ARYL HYDROCARBON RECEPTOR SUPPRESSES SPONTANEOUS NEOPLASMS AND EXTENDS LIFE SPAN: POSSIBLE MECHANISM IMPLIED BY HEMATOPOIETIC STEM CELL KINETICS

Hirabayashi Y[1], Yoon BI [1], Li GX [1], Kaneko T[1], Kanno J[1], Fujii-Kuriyama Y[2], Inoue T [3]
[1] Division of Cellular and Molecular Toxicology, Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158-8501, Japan; [2] Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba 305-8577, Japan; [3] Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158-8501, Japan.

Abstract
The aryl hydrocarbon receptor (AhR) is an orphan receptor whose original physiological function remains unknown. Since AhR-knockout (KO) mice were found to show an earlier onset of spontaneous neoplasms than wild-type mice, AhR was assumed to play a suppressor gene function (Hirabayashi, 2006). However, because not all AhR-KO ($AhR^{-/-}$) mice or wild-type mice die of spontaneous neoplasms, the function of wild-type AhR may also be associated with a possible genomic stabilization, thereby consequently extending the life span of mice simultaneously. What are the underlying mechanisms that contribute to these suppressed cell cycle and extended longevity? The result of the evaluation of a reactive oxygen species (ROS) using a DCFH-DA dye showed a prominent increase in oxidative stress in unfractionated bone marrow cells as well as in hematopoietic progenitor cells in the $AhR^{-/-}$ mice. Hematopoietic progenitor cells are quiescent in an anoxic environment, and are regulated by a weak oxidative stimulation. Thus, the higher reactivity of the fraction to the DCFH-DA dye in the $AhR^{-/-}$ mice is in good agreement with the underlying mechanism of genomic stabilization under a low oxidative tension in combination with the suppressor gene function and the consequent longevity observed in wild-type, $AhR^{+/+}$ mice.

Introduction
We found that aryl hydrocarbon receptor (AhR) knockout (KO) mice show an earlier onset of spontaneous neoplasms than wild-type mice [1]. Thus, it is plausible that AhR functions as a tumor suppressor gene in the steady state. Furthermore, since not all of these mice die of malignant neoplasms, AhR may also extend the life span of these mice, i.e., the "longevity" function. Such biological plausible functions are possible reasons for the molecular evolution of *AhR* from equivalent homologues in invertebrates, such as nematodes, to those in vertebrates. However, one question remains: Why do AhR-KO mice show early onset of spontaneous neoplasms? Supposedly these mice should exhibit unfavorable xenobiotic responses when AhR is knocked out [2,3]. Furthermore, the mechanisms of the possible suppressive function of wild-type *AhR* remain to be elucidated. To answer the question, we extensively analyzed spontaneous neoplasms with respect to Gompertzean survival [4] in AhR-deficient mice and other wild-type mouse strains known to carry a mutated AhR structure, as well as the implication of differences in the type of spontaneous neoplasms, life spans and AhR function. Furthermore, we found a possible function of eliminating reactive oxygen species (ROS) in the wild-type progenitor cells, which may be a key mechanism to support the above-mentioned AhR function for longevity.

Materials and Methods
Experimental animals. The generation of homozygous AhR-KO ($AhR^{-/-}$) mice of the 129/SvJ strain has been described elsewhere [2,5]. The animal experiment using $AhR^{-/-}$ mice was approved by the Interdisciplinary Monitoring Committee for the Proper Use and Welfare of Experimental Animals. The mice were maintained in a board-approved laboratory animal facility of the National Institutes of Health Sciences (NIHS) of Japan. Heterozygous AhR-KO ($AhR^{+/-}$) males were backcrossed with C57BL/6 females. The breeding of 14 generations of heterozygous $AhR^{+/-}$ males with $AhR^{+/-}$ females generated wild-type ($AhR^{+/+}$), $AhR^{+/-}$, and $AhR^{-/-}$mice, although the proportion of $AhR^{-/-}$mice was slightly lower than the Mendelian estimate. The neonates were genotyped by the polymerase chain reaction (PCR) screening of DNA from the tail. All the mice were housed under specific pathogen-free conditions at 24 ± 1°C and 55 ± 10%, room temperature and humidity, using a 12-hr light-dark cycle. Autoclaved tap water and food pellets were provided *ad libitum*.
Life-time observation. Twenty-four $AhR^{+/+}$, 23 $AhR^{+/-}$, and 18 $AhR^{-/-}$male mice were used in this study. All the

mice were monitored at least twice daily throughout their lifetime. Those showing symptoms of advanced leukemia such as anemia and palpable splenomegaly were euthanized at the agonal period and then examined hematopathologically. In addition, mice that died were subjected to gross and microscopic examinations.

Histopathological examination. To evaluate hematopoietic malignancies, mice from each group were sacrificed under ethyl ether anesthesia and autopsied. For histopathological examination, all the visceral organs of the mice including the thymus, spleen, sternum, and femoral bone marrow (BM) were fixed in 10% neutral-buffered formalin for 24 h. The sternum and femoral BM were decalcified in 7.5% formic acid for 72 h. After conventional processing for dehydration, paraffin-embedded sections were stained with hematoxylin and eosin (H and E) and then examined histopathologically under a light microscope [6, 7].

Measurement of ROS-production in BM cells. In the assessment of ROS-production for each mouse genotype group, we loaded BM cells with a 25 μM fluorescent probe, 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma, St. Louis, MO) and analyzed their intracellular fluorescence intensity by flow cytometry [8-10] (FACS-Calibur, BD Biosciences, San Jose, CA). Briefly, femoral BM cells from each mouse genotype were incubated with 25 μM DCFH-DA dye at 37°C for 25 min in a humidified atmosphere of 5% CO_2. After incubation, the cell suspension was measured of fluorescence intensity from DCFH-DA as pretreatment for establishing a baseline, and the cells were then treated with or without 3 μM H_2O_2 (Sigma) to increase ROS-production. The size range of the gate of the cell sorter was set for large mononuclear cells by corresponding forward scatter over small lymphocytes and by perpendicular side scatter over granulocytes, followed by the evaluation of fluorescence intensity from DCFH-DA. A 488-nm argon laser beam was used for excitation. The histograms and dot-plotted mean fluorescence intensity were analyzed using Cell Quest™ software (BD Biosciences) [11, 12].

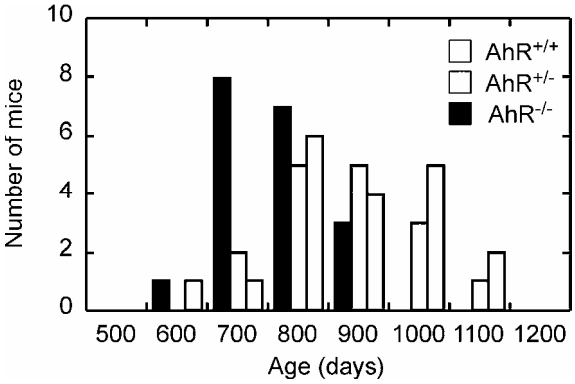

Figure 1: Incidences of spontaneous malignant lymphomas at unit time intervals.

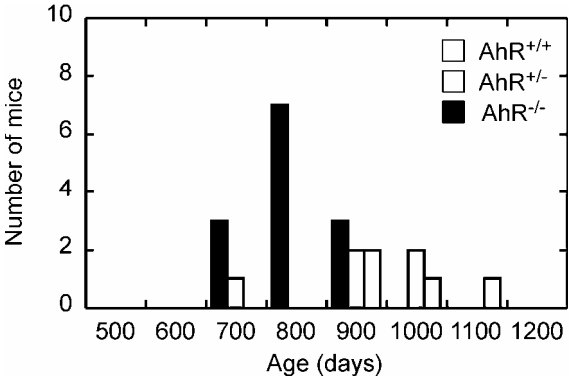

Figure 2: Incidences of spontaneous hepatomas at unit time intervals.

Results and Discussion

AhR$^{-/-}$ mice show earlier onset of spontaneous neoplasms − Gompertzean accelerated aging. Figure 1 shows the incidence of spontaneous malignant lymphomas in each genotype group for *AhR* plotted along the ordinate axis vs age in days plotted along the horizontal axis. The incidence of lymphomas in the AhR$^{-/-}$group (dark-shaded columns) starts earlier than those in the AhR$^{+/-}$ (light-shaded columns) and wild-type (open columns) groups. The peak of lymphomas in the AhR$^{-/-}$group is observed after 700 days, whereas those in the AhR$^{+/-}$ and wild-type groups are observed at 850 and 1000 days, respectively. Similarly, the incidences of spontaneous

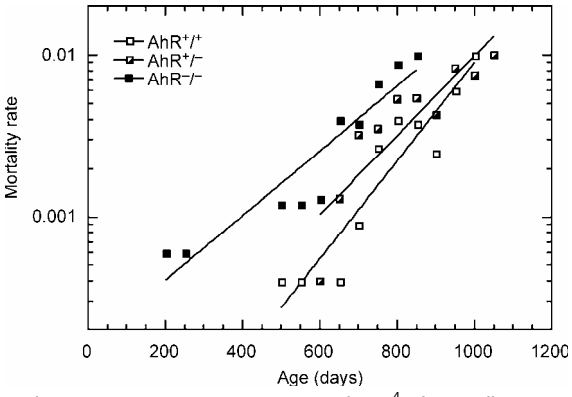

Figure 3: Gompertzean expressions [4] of mortality rates of mice of different genotypes; AhR$^{+/+}$, open squares; AhR$^{+/-}$, half-closed squares; AhR$^{-/-}$, closed squares.

hepatomas in each genotype group are plotted in Figure 2. In this figure, spontaneous hepatomas in the $AhR^{-/-}$ group appear at 700 days and those in the wild-type group appear much later (at 900 days) at significantly lower incidences. In Figure 3, the mortality rate/unit time interval in each genotype group is plotted. Mortality rate/unit time interval is shown in the ordinate on a logarithmic scale and age in days is plotted along the horizontal scale (Gompertzean expression[4]). In this figure, the line shown by closed squares for the $AhR^{-/-}$ mice shows much advanced curve with a lower and flatter slope than the line for the wild-type group shown by open squares; thus, the mortality rate of the $AhR^{-/-}$ group can be concluded to indicate "accelerated aging". The shortened lifespan observed in the AhR-KO mice may be due to the impairment of a possible suppression gene in the KO mice. However, some mice for each survival curve are non-tumor-bearing mice. The mechanism of this accelerated aging may not be as simple as that involving a tumor suppressor and remains to be elucidated. In other words, the role of AhR in tumor suppression or longevity remains unknown.

Comparisons of different mouse strains in terms of* AhR *function, receptor concentration, and life span – Review of literature. Because mice of different strains show different spontaneous neoplastic propensities, sometimes, possess different AhR structures, and have different survival life spans, available databases for such parameters were compared. The results obtained are shown in Table 1.

Table 1. AhR Binding Affinities and Receptor Activities, Cell Cycles, and Life Spans among Murine Species

STRAIN	AHR-AFFINITY [a)]	CYP1A2 ENZYME ACTIVITY [b)]	RECEPTOR CONCENT-RATION [c)]	CELL CYCLE [d)]	LIFE SPANS [e)] (DAYS)	NOTES
C3H/He	High*	Low to mid	86 ± 23	High	500	* low signal induction
DBA/2J	Low	Low	-------	High	710*	* 708.7 days in other litr.
C57BL/6J	High	High	151 ± 26	Low	789*	*860.8 days in other litr.

[a)] Murine Ah receptor specified by the Ah^d and Ah^{b-3} alleles is compared [13]. [b)] Activity for methoxyresorufin *o*-demethylation (MOD) and pentoxyresorufin *o*-dealkylation, and metabolic activation of IQ for phenobarbital were tested. For activity in DBA/2J, high in male, but low in female for CYP1A2. Activity for MOD was significantly low for both genders [14]. [c)] Murine Ah locus (mg/protein) [15]. [d)] Scored using hematopoietic cobblestone area-forming cell assay [16]. [e)] Data cited from Dear et al.[17] and Forster MJ et al.[18].

In this table, two strains, namely, C3H/He and DBA/2, showed a low affinity or a low enzyme activity for constitutively activated CYP1A2, whereas C57BL/6J, showed a high affinity or a high enzymatic activity for CYP1A2 [14]. Although C3H/He mice showed a high affinity, the receptor concentration (mg/protein) measured was low [15], thus, the total activity in the literature is low. It is interesting to determine correlations between AhR activity and stage of cell cycle or life span, because the mice with a high activity for AhR, i.e., C57BL/6J, seem to show a suppressed cell cycle and longer life span, whereas the those mice with low activity for AhR, i.e., C3H/He, seem to show an accelerated cell cycle with a shortened life span [16-18]. No comparable data on genomic stability (or instability) fully supportive of the above AhR activity are available. However, the correlations between AhR activity and the stage of cell cycle or life span seem to be plausible and compatible with the results of our present study under experimental induction of AhR deficiencies.

AhR suppresses oxidative stress − possible mechanism for tumor suppression and longevity. By analyzing antioxidative responses to thioredoxin concerns, we have recently found that benzene-induced xenobiotic responses are associated with antioxidative signaling [19]. Thus the increased incidence of spontaneous neoplasms and accelerated aging observed in the AhR-KO mice can be hypothesized as a consequent genomic instability possibly due to the absence of xenobiotic or antioxidative responses. ROSs in hematopoietic tissues, in both AhR-KO and wild-type mice, were evaluated using the DCFH-DA dye by flow cytometry, followed by the fractionation of hematopoietic progenitor cells. The hematopoietic tissues from the $AhR^{-/-}$ mice showed a high reactivity to DCFH-DA, as shown in Figure 4. Because the AhR expression level is high in primitive hematopoietic progenitor cells [20, 21], fractionated primitive hematopoietic progenitor cells were also evaluated of their reactivity to DCFH-DA, which was found to be higher than the reactivity of other unfractionated bone

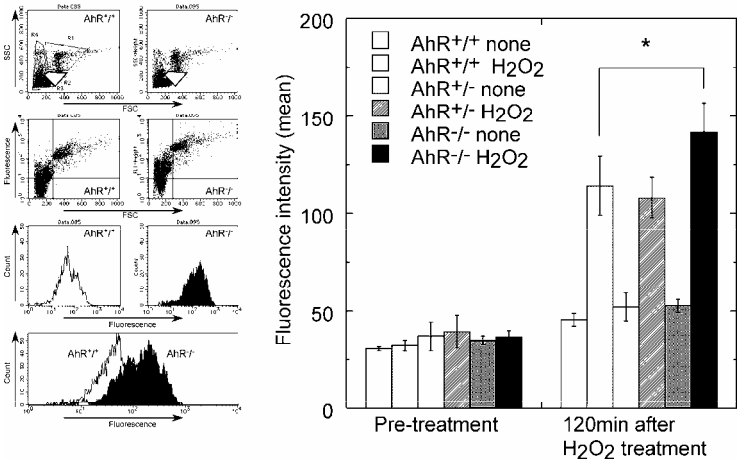

Figure 4: The primitive BM cell fraction of the $AhR^{-/-}$ mice shows a higher fluorescence intensity than that of the $AhR^{+/+}$ mice after H_2O_2 treatment. (*: $p<0.05$)

marrow cells. Hematopoietic progenitor cells, i.e., $lin^-/c\text{-}kit^+/Sca1^+$ cell fraction, are quiescent in an anoxic environment; further, their activity is regulated by weak oxidative tensions. Thus, the higher reactivity of the hematopoietic progenitor cell fraction in $AhR^{-/-}$ mice to the DCFH-DA dye is considered to be in good agreement with the underlying mechanism of genomic stability of wild-type $AhR^{+/+}$ mice, which may be linked to the suppressive function and the consequent longevity.

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