DIVISION OF BEHAVIORAL BIOLOGY (ADJUNCT)

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In mammals, several social behaviors are dependent on sex. These sex-dependent patterns of behavior must be acquired through highly irreversible processes during development. We hypothesize that the long-term effects of sex steroids at the perinatal stage on behaviors after puberty are somehow marked at the genome level. We are currently investigating the epigenetic status of the discrete brain areas responsible for the sex difference of the behaviors.

I. DNA methylation of sex steroid receptor genes

In mammals, DNA methylation, mainly occurring on CG dinucleotides, is a fundamental mechanism that differentiates the gene expression pattern in respective cells. DNA methylation is a restraint of the pluripotency because once the pattern is established during development it is maintained through cell division. On the other hand, for example, some fish that contain much less methylation activity are found to easily and reversibly change their sex status according to the environmental context. In rodents, endocrine disturbance at the fetal and/or postnatal stage irreversibly changes behaviors such as the lordosis (in females) and the mounting (in males) after the pubertal stage that are normally dependent on genetic sex. In some cases, lordosis can be observed even in males and *vice versa*. These clearly indicate that sex-dependent patterns of behavior are not directly dependent on ìs ex-specificî genes but rather established through epigenetic processes. We have found the sex- and brain area-dependent DNA methylation pattern of rodent steroid receptor genes (estrogen receptor α, *ERα*; androgen receptor, *AR*; progesterone receptor, *PR*) that can be modulated by the novel noncoding RNAs, named *ERαas, ARas* and *PRas*. We report herein epigenetic, expressional, endocrinological, and behavioral analyses of transgenic (Tg) mice constitutively expressing these noncoding RNAs.

1-1 Association of the noncoding RNA with the coding transcripts through epigenetic mechanisms in the brain

The number of mammalian noncoding RNA genes is rapidly expanding, necessitating the evaluation of their RNA function. Neither of the noncoding RNAs we found showed obvious open reading frames or possible amino acid motifs, raising the possibility that these function without translation. Each RNA overlapped with the respective promoter region including the sex-dependent differentially methylated region. These structural features led us to investigate the possible involvement of these noncoding RNAs on the establishment of the specific DNA methylation pattern *in vivo*.

We generated *ERαas*- and *ARas*-Tg mice. Most of the hypermethylated CG sites located on the sex-dependent differentially methylated region of *ERα* in intact adult females became hypomethylated in sex-matched *ERαas*-Tg mice (Figure 1). This effect seemed gene-specific, because DNA methylation status of *AR* did not differ between intact and *ERαas*-Tg females. Similarly, specific reduction of DNA methylation level in AR promoter region was observed in *ARas*-Tg mice (Figure 1). These findings conform to the previous *in vitro* experiment in which transient overexpression of *ERαas* or *ARas* fragment forced demethylation in a sequence-specific manner.

In general, the binding affinity of transcription factors is reduced by the hypermethylation that is frequently observed on the promoter region of either *ERα,* or *AR* in tumor cells. Therefore, we analyzed the expression of these coding transcripts. The results showed that *ERα* was expressed significantly higher in *ERαas*-Tg mice, but not

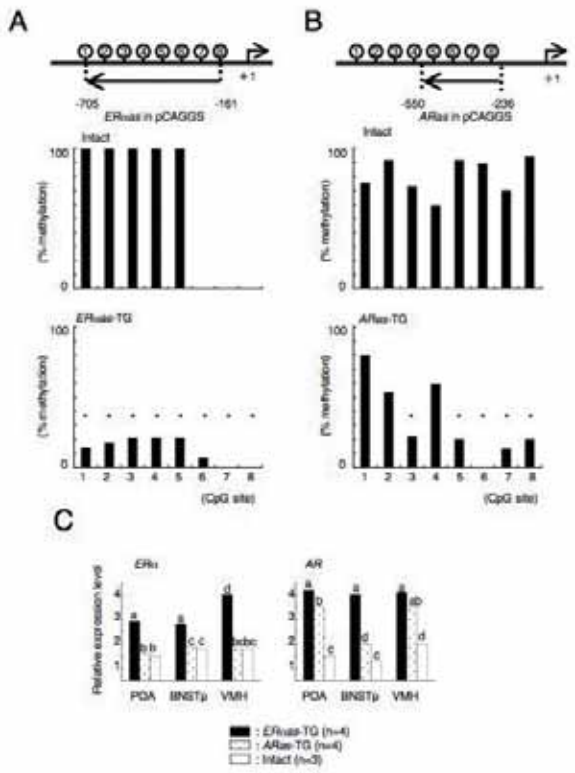

Figure 1. Deregulated epigenetic control of sex steroid receptor genes in *ERαas*- and *ARas*-Tg mice. (A) DNA methylation status of the *ERα* promoter in the BNSTp of intact and *ERαas*-Tg females. (B) DNA methylation status of the *AR* promoter in the VMH of intact and *ARas*-Tg females. BNSTp and VMH are the target brain areas for sex-dependent DNA methylation of *ERα* and *AR*, respectively. Asterisks denote the significant difference between intact and Tg mice. (C) *ERα* and *AR* expression in intact and Tg females analyzed by quantitative RT-PCR. Values with different letters indicate significant difference ($p<0.01$). POA, preoptic area; BNSTp, principal portion of bed nucleus of the stria terminalis; VMH, ventromedial hypothalamus.

in *ARas*-Tg mice, when compared with sex-matched intact mice (Figure 1), strongly suggesting that induction of DNA demethylation by promoter noncoding RNA upregulated the associated coding transcript. For *AR*, the results were a little complicated. *AR* was expressed higher in both *ERαas*- and *ARas*-Tg mice than in sex-matched intact mice. Since estrogen signaling has been realized to predominate the androgen receptor-mediated pathways in the masculinization of the rodent brain, this could explain the observed upregulation of *AR* not only in *ARas*-Tg but also in *ERαas*-Tg mice.

1-2 Prevailing effect of promoter noncoding RNA expression *in vivo*

ERαas- and *ARas*-Tg mice were anticipated to show the deregulated endocrinological and behavioral status. We investigated the female- and male-specific sexual behaviors by analyzing lordosis quotients and the number of mounts in these mice. Both Tg mice showed a significant reduction of lordosis quotients. Furthermore, females of *ARas*-Tg mice did mounting even though exogenous androgen treatment was not performed. We also measured the circulating testosterone and estrogen in these Tg mice and found that the level of these steroid hormones altered when compared with intact animals. We now postulate that the observed effects caused by the forced expression of *ERαas* and *ARas* reflect the impact of the corresponding endogenous noncoding RNAs. We are currently investigating the developmental aspect of the neural cells expressing/repressing these noncoding RNAs.

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