DIVISION FOR SEX DIFFERENTIATION

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Sexual dimorphism manifests most obviously in the gonads (testis and ovary), and is thereafter observed in other parts of the body such as the external genitalia, muscle, and brain. This process of sex differentiation is divided into three steps. The first step occurs at fertilization, during which the sexes of fertilized eggs are determined genetically according to a combination of sex chromosomes. During the second step, mammals carrying XY and XX sex chromosomes develop the testis and ovary, respectively. This gonad sex differentiation usually proceeds during fetal stages, and subsequently sex steroids synthesized in the sexually differentiated gonads control the sexes of the other tissues. Therefore, the gonad sexes are quite important for the sex differentiation of animals.

A number of transcription factors are known to play crucial roles in the process of gonad differentiation. Some of these genes, such as *SRY*, *WT1*, *DAX-1*, *SOX9* and *ARX,* were identified as the genes responsible for human diseases that display structural and functional defects in the gonads. Functions of the other genes such as *Ad4BP/SF-1*, *Emx2*, *M33*, and *Lhx9* were elucidated by the phenotypes of the gene-disrupted mice. In addition, their expression profiles in the sexually differentiating gonad strongly suggested their functional significance at the early stage of gonad differentiation. However, it remains to be elucidated how the genes are expressed by upstream regulators. Studies considering this aspect of sex differentiation are quite important in order to define the gene regulatory cascade and the molecular mechanisms mediating sex differentiation of the gonad.

Tentatively, we have hypothesized that the sexually indifferent gonads determine their sexes under the control of two opposite signals: the signal for male (testicular) differentiation and the signal for female (ovarian) differentiation. It is possible to assume that the signals are transcriptional activities driven by the transcription factors expressed in the sexually differentiating gonads or other types of growth factors. This divisionís research has focused primarily on the transcriptional control of the genes implicated in gonad sex differentiation.

I. Structure and Function of *Ad4BP/SF-1* gene locus

Ad4BP/SF-1 is expressed in the testicular Leydig and Sertoli cells, ovarian theca and granulosa cells, adrenocortical cells, pituitary gonadotropes, and ventromedial hypothalamic nucleus, and the expressions are tightly controlled during the tissue development processes. It is well accepted that the spatial and temporal control of gene expression is essential for the establishment of cell fates, and gene transcription is thought to be controlled through appropriate interaction between enhancers and the basal promoter. In the case of *Ad4BP/SF-1*, the tissue-specific enhancers should be localized somewhere in the gene locus and functionally correlated with the basal promoter localized upstream of the first exon. Evidently, functional interaction between the enhancers and the basal promoter is one of the fundamental elements required for tissue specific expression. Therefore, it was expected that the primary structure of the enhancer would provide information to understand the mechanisms underlying tissue development and gonad sex differentiation. Based on this concept, we have conducted transgenic mouse assay (TG mouse assay) with fragments prepared from the *Ad4BP/SF-1* gene locus and bacterial *lacZ* gene as the reporter. As indicated in Figure 1, we analyzed whether 20-30 kb long fragments prepared from a mouse BAC clone contain enhancer sequences or not. Two fragments, cGcnf5 and cIA3, gave lacZ signal in the fetal adrenal, ventromedial hypothalamus, and pituitary. Positive fragments were further examined through the TG mouse assays using deletion mutants. Consequently, we confirmed the location of the enhancer sequences for the fetal adrenal cortex in the fourth intron while those for the pituitary gonadotrope and ventromedial hypothalamic nucleus were located in the sixth intron.

In general, it is well known that functional genomic sequences such as exon, basal promoter, and splicing site are structurally conserved among animal species. We asked whether the functional enhancer sequences are conserved among vertebrate animal species. As expected, sequence comparisons revealed that the sequences were actually conserved in such mammalian species as human, mouse, rat, etc. Since the conserved region should contain core sequences to be recognized by certain transcription factors, the conserved regions were further analyzed by introducing nucleotide substitution. These constructs were subjected to the TG mouse assays and thus the functionally active core sequences composing the tissue-specific enhancers were finally identified.

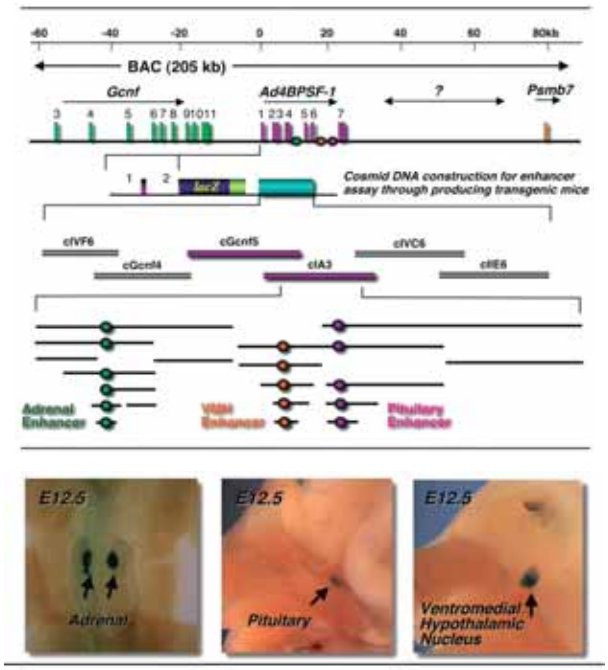

Figure 1, Identification of enhancers for the fetal adrenal, ventromedial hypothalamus (VMH), and pituitary of the mouse Ad4BP/SF-1 gene. (upper panel) The Ad4BP/SF-1 gene consists of seven exons (red boxes). Two other genes, Nr6a1 (green boxed) and Gpr144 (region indicated by?), are localized 5í upstream and 3í downstream of the Ad4BP/SF-1 gene, respectively. Positions and structures of these genes were obtained from the NCBI database (Entrez Gene). A 6-kb fragment of the Ad4BP/SF-1 promoter region from the initiation methionine in exon 2 was used to generate the basal vector by ligation to the bacterial lacZ gene followed by SV40 poly (A) signal. Various DNA fragments were inserted downstream of lacZ, and subjected to transgenic mouse assays. Among the clones covering the Ad4BP/SF-1 locus, cIA3 induced lacZ expression in fetal adrenal, ventral diencephalon (VMH), and pituitary, while cGcnf5 induced lacZ expression only in the fetal adrenal. cIVC6 induced no lacZ expression. These active fragments were further fragmented into smaller pieces and subjected to the TG mouse assays. Finally, the enhancers for the fetal adrenal was localized in the fourth intron, while those for the VMH and pituitary were in the sixth intron. (lower panel) Representative expression patterns of lacZ at embryonic day 12.5 are shown.

II. Two-Step Regulation by Fetal Adrenal Enhancer

In order to identify the sequences responsible for the fetal adrenal enhancer function, we compared the nucleotide sequences of the enhancer regions of mice and humans. Among the sequences conserved between the two animals, we noted the presence of two potential Ad4BP/SF-1 binding sites. When analyzed by electrophoretic mobility shift assays, Ad4BP/SF-1 associated with the potential binding sites. Since Ad4BP/SF-1 activates gene expression by binding to recognition sites, the presence of the Ad4 sites in the fetal adrenal enhancer suggested that Ad4BP/SF-1 has a role in the transcriptional regulation of gene expression in the fetal adrenal gland. To examine this hypothesis, we generated TG mice harboring a construct carrying mutations in the two Ad4 binding sites. When we examined TG mouse fetuses at E11.5, lacZ expression in the mutant construct was similar to the wild-type construct. However, at E17.5, the lacZ signals disappeared from the TG fetuses with the mutated construct. To determine whether Ad4 sites were responsible for lacZ expression at E17.5, mutations were introduced in both Ad4 sites. TG assays with these constructs revealed that the Ad4 sites are essential for this expression pattern, and we subsequently examined the enhancer activities of constructs with a single site mutation. Since neither mutation led to the disappearance of the lacZ signal, a single site is sufficient to drive lacZ expression in the fetal adrenal gland. These results strongly suggest that Ad4BP/SF-1 binding to the Ad4 sites in the fetal adrenal enhancer participate in the autoregulation of *Ad4BP/SF-1* gene expression in the adrenal cortex at later stages of fetal development.

The data above suggests that Ad4BP/SF-1 maintains its own expression at later stages of development, but it remains unclear how Ad4BP/SF-1 expression is controlled prior to the maintenance phase of transcription. To address this question, we attempted to identify functional *cis*-elements other than the Ad4 sites present in the fetal adrenal enhancer. When the sequence of this DNA fragment was examined in detail, we found potential binding sites for the Pbx/Prep and Pbx/Hox heterodimers present in the upstream region proximal to the Ad4 site (Figure 2). Since both sites are conserved in human and chick, we examined whether those factors are expressed in the adrenal primordium. RNAs prepared from the isolated adrenal primordium were used for RT-PCR analyses to examine candidate gene expression.

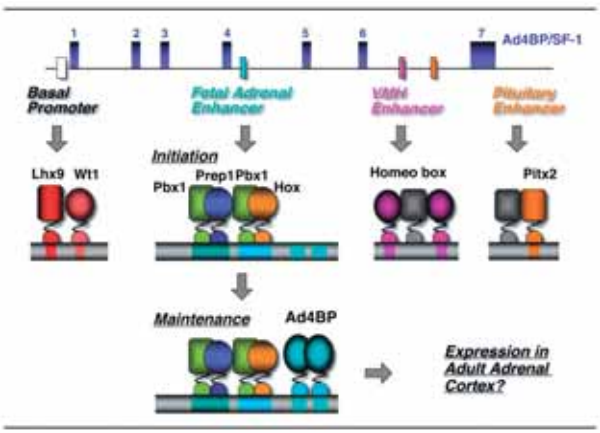

Figure 2, Schematic presentation of structures and functions of the tissue-specific enhancers and basal promoter of the Ad4BP/SF-1 gene. Transcription factors, Lhx9 and Wt1, are thought to bind the basal promoter, while Pbx1, Prep1, and Hox proteins are able to bind the fetal adrenal enhancer as a ternary complex to initiate transcription at the adrenal primodial cells. At a later stage, the gene product, Ad4BP/SF-1, binds to the enhancer to maintain the expression. Homeobox-containing proteins together with unidentified protein bind to VMH enhancer while Pitx2 acts as an essential factor to drive the pituitary enhancer. Although still unclear, a factor functionally correlated with Pitx2 is required for the enhancer function.

As expected, RT-PCR revealed that all candidate factors were successfully detected in the early adrenal primordium. Thus, we examined whether the Pbx/Prep and/or Pbx/Hox binding sites are functional or not. When either the Pbx/Hox or Pbx/Prep binding sites was mutated, weak lacZ signals were still observed in the fetal adrenal tissue. As expected, when both the Pbx/Hox and Pbx/Prep sites were mutated, lacZ expression was completely absent from the adrenal primordia. Importantly, this transcriptional activity driven by the Pbx/Hox and Pbx/Prep sites was active even though Ad4 sites were mutated.

Hox transcription factors direct the patterning of a variety of structures during the embryonic development of vertebrates and invertebrates through regulating numerous target genes. Based on the expression profile and binding specificity, *Hoxb5*, *Hoxb9*, *Hoxc5*, and possibly *Hoxc6* were thought to regulate the *Ad4BP/SF-1* expression in the fetal adrenal. The adrenal cortex is known to be derived from a certain part of the intermediate mesoderm lying along the anterior to posterior axis. To specify the adrenal region, these *Hox* gene products are thought to induce Ad4BP/SF-1 expression at that particular region of the mesoderm. As it has been established that *Hox* genes control anterior to posterior axial identity through regulating target gene expression, the location of the adrenal cortex could be determined by combined expression of the particular set of the *Hox* genes.

III. Asymmetric ovarian development in birds

Mammalian gonads develop bilaterally, and this is achieved through the orchestrated action of a number of genes. Many of these genes have been identified through the study of knockout mice as well as patients suffering from gonad developmental abnormalities. However, no identified gonad defects exhibit clear LñR asymmetry, and no genes involved in gonad development are expressed asymmetrically in the L-R axis. In contrast to the situation with mammals, most female birds develop ovaries only on their left side, while males develop bilateral testes. During the early sexually-indifferent stage, chick embryonic gonads show no obvious morphological LñR asymmetry, and they consist of two components, the cortex and medulla. After sexual differentiation, testicular development occurs bilaterally in the male (genetically ZZ). The testicular cords appear in the medulla where Sertoli and Leydig cells differentiate, while the cortex regresses and eventually disappears. In female birds (genetically ZW), the left cortex proliferates and develops into the ovary, while the right cortex disappears. Such asymmetric gonad development has not been described in other vertebrates, and the process regulating avian gonad development is interesting from both evolutionary and developmental perspectives. We have studied the molecular mechanisms for this asymmetric ovarian development in collaboration with Prof. Yoshioka (Hyogo Univ. of Teacher Education).

The study revealed that homeobox gene *PITX2* is expressed asymmetrically in the left presumptive gonad and this asymmetric expression induces the asymmetric expression of the retinoic-acid-catabolizing and -synthesizing enzymes, *CYP26A1* and *RALDH2*, respectively. Subsequently, retinoic acid suppresses the expression of *Ad4BP/SF-1* in the right ovarian primordium. Conversely, Ad4BP/SF-1 expressed in the left ovarian primordium asymmetrically upregulates *cyclin D1* to stimulate cell proliferation. Leftñright nodal asymmetry is transferred to the left lateral plate mesoderm through induction of *PITX2*. Interestingly, avian but not mouse *PITX2*/*Pitx2* expression expands throughout the left presumptive gonad derived from the lateral plate mesoderm, most likely ultimately leading to the observed ovarian asymmetry. We thus provided a mechanism linking early embryonic *PITX2* expression with subsequent asymmetric visceral organ development with particular emphasis on bird ovarian development.

Publication List:

Original papers

Zubair, M., Ishihara, S., Oka, S., Okumura, K., and Morohashi, K. (2006). Two-step regulation of *Ad4BP/SF-1* gene transcription during fetal adrenal development; initiation by a Hox-Pbx1-Prep1 complex and maintenance via autoregulation by Ad4BP/SF-1. Mol. Cell. Biol. *26*, 4111-4121.

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