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 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 division's research has focused primarily on the transcriptional control of the genes implicated in gonad sex differentiation.

Ⅰ. Function of Forkhead transcription factor Fkhl18 during testicular vasculature development

There is a general agreement that gonad sex determination in mammals is a process initiated by *Sry* gene. Downstream of *Sry*, *Sox9* specifies Sertoli cell lineages, organization of the testicular cord, and production of male hormones. In addition, the vasculature system develops differentially between the testis and ovary. Before the actions of *Sry* are evoked at around embryonic day 11.0 (E11.0), the structure of the primitive vasculature in the genital ridge is similar irrespective of sex. During the early phase of gonad sex differentiation, however, the mesonephric cells migrate vigorously into the developing testis to form a vasculature structure characteristic of the testis. In contrast, no such active cell migration is observed in the developing fetal ovary. This difference gives rise to sexually dimorphic vascular patterns in the gonads. Especially, in the testis, a large artery is formed at the coelomic surface at around E12.5. This male-specific vascular system that develops during fetal life is thought to be required for the export of testosterone from the testis to the rest of the fetus to ensure masculinization.

Vasculogenesis starts during fetal development. Precursor cells for blood vessel endothelia, which share their origins with hematopoietic progenitors, assemble into a primitive vascular network of small capillaries. Subsequently, the vascular plexus progressively expands by sprouting and matures into stable blood vessels. During this phase of angiogenesis and arteriogenesis, nascent endothelial cells become covered by periendothelial cells (pericytes and smooth muscle cells) and association with these cells is required to regulate proliferation, survival, migration, differentiation, vascular branching, blood flow and vascular permeability.

Forkhead transcription factors are characterized by a winged helix DNA binding domain, and the members of this family are classified into 20 subclasses by phylogenetic analyses. *Fkhl18* is structurally unique, and is classified in the FoxS subfamily. We found *Fkhl18* expression in periendothelial cells of the developing mouse fetal testis. In an attempt to clarify its function, we generated mice with *Fkhl18* gene disruption. Although KO mice developed normally and were fertile in both sexes, we frequently noticed unusual blood accumulation in the fetal testis. Electron microscopic analysis demonstrated frequent gaps, measuring 100-400 nm, between the endothelial cells of blood vessels. To visualize the entire structure of the testicular vasculature system, we injected carbon ink into the umbilical vein of E14.5 fetuses. Although the whole view of branches of the vasculature system was indistinguishable

between the wild-type and *Fkhl18* KO testes (Figure 1a, b), the area around the vasculature looked dark and blurred in the *Fkhl18* KO testes. The gonads were subsequently sectioned, and we found leaking of the injected carbon ink from the testicular vessels and coelomic vessel. The leakage of the carbon ink suggested a defect in the sealing structure of the vasculature in the *Fkhl18* KO mice.

These gaps probably represented ectopic apoptosis of testicular periendothelial cells, identified by caspase-3 expression, in KO fetuses. No apoptosis of endothelial cells was noted. *Fkhl18* suppressed the transcriptional activity of *FoxO3a* and *FoxO4*. Considering that *Fas ligand* gene expression is activated by Foxs, the elevated activity of *FoxOs* in the absence of Fkhl18 probably explains the marked apoptosis of periendothelial cells in *Fkhl18*-KO mice.

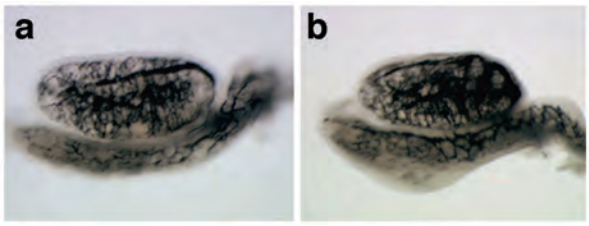

Figure 1. Visualization of the vasculature structure using carbon ink. Carbon ink was injected into the testes through the umbilical vein. The whole views of the wild-type (a) and *Fkhl18* KO testes (b) are shown.

Ⅱ. Function of Dioxin Receptor (AhR) in the Male Reproductive Tract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix (bHLH)-PAS super-gene family. Since AhR can bind with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the molecular properties of AhR as a transcription factor have been extensively studied. Recently, the intrinsic functions of AhR have been investigated with regards to animal reproduction. Indeed, our recent study in *AhR*(-/-) mice demonstrated that AhR is involved in female reproduction by regulating estradiol synthesizing *Cyp19* (*P450 aromatase*) gene expression. Based on the essential functions of estradiol in the female reproductive process such as folliculogenesis, ovulation, and implantation, it was concluded that AhR plays an indispensable function in female reproduction. Its function in male reproduction, however, remained unknown.

The accessory internal reproductive systems, derived from the Wolffian duct for males and from the Mullerian duct for females, are clearly different between the two sexes. The male internal reproductive system consists of multiple tissues such as the epididymis, the deferens duct, the seminal vesicle, the coagulating gland, and the ejaculatory duct. Developmentally, all these tissues are known to be regulated by androgen signaling. The mature seminal vesicle consists of numerous outpouchings of alveolar glands that empty into the ejaculatory duct. Although semen mostly contains materials secreted from the seminal vesicle, a definite functional relationship linking the seminal vesicle to male fertility has yet to be elucidated. The coagulating gland secretes a substance that, when mixed with the secretions from the seminal vesicle, forms a vaginal plug, and it has been thought that the vaginal plug is required for efficient pregnancy after insemination.

We showed age-dependent regression of the seminal vesicles, probably together with the coagulating gland, in *AhR*(-/-) male mice. The KO mice had abnormal vaginal plugs, low sperm counts in the epididymis, and low fertility. Moreover, serum testosterone concentrations and expression of steroidogenic 3ß hydroxysteroiddehydrogenase (3ßHsd) and steroidogenic acute regulatory protein (StAR) in testicular Leydig cells were decreased in *AhR*(-/-) males. Taken together, our results suggest that impaired testosterone synthesis in aged mice induces regression of seminal vesicles and the coagulating glands. Such tissue disappearance likely resulted in abnormal vaginal plug formation, and eventually in low fertility. Together with previous findings demonstrating AhR function in female reproduction, AhR has essential functions in animal reproduction in both sexes.

Publication List

〔Original papers〕

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