博士論文（要約）

Dioxin induces stable epigenetic modulation at the mouse *Cyp1a1* promoter

(ダイオキシン誘発性のマウス *Cyp1a1* プロモーター上の安定した
エピジェネティックな変化)

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論文の内容の要旨

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Introduction: Epigenetic regulation is important for transcriptional control and cellular function. Transcription factors have the potential to target epigenetic modulators to specific genomic sites in order to regulate target gene expression. Aryl hydrocarbon receptor (Ahr) is a highly conserved nuclear receptor that is involved in toxic response to halogenated aromatic hydrocarbons. Emerging evidence also places Ahr as an important regulator of hemopoiesis, cellular differentiation, immune response and cardiovascular development. As such, it is anticipated that epigenetic mechanisms may have a central role in Ahr downstream signaling.

Accumulating evidence also indicates that the epigenome is sensitive to environmental cues, such that factors such as chemicals, diet and stress can lead to epigenetic changes and consequent alterations in gene expression patterns. Recently, paternal exposure to chemicals has been reported to affect the germline epigenome which can promote propagation of epimutations to the offspring. Additionally, developmental exposure to chemicals can also induce transgenerational inheritable epigenetic changes which have significant roles in adult-onset disease susceptibility.

I thus hypothesized that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent Ahr ligand, may elicit robust epigenetic switching at the Ahr target gene *Cyp1a1*. Given that epigenetic changes can be stably passed through mitotic divisions I hypothesized that TCDD-induced epigenetic alterations can be maintained long after TCDD exposure, and these may have a significant impact on *Cyp1a1* inducibility. In the second study, I sought to analyze the transgenerational effect of developmental (*in utero*) as well as adult TCDD exposure on the *Cyp1a1* promoter methylation across three generations.

Materials and methods: In the first study, wild type ($Ahr^{+/+}$) female mice aged 10 weeks were orally treated with a single dose of TCDD (3 μg/kg bw) or corn oil. The liver, kidney and brain samples were obtained at 6 hrs, 12 hrs, 24 hrs, 7 days, 20 days and 40 days post dioxin treatment. Similarly, age matched Ahr knockout ($Ahr^{-/-}$) mice were given a single dose of TCDD (3 μg/kg bw) or corn oil. Liver samples were collected 24 hrs after TCDD treatment. DNA methylation was analyzed by methylation-sensitive enzyme restriction-qPCR (MSRE-qPCR) and

histone modifications by ChIP assay. In the second experiment of this study, two groups of female mice were either treated with 3 μg/kg TCDD orally or given corn oil. Forty days after the initial dose, 100 ng/kg bw of TCDD was re-administered to both the TCDD pretreated group and the control group. Liver samples were collected 24 hrs after dioxin treatment for *Cyp1a1* expression analysis. For the *in vitro* DNA demethylation assay, an artificially methylated *Cyp1a1* reporter plasmid and siRNA knockdown were utilized to analyze functional involvement of the DNA demethylation proteins in *Cyp1a1* promoter demethylation.

In the second study, pregnant female mice were orally treated with 3 μg/kg bw of TCDD on E12.5. The *in utero* TCDD exposed F1 males were bred to sexually naïve unexposed females at postnatal day 60. Further breeding was carried on until the F4 generation. For the second experiment, sexually naïve adult male mice received 3 μg/kg bw of TCDD orally at 10 weeks of age, and were designated as the F0. Breeding was then done 50 days after TCDD administration, with sexually naïve, untreated females. Further breeding was carried on until the F3 generation. Control animals that had been treated with corn oil were bred the same way as the treatment groups. To understand the biological consequence of the transmitted epigenetic phenotype, I repeat-challenged the progeny that were not directly exposed to TCDD, i.e., F1, F2 and F3 for the adult exposed (aTCDD) and F2, F3 and F4 for the *in utero* exposed group (*iu*TCDD). The repeat-treatment dose was 100 ng/kg bw TCDD, similar to Study1. The liver was harvested 24 hrs post treatment for *Cyp1a1* expression analysis. The liver and sperm DNA methylation was analyzed by MSRE-qPCR and gene expression by RT-qPCR.

Results: A single dose of TCDD elicited *Cyp1a1* transcriptional memory in the adult mouse liver, as evidenced by a three-fold super-induction of *Cyp1a1* in dioxin pre-treated animals. Notably, acute Ahr activation led to *Cyp1a1* promoter DNA demethylation, increases in H3K4me3, H4ac and a loss of H4K20me3 in $Ahr^{+/+}$ but not in $Ahr^{-/-}$ mice. These epigenetic changes persisted to 40 days post TCDD treatment, and constituted the epigenetic memory of initial TCDD exposure. In addition, Ahr recruited thymine DNA glycosylase (Tdg), an active demethylation factor, to the *Cyp1a1*promoter within 24 hrs after dioxin exposure. Further analysis using siRNA knockdown revealed that Ahr was required alongside the demethylation proteins (ten-eleven translocation methyldioxygenases) Tet2, Tet3 and Tdg in the initial *Cyp1a1* promoter DNA demethylation.

In the second study, there was liver *Cyp1a1* hypomethylation in directly exposed F0 aTCDD males. However, this epigenetic change was reset in the F1, F2 and F3 generations. Similarly, in the *iu*TCDD group, there was *Cyp1a1* promoter hypomethylation in the F1 liver, which was followed by methylation increase in the F2 progeny and recovery of the methylation levels to the control group levels in the F3 and F4 generations. The biological consequence of these epigenetic changes in the liver was a diminished *Cyp1a1* response to a second dose of dioxin up to the F2 generation of the aTCDD group, and the F4 of the *iu*TCDD group. To understand whether these phenomena correlated with sperm epigenetic changes, I measured the sperm *Cyp1a1* promoter methylation across all generations. Only significant hypomethylation in the F1

aTCDD progeny was observed in the sperm.

Discussion and conclusions: The first study provides novel evidence that Ahr drives epigenetic modulation and memorization at the *Cyp1a1* promoter and suggests that *Cyp1a1* transcriptional memory may play a role in adaptive response to dioxin exposure. Ahr activation by dioxin led to rapid, Ahr-dependent histone modification and active DNA demethylation of the *Cyp1a1* proximal promoter in the adult mouse liver. Ahr recruited Tdg to the *Cyp1a1* promoter, in addition to apurinic/apyrimidinic endonuclease 1 (Apex1) and Tet3 and resulted in a steady decline in 5-hydroxylmethylcytosine, an active demethylation pathway intermediate. Maintenance of this hypomethylated state and open chromatin conformation contributed to *Cyp1a1* transcriptional memory. Therefore, the histone modifications as well as DNA hypomethylation co-existed as epigenetic bookmarks for *Cyp1a1* super-induction in dioxin re-exposed animals. Transcriptional memory of the *Cyp1a1* gene may imply increased detoxification of endogenous *Cyp1a1* substrates or increased activation of procarcinogens (such as benzo(a)pyrene) to carcinogenic metabolites. Since Ahr directed the active DNA demethylation machinery to the *Cyp1a1* promoter, it can be anticipated that there is widespread epigenetic modulation of other Ahr target genes, although in a cell or tissue specific manner. Taken together, these findings highlight the importance of epigenetic mechanisms in downstream Ahr signaling and target gene regulation and indicate the possibility of wider roles of epigenetic memory in xenobiotic tolerance.

In the second study, my results bring to focus the impact of dioxin on the somatic and germline epigenome and its implications on physiological response and possibly disease susceptibility in subsequent generations. The observed methylation change in *iu*TCDD F2 can be considered to have resulted from an overcompensatory homeostatic response to reset the epigenetic terrain at the *Cyp1a1* promoter, resulting in partial reduction in xenobiotic response to dioxin. These results were in contrast with the Study1 findings where a single adult dose induced DNA demethylation and transcriptional memory that was characterized by stronger *Cyp1a1* induction upon repeat treatment with TCDD. This study provides some evidence of dioxin-induced epigenetic modulation at the *Cyp1a1* promoter. While the epigenetic changes do not appear to be transgenerational, the reduced response to subsequent dioxin exposure is sustained through generations and suggests molecular archiving of ancestral xenobiotic exposure.

In conclusion, both studies give evidence of both short-term epigenetic modulation and long-term epigenetic memory at the *Cyp1a1* promoter resulting in transcriptional memory. Across generations, I observe a contrariwise response, characterized by diminished *Cyp1a1* inducibility to subsequent dioxin exposure. Both observations, however, are indicative of short-term adaptive response to xenobiotic stress and an overcompensatory countermeasure to overwrite the TCDD induced epigenetic memory in descendant generations.