposure, with respect to each sham-exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed line indicates the mean (100%) and the solid lines indicate the standard deviation of the mean (100%) from the sham-exposure control Wt group. The dashed line at the bottom indicates benzene exposure duration. *: Significant difference between sham-exposure group and benzene-exposed group determined by *t*-test at $p < 0.05$. †: Significant difference between Wt mice and AhR-KO mice.

15, three days after cessation of benzene treatment. In this figure, interestingly, the benzene-induced decrease in the number of CFU-GM in the BM of mice repopulated with the AhR-KO BM cells (closed symbols) is clearly nullified for the Wt BM cells (open symbols), and the number stays within the range found for the sham-exposure. The reason for this much prominent decrease observed in the number of CFU-GM in the case of benzene exposure may be due, in part, to the expression of AhR, whose level is significantly high in primitive hematopoietic progenitor cells (Hirabayashi et al., 2002b, 2005b), the KO of which nullified the decrease in the number of CFU-GM much more significantly than the decrease in peripheral blood parameters.

4. Conclusions

Mice that had been lethally irradiated and repopulated with BM cells from AhR-KO mice essentially did not show any benzene-induced hematotoxicity. The present study elucidated the following: first, benzene-induced decrease in BM cellularity was clearly nullified by BM cells in mice that had been repopulated with AhR-KO BM cells. Second, we observed some differences in toxicologic phenotypes depending on the exposure route, that is, intraperitoneal, used in the previous study, or intragastric, in this study; that is, the former route induced attenuation of significant decreases in RBC number, spleen weight, and CFU-GM number, whereas the latter route induced attenuation of decrease in BM cellularity and CFU-GM number as compared with the Wt mice, respectively. Third, the marked decrease in the number of CFU-GM following benzene treatment and its nullification by repopulation with AhR-KO BM cells are the essential key discoveries of this study that may be related to the expression of AhR in primitive hematopoietic progenitor cells. Together with our previous observation and the report on the expression of CYP2E1 (Yoon et al., 2003; Ivanova et al., 2002), findings in this study may be related to the expression of CYP2E1 in BM cells.

Acknowledgments

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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

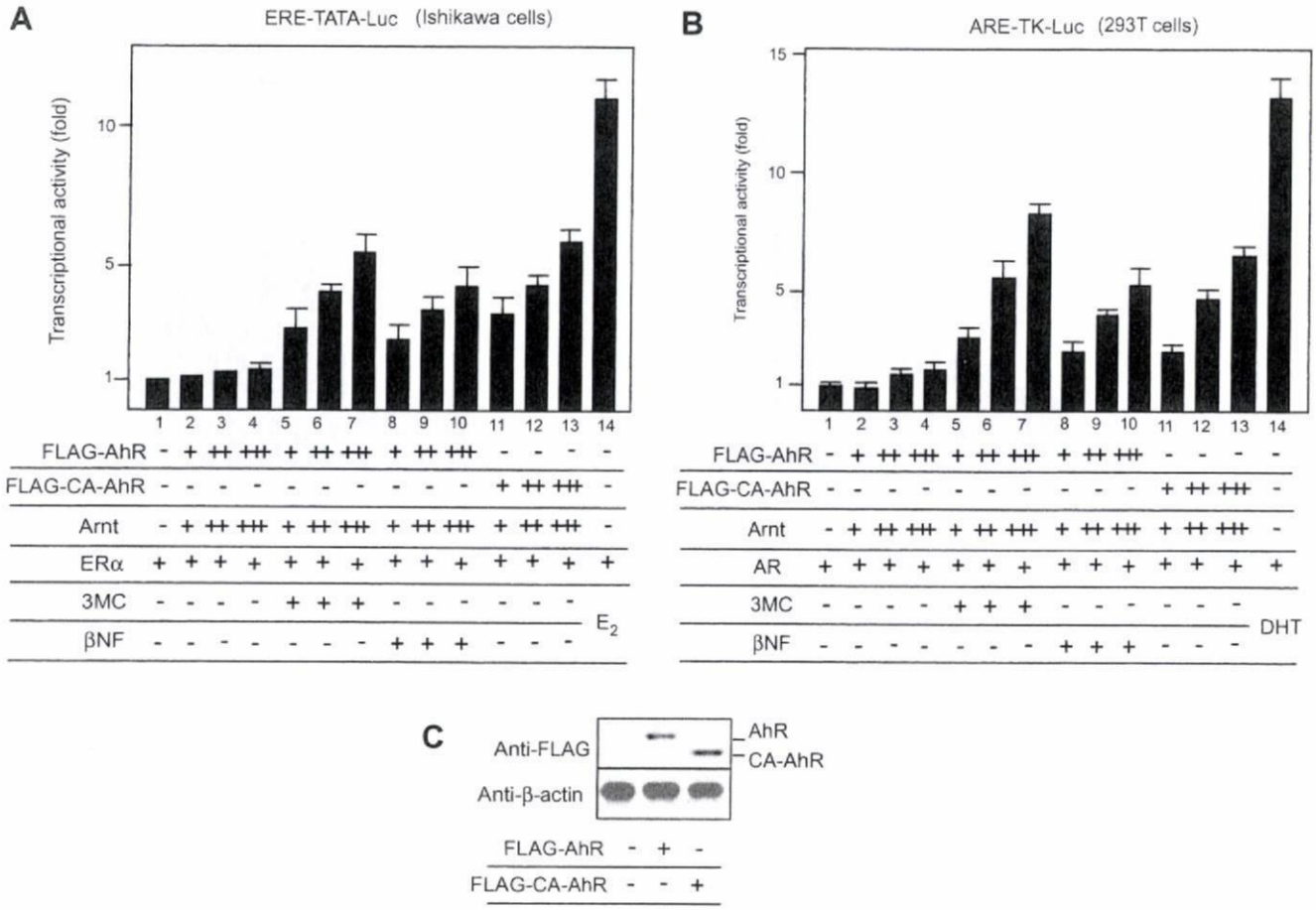

Fig. 1. The constitutively active AhR activates transcription through the unliganded ERα and AR in the absence of AhR-ligands. Ishikawa cells (A) or 293T cells (B) were transfected with the reporter plasmid bearing ERE (A) or ARE (B) together with the indicated expression plasmids (AhR/Arnt; +, 0.05 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-

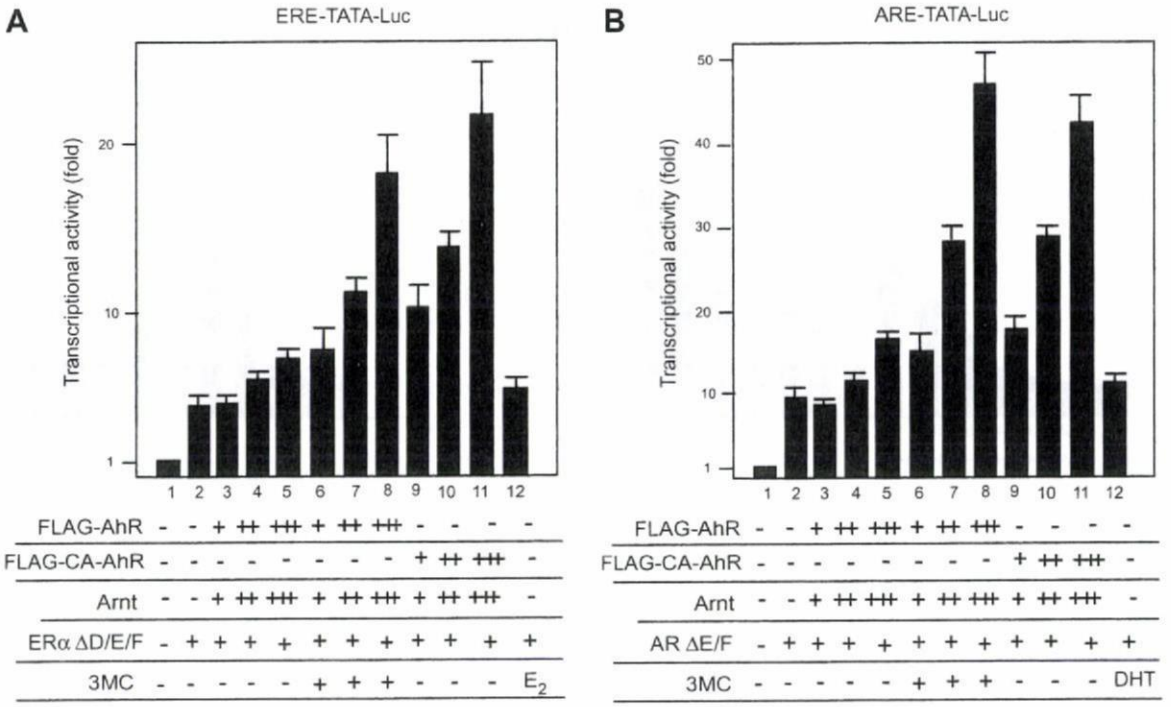

Fig. 2. CA-AhR activates mutants of ERα and AR which lack ligand-binding domains. Ishikawa cells (A) or 293T cells (B) were transfected with the indicated plasmids, and the Luciferase assay was performed as in Fig. 1. Data are means ± SD of three independent experiments. The data show that CA-AhR modulates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

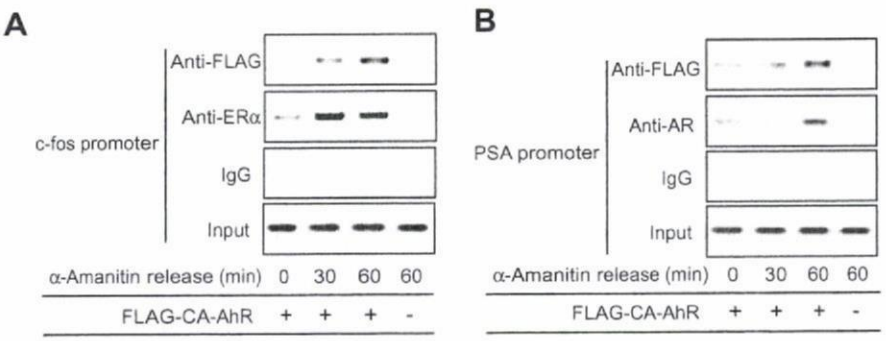

Fig. 3. Promoter occupancy of CA-AhR together with ERα or the AR in the absence of AhR-ligands. MCF-7 cells (A) or LNCaP cells (B), in which endogenous ERα (A) or the AR (B) was functional, were transfected with FLAG-tagged AhR or CA-AhR as indicated. The cells were treated with 10 μM α-amanitin for 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-

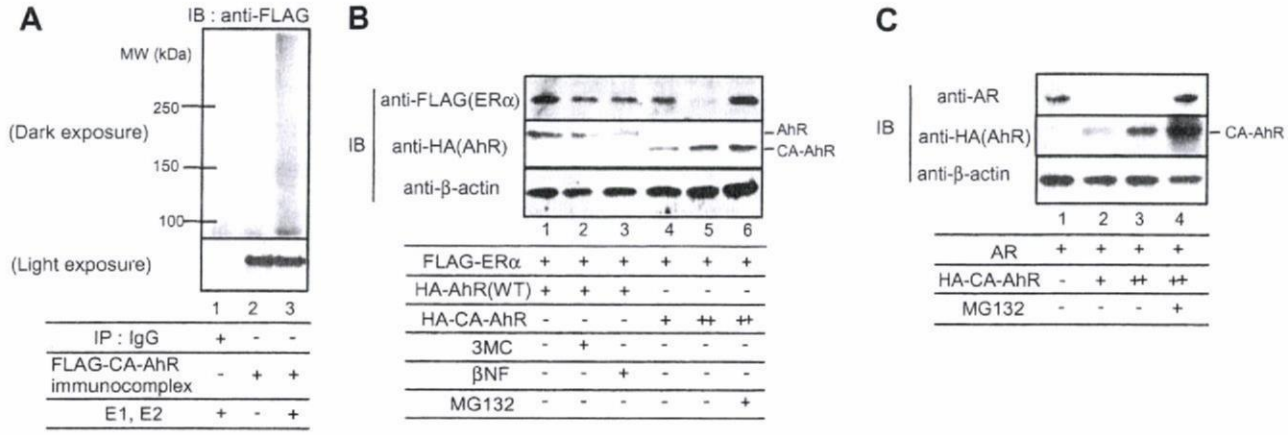

Fig. 4. CA-AhR promotes proteasomal degradation of ERα and the AR. (A) A FLAG-CA-AhR immunocomplex and IgG immunocomplex, prepared from MCF-7 cells, were subjected to the *in vitro* ubiquitination assay. The self-ubiquitination of CA-AhR was detected by Western blotting as indicated. (B,C) MCF-7 cells (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

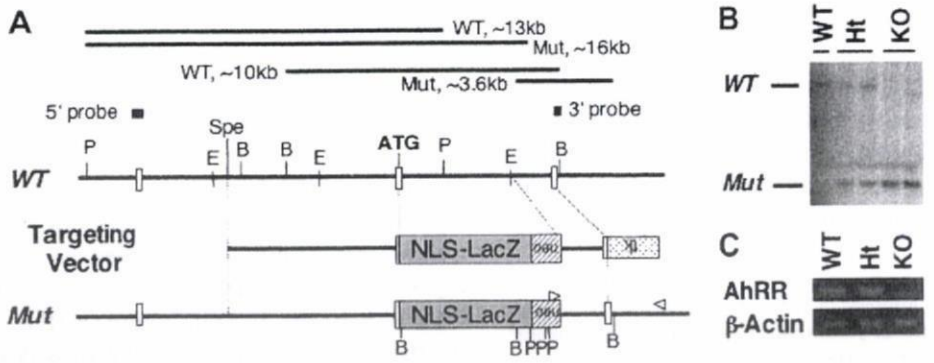

Fig. 1. Targeted disruption of the mouse *AhRR* gene. (A) Schematic representation of the targeting vector, *AhRR*-WT and Mut alleles. Cleavage sites for the restriction enzymes are indicated by E (EcoRI), B (BamHI), Sp (SpeI) and P (PstI). The locations of the 5′ and 3′ probes used for the DNA blot 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

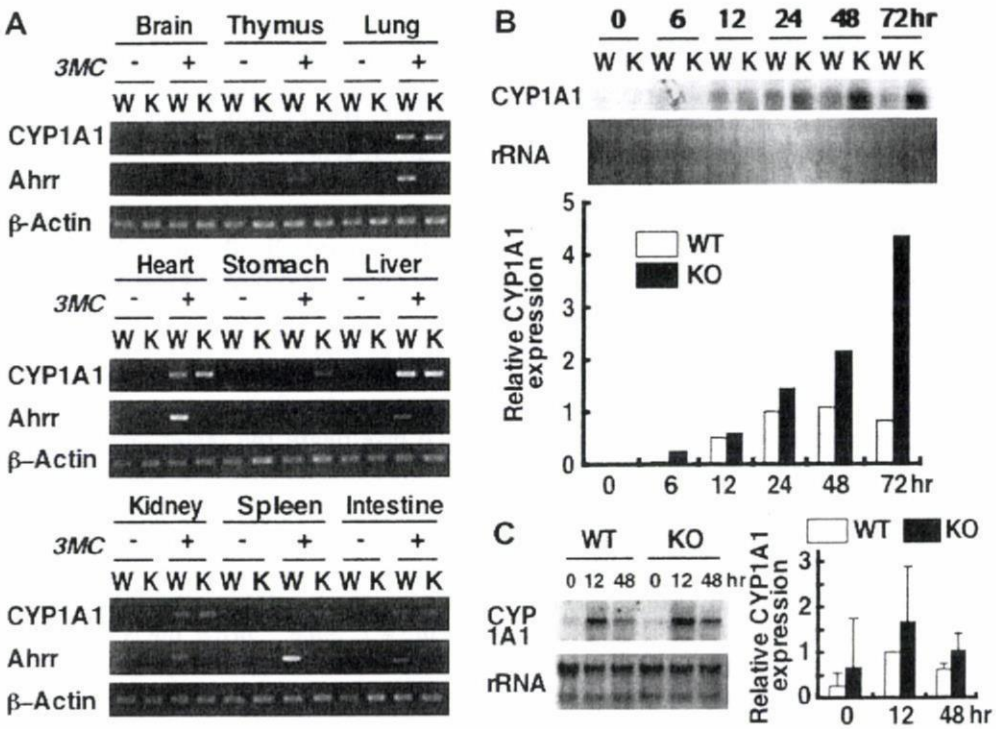

Fig. 2. Inducibility of *AhRR* and *Cyp1a1* mRNA in various tissues. (A) Expression of *Cyp1a1* and *AhRR* mRNA in various tissues of wild type (W) and *AhRR(−/−)* (K) mice. The mice were intraperitoneally injected with 3MC (80 mg/kg body weight). After 24 h, *Cyp1a1* and *AhRR* mRNA expression levels 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-

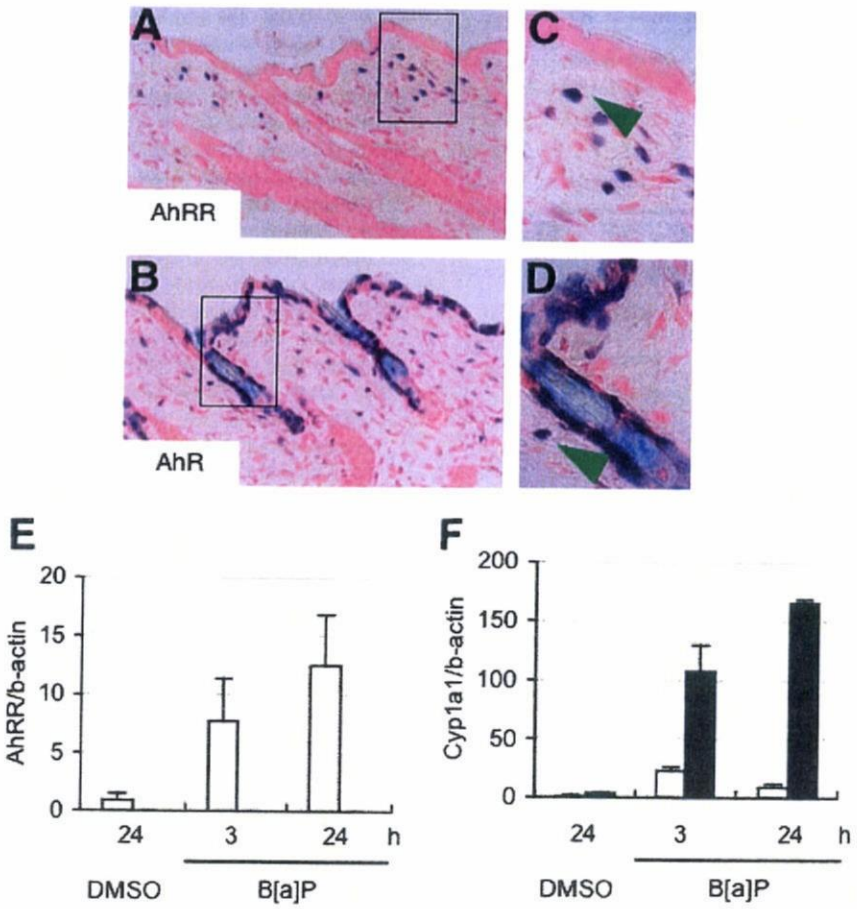

Fig. 3. Cyp1a1 and AhRR expressions in the skin and the skin fibroblast cells of wild-type and *AhRR(−/−)* mice. (A–D) AhRR and AhR expression in the skin. Skin sections prepared from *AhRR(−/−)* (A,C) or *AhR(+/−)* mice (B,D) were subjected to β-galactosidase staining for detection of AhRR or AhR expressing 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]. *AhRR(−/−)* 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

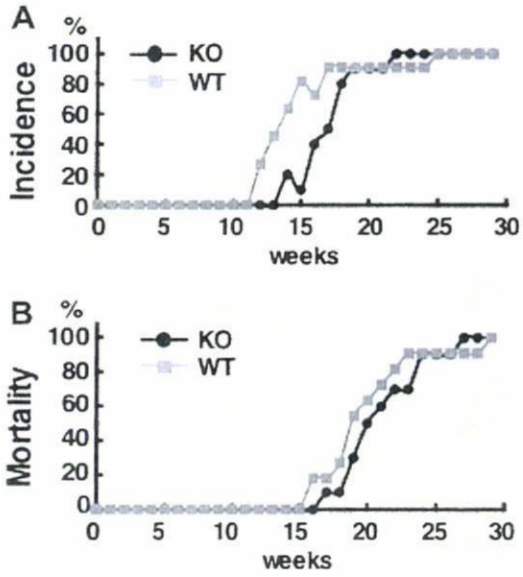

Fig. 4. B[*a*]P-induced tumor incidence and mortality of *AhRR(−/−)* mice. (A) WT (black; $n = 11$) and *AhRR*-KO (magenta; $n = 10$) mice were injected B[*a*]P subcutaneously twice. Tumor formation (A) and mortality (B) were observed as described in Materials and methods and shown as a percentage of 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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that this novel protein inhibits AhR-dependent transactivation of the XRE-driven gene, and thus designated it AhR repressor (AhRR) [10].

Recently, AhRR orthologs have been reported in human [11,12], rat [11,13], and fish [14,15] and their genes have three conserved XRE sequences in the 5′-flanking promoter region. Accordingly, it has been reported that the AhRR expression is activated by the AhR/Arnt heterodimer in some cell lines [11,12] and multiple tissues of various species [10,12–14], indicating that AhRR participates in a negative feedback loop in the AhR signaling pathway [10,14,15].

Although the precise mechanism of inhibitory activity of AhRR remains to be elucidated, it has been proposed that AhRR competes with AhR for heterodimerization with Arnt and binding to the XRE sequence, a property that is likely to be mediated by N-terminal bHLH-PAS domains of these proteins [10].

In this study, we report that AhRR has a transcriptional repression domain within its C-terminal region, which exhibits a trichostatin A (TSA)-sensitive HDAC activity. By Cytotrap yeast two-hybrid screening with the C-terminal sequence of AhRR used as bait, we isolated Ankyrin-repeat protein2 (ANKRA2) as a binding partner to the AhRR C-terminal sequence.

Materials and methods

Plasmids. Fragments for AhRR(1–701 a.a.), AhRR(1–342 a.a.), AhRR(342–701 a.a.), AhRR(342–478 a.a.), AhRR(478–701 a.a.), AhRR(478–555 a.a.), AhRR(555–701 a.a.) were excised from pBOS-AhRR [10] and cloned into the pBOSGAL4DBD vector [16]. pG3TK-Luc was produced by inserting three copies of the GAL4 binding site excised from pG5EC and TK promoter sequence excised from pBSCAT2 into the XhoI site of pGL3 vector (Clontech). pBOSHA-AhRR was constructed as follows: pBOST7HA vector was constructed by inserting the blunt-ended BglII/PstI fragment of pGADT7 vector containing T7 promoter and HA epitope Tag into blunt-ended BamHI site of pEFBOS vector [17]. The EcoRI/SalI fragment excised from pBSKAhRR (Mimura, unpublished data) was inserted into the EcoRI and SalI site of pBOST7HA. pBOS-FLAG-ANKRA2 was constructed as follows: pBOST7FLAG vector was constructed by inserting the fragment containing the T7 promoter and FLAG epitope Tag cleaved from pGADT7FLAG vector into blunt-ended BamHI site of pEFBOS vector. Mouse ANKRA2 cDNA was amplified with a pair of primers, 5′-CatcgatACATGGCTACATCTGCAAAT-3′ and 5′-CggatccTCACTCCCTGATGTTCTGAA-3′ as the 5′ and 3′ primers, respectively. The amplified cDNA fragment was digested with ClaI and BamHI, and inserted into the ClaI and BamHI site of pBOST7FLAG. Expression plasmids encoding HDAC4-FLAG and HDAC5-FLAG were kindly provided by Dr. Stuart L. Schreiber (Harvard University, MA, USA).

Antibody production. Recombinant glutathione S-transferase (GST)-tagged mouse AhRR (342–701 a.a.) and recombinant maltose-binding protein (MBP)-tagged mouse AhRR (342–701 a.a.) were expressed in *Escherichia coli* and purified with Glutathione Sepharose 4B (Amersham Biosciences) and amylose resin (New England Biolabs), respectively, according to the manufacturer's protocols. Polyclonal rabbit antisera were raised against the recombinant GST-AhRR (342–701 a.a.) and further affinity-purified with the recombinant MBP-AhRR (342–701 a.a.) (Hokudo Inc., Japan).

Cell culture. Mouse embryonic fibroblast (MEF) cells were isolated from C57B/6J mice. COS-7, MEF, and Hepa-1c1c7 (Hepa-1) cells were maintained in high glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Invitrogen) under 5.0% CO_2 at 37 °C.

Luciferase assay. Hepa-1 cells (5.0×10^4 cells/well) were grown in 24-well dishes for 24 h and were transfected with the expression plasmids indicated in the figure legends, pG3TK-Luc and the expression plasmids for sea pansy luciferase as an internal control using Lipofectamine™ (Invitrogen). Forty-eight hours after transfection, the cells were harvested and luciferase was quantified by using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocols. For control of transfection efficiency, firefly luciferase activity was normalized to cotransfected sea pansy luciferase activity as a standard.

Coimmunoprecipitation and immunoblot analysis. Cell lysates from the transfected COS-7 and MEF cells were prepared as described and used for immunoblot analysis either directly or after immunoprecipitation. Immunoprecipitation with anti-Flag M2 agarose (Sigma) or anti-HA agarose (Sigma) was performed for 12 h and the immunoprecipitates were washed according to the published procedure for immunoblot analysis. Immunoblot analysis was performed as described [7] using anti-FLAG (Sigma), anti-HA (Sigma), anti-Tubulin (Sigma), and anti AhRR antibodies.

RNA interference experiments. The siRNAs for mouse ANKRA2 or mouse AhRR were designed and synthesized by B-Bridge International Inc. The coding sequences were: ANKRA2, (5′-AGGAAAAGGUCGAG AAAGUdTdT-3′) and AhRR, (5′-GGAAAGGCCUUGUGGCUAAdT dT-3′). Hepa-1 cells or MEF cells (5.0×10^4 cells/well) were transfected with siRNA for ANKRA2 (20 pmol) or AhRR (50 pmol) with or without expression plasmids by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Quantification of mRNA. Total RNAs are extracted from cells using Isogen (Nippon Gene, Tokyo) and reverse transcribed by Superscript II (Invitrogen). Real-time PCR was carried out in ABI PRISM 7700 sequence detection system using the following primer sets: ANKRA2, forward (5′-TCTACCACACCTCTGTTAGC-3′) and reverse (5′-GCA CTTTCTCGACCTTTTCC-3′); AhRR, forward (5′-GCTTTCTGTCCT GCGCCTC-3′) and reverse (5′-TCCTTCCTGCACGGGGAAC-3′); CY P1A1, forward (5′-GGACATTTGAGAAGGGCCAC-3′) and reverse (5′-CGTCCAGCTTCCTGTCCTGA-3′); actin, forward (5′-GACAGGAT GCAGAAGGAGAT-3′) and reverse (5′-TTGCTGATCCACATCTGC TG-3′).

Cytotrap yeast two-hybrid assay. The CytoTrap™ (Stratagene) yeast screening was performed with a murine thymus cDNA library (Stratagene) and pSos-AhRR (342–701) as prey and bait, respectively, according to the manufacturer's instructions.

Results

Functional characterization of the AhRR transcriptional repression domain

We previously reported that AhRR inhibits the transcription activity of Arnt [10]. In order to confirm transcriptional repression activity and to localize the transcriptional repression domain of AhRR, we fused a series of AhRR deletion mutants to the GAL4 DNA binding domain (GAL) (Fig. 1A). These fragments were transfected into Hepa-1 cells, along with a luciferase reporter gene driven by three GAL4 binding sites and the TK promoter (Fig. 1A). The luciferase activity driven by the TK promoter was repressed 5.2-fold by the transfection with plasmid, GAL4DBD-AhRR (1–701) (Fig. 1B). Plasmids encoding the fusion proteins, GAL4DBD-AhRR (342–701), (478–701), and (555–701) also repressed luciferase expression 5.6-, 4.9- and 3.7-fold, respectively, while plasmids of AhRR (1–342), AhRR (342–478), and AhRR

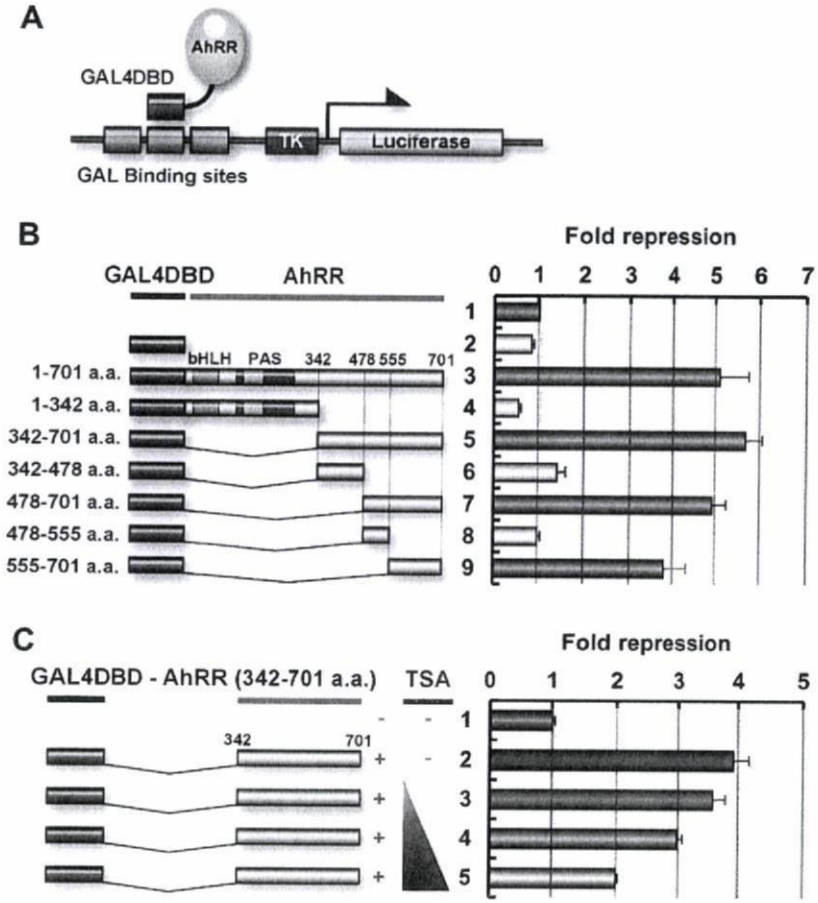

Fig. 1. The carboxy-terminal half of AhRR shows a repressor activity, which is sensitive to the HDAC inhibitor, TSA. (A) Schematic representation of luciferase reporter assay. (B) Transcriptional repression activity of AhRR. Hepa-1 cells were transiently transfected with the expression plasmids of GAL4DBD-AhRR and GAL-TK-Luc reporter gene containing three GAL binding sites. Cell extracts were prepared 48 h after transfection and used for luciferase assays. The fold repression is relative to the reporter gene alone. (C) Effects of TSA on AhRR-mediated transcriptional repression. The transfections were performed as described in (B), Cells were treated with increasing amounts (2, 4, and 8 ng/ml) of TSA 24 h after transfection and then, 12 h later, whole cell extracts were prepared and used for luciferase assays.

(478–555) did not significantly repress luciferase expression. These data localized a core region needed for transcriptional repression by AhRR to the sequence of amino acid 555–701.

To investigate how the repression activity of the AhRR fragment (342–701) functions in the TK promoter-driven reporter system, we used the HDAC inhibitor, TSA, which reversed the repression of reporter gene expression by the AhRR fragment (342–701) in a dose-dependent manner (Fig. 1C). These results suggest that the repression activity of the AhRR C-terminus is due to an HDAC activity. Since the C-terminal sequence of AhRR is well conserved among multiple mammalian species (Supplementary Fig. S1), we next searched for transcriptional corepressor, which interact with the AhRR C-terminus.

Isolation of ANKRA2 as a factor interacting with AhRR and interaction of AhRR with ANKRA2 and HDAC4 or HDAC5

To isolate a corepressor of AhRR, we performed a Cytotrap yeast two-hybrid screen with the C-terminal fragment of AhRR (342–701) used as bait (Fig. 2A) and isolated several clones including Dhx8, EB1, EB3, p21, Prostaglandin E receptor, EGF-containing fibrin-like extracellular matrix protein1, and ANKRA2. We chose ANKRA2 for further work in this paper, because ANKRA2 is reported to interact with HDAC4 and HDAC5 [18]. Recently, its mammalian paralogue, RFXANK has also been reported to interact with HDAC4 and HDAC5 [18] and to repress MHC class II promoter activation through association with HDAC4 and HDAC5 [19]. Taken together, these results suggest a potential role of ANKRA2 as mediator in transcriptional repression. ANKRA2 is a protein of 312 amino acids with consecutive 3 ankyrin repeats and the cDNA encoded a C-terminal fragment, amino acid 117–312 (Fig. 2A).

To address whether a physical interaction occurs between AhRR and ANKRA2, whole cell extracts from COS-7 cells cotransfected with expression plasmids for HA-tagged AhRR and FLAG-tagged ANKRA2 were used for *in vivo* coimmunoprecipitation studies. As expected, when the cell extracts were immunoprecipitated with an anti-FLAG antibody, AhRR was coimmunoprecipitated

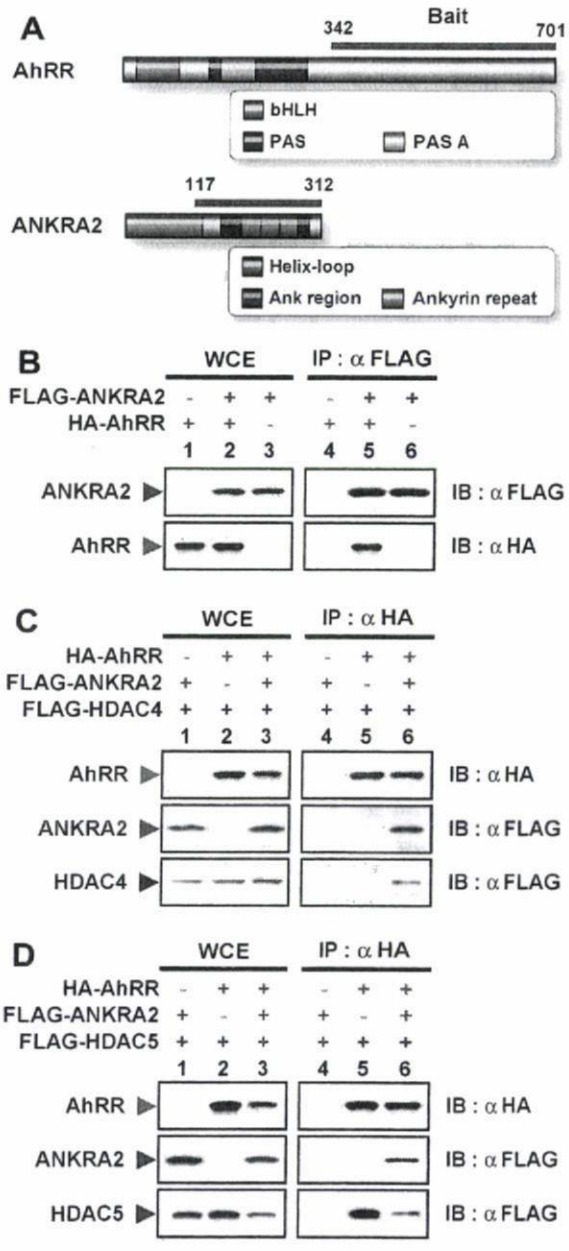

Fig. 2. Physical interaction of AhRR with ANKRA2 and HDAC4 or HDAC5. (A) Schematic diagrams of AhRR and ANKRA2. The carboxy-terminal half of AhRR was used as bait in a yeast two-hybrid screen. The AhRR bait interacted with a fragment (117–312 amino acid) of ANKRA2. ANKRA2 is a protein of 312 amino acids that contains an amino-terminal helix-loop-helix domain and four ankyrin repeats at the C terminus. (B) COS-7 cells were cotransfected with expression plasmids of HA-tagged AhRR and FLAG-tagged ANKRA2. Whole cell extracts were prepared 48 h after transfection and immunoprecipitated (IP) with anti-FLAG antibodies. Immunoprecipitates were analyzed by immunoblot with the indicated antibodies. Crude lysates were analyzed by immunoblot to control protein expressions (WCE). (C) COS-7 cells were cotransfected with expression plasmids of HA-tagged AhRR, FLAG-tagged ANKRA2 and either FLAG-tagged HDAC4 or HDAC5. Cell extracts were prepared 48 h after transfection and immunoprecipitated (IP) with anti-HA antibodies. Proteins were analyzed by immunoblot as described above.

with ANKRA2 (Fig. 2B, lane 5). We next asked if HDAC4 and HDAC5 also interact with AhRR. Whole cell extracts from COS-7 cells cotransfected with expression plasmids for HA-tagged AhRR and FLAG-tagged ANKRA2 together with either FLAG-tagged HDAC4 or FLAG-tagged HDAC5 were immunoprecipitated with an anti-HA antibody. HDAC4 was coimmunoprecipitated with AhRR only when ANKRA2 was cotransfected (Fig. 2C, lane 6). On the other hand, HDAC5 was also coimmunoprecipitated with AhRR even in the absence of ANKRA2, indicating that HDAC5 interacts with AhRR either directly or through ANKRA2 (Fig. 2D, lanes 5 and 6).

Depletion of ANKRA2 by siRNA attenuates transcriptional repression activity of AhRR

If ANKRA2 is a corepressor for AhRR, downregulation of the endogenous level of ANKRA2 by siRNA against ANKRA2 should reverse the transcriptional repression activity of AhRR. To confirm the requirement of ANKRA2 for the repression activity of AhRR, Hepa-1 cells were transiently transfected with expression plasmids for GAL-AhRR together with ANKRA2 siRNA or control siRNA. As shown in Fig. 3A, ANKRA2 siRNA transfection significantly reduced the expression of ANKRA2 mRNA. As expected, when the cells were treated with ANKRA2 siRNA, the repression activity of AhRR was significantly reversed as compared with that observed in cells treated with control siRNA (Fig. 3B).

To further investigate whether ANKRA2 is required for the endogenous AhRR/Arnt heterodimer to repress the XRE-driven transcription of endogenous target genes such as *CYP1A1*, we conducted RNA interference experiments in MEF cells. When MEF cells were transfected with ANKRA2 siRNA, endogenous ANKRA2 mRNA levels were significantly lowered both in the presence or absence of an AhR ligand, 3MC (Fig. 3C). Under normal conditions, CYP1A1 mRNA expression was barely detectable, but knockdown of ANKRA2 by ANKRA2 siRNA caused a small, but significant increase in basal CYP1A1 mRNA levels (Fig. 3D, lane 1). When treated with 3MC for 6 h, MEF cells inducibly expressed CYP1A1 mRNA to a similar level in the presence and absence of ANKRA2 siRNA (Fig. 3D, lane 2). These results suggest that in the silent state of MEF cells, the AhRR-ANKRA2 suppressor complex is involved in silencing the CYP1A1 basal expression, while under inducing conditions, ANKRA2 does not greatly affect the inducible expression of *CYP1A1* gene.

To confirm that the AhRR–ANKRA2 repressor complex silences the transcription of endogenous CYP1A1 in MEF cells, we used siRNA against AhRR. As previously reported in many other cell lines and tissues, AhRR mRNA and protein was constitutively expressed in MEF cells under normal conditions and was further enhanced in response to 3MC (Fig. 3E, lanes 1 and 2, and F). When MEF cells were transfected with AhRR siRNA, both mRNA and protein levels of AhRR were markedly downregulated (Fig. 3E, lanes 1 and 3, and F). In agreement with the result of the ANKRA2 siRNA experiment, treatment of AhRR siRNA increased CYP1A1 mRNA under normal