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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

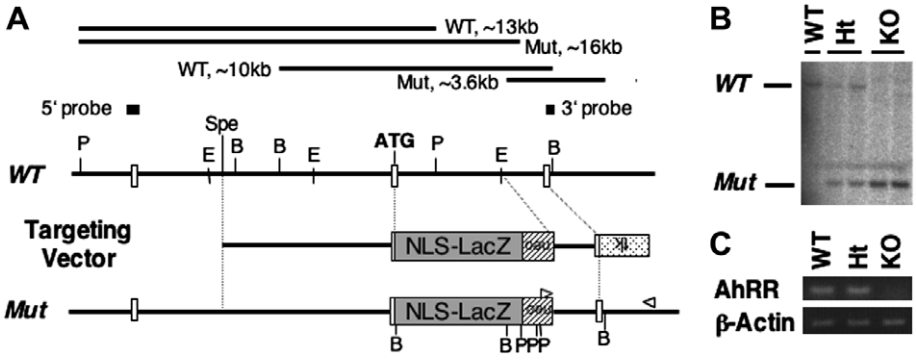

Fig. 1. Targeted disruption of the mouse *AhRR* gene. (A) Schematic representation of the targeting vector, *AhRR*-WT and Mut alleles. Cleavage sites for the restriction enzymes are indicated by E (EcoRI), B (BamHI), Sp (SpeI) and P (PstI). The locations of the 5′ and 3′ probes used for the DNA blot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