学位論文内容の要旨

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学 位 論 文 題 目

Role of follicle-stimulating hormone in gonadal sex differentiation in Nile tilapia, *Oreochromis niloticus*

ナイルティラピア性分化における濾胞刺激ホルモンの役割に関する研究

The beginning of Estradiol-17β (E2) production is the key event for the initiation of ovarian differentiation in Nile tilapia. Aromatase encoded by *cyp19a1a* gene is the enzyme that converts testosterone into E2. Although two transcriptional factors such as *foxl2* and *ad4bp*/*sf1* are involved in *cyp19a1a* expression in the Nile tilapia, follicle stimulating hormone (FSH) is also considered an additional factor to induce the expression of *cyp19a1a*. The expression levels of FSH were similar in pituitaries between genetic female (XX) and genetic male (XY) during 3-25 days after hatching (dah), while FSH receptor (*fshr)* showed significant higher expression in XX undifferentiated gonads than XY undifferentiated gonads in this period. Therefore, FSH signaling is possible to involve in the ovarian differentiation via inducing *cyp19a1a* expression.

In the second chapter, to investigate whether FSH signaling has roles for E2 production through expressions of steroidogenic enzymes and sex differentiation-related genes, recombinant FSH (rFsh) was injected into XX and XY larvae in 5-6 dah, 7-8 dah, and 9-10 dah during the early molecular sex differentiation period. For injection experiments, rFsh was dissolved in PBS buffer. As control groups, two groups were set. One was the no injection group and another was the PBS injection group. After two rounds rFsh injections, the expression levels of possible sex differentiation-related genes in the isolated undifferentiated gonads were measured by qPCR. The expression level of *hsd3b* in XX and XY undifferentiated gonads was significantly higher in the rFsh injection group than in the no injection and the PBS injection groups. The expression levels of *cyp11a1* and *cyp17a1* tended to be higher in rFsh injection group than in other control groups in XX and XY undifferentiated gonads. The expression level of *cyp19a1a* tended to be higher only in XX undifferentiated gonads. These results suggested that FSH signaling may play roles to induce expression of *cyp11a1*, *hsd3b*, *cyp17a1* and *cyp19a1a* and then contribute E2 production to initiate ovarian differentiation in XX Nile tilapia. However, those levels induced by FSH stimuli seemed not very strong, therefore, the role of FSH signaling on E2 production may be limited, suggesting a supplemental role for activation of steroidogenesis during the molecular sex differentiation period in XX tilapia. Moreover, FSH signaling is involved in the expression of *cyp11a1*, *hsd3b*, and *cyp17a1* to contribute androgen production in testis development in XY Nile tilapia. Although FSH signaling may be involved in androgen production, it is not a key role for the activation of steroidogenesis during the molecular sex differentiation period due to the low expression levels of *fshr* and genes related with steroidogenesis in XY tilapia.

The expression patterns of *foxl2* and *cyp19a1a* are similar with sexually dimorphic. Furthermore, the expression levels of *ad4bp*/*sf1* and *fshr* are higher in XX gonads than XY gonads during 5-7 dah. The third chapter investigated the plausible role of FSH signaling in ovarian differentiation through

transcriptional regulation of *cyp19a1a* in Nile tilapia. Plasmids encoding *foxl2* and *ad4bp*/*sf1* from Nile tilapia ovary cDNA and *cyp19a1a* promoter region were cloned from genomic DNA to determine the function of FSH signaling in *cyp19a1a* regulation and relationships among FSH signaling, *foxl2*, *ad4bp*/*sf1* and *cyp19a1a*. Under *in vitro* conditions in HEK293T cells, *foxl2* can interact with *ad4bp*/*sf1* to form a heterodimer and enhance the *ad4bp*/*sf1* mediated *cyp19a1a* transcription. Although FSH signaling alone cannot induce the expression of *cyp19a1a*, FSH signaling can enhance *cyp19a1a* transcription in HEK293T cells expressing *foxl2* and *ad4bp*/*sf1*. Altogether, these results suggest that FSH signaling plays a role in the ovarian differentiation of the Nile tilapia by regulating aromatase expression and possibly the entire steroidogenic pathway.

In Nile tilapia, FSH secreted from the pituitary gland is expressed in both genetic male and female from 3 dah. On the other hand, the expression level of *fshr* in the undifferentiated gonads is higher in genetic XX than in genetic XY from 5 dah. Therefore, FSH signaling is thought to play a role in initiating ovarian differentiation. However, it is not clear how FSH signaling affects the expression of sex differentiation-related genes, and no clear conclusion has been reached regarding the involvement of FSH signaling in sex differentiation. In the fourth chapter, to clarify the involvement of FSH signaling in Nile tilapia ovarian differentiation, it was necessary to generate genetic XX *fshr* mutant Nile tilapia by CRISPR/Cas9. First, guide RNA specific to the target gene *fshr* was microinjected into XX 1-2 cell embryos, resulting in the production of five mutant F0 individuals. Testis-differentiated genetic XX mutant F0 individuals were crossed with wild-type XX individuals to obtain genetic XX F1 individuals. Subsequently, genetic XX F1 individuals were crossed with each other to produce homozygous mutant genetic XX F2 individuals. Gonads of genetic XX F2 wild-type individuals, heterozygous mutant individuals, and homozygous mutant individuals were sampled, and then histological observation of them was conducted. In addition, the mRNA expression levels of sex differentiation-related genes in these gonads were measured by qPCR. In the F2, among the 24 heterozygous XX individuals, seventeen had normal ovaries and seven had testes. In the F2 heterozygous mutant female individuals, the mRNA expression levels of sex differentiation-related genes were not significantly different from those of the F2 wild-type XX individuals. On the other hand, in F2 heterozygous mutant male individuals, the mRNA expression levels of *cyp11a1*, *hsd3b*, *cyp17a1*, *ad4bp*, *lhr*, *gsdf* and *dmrt1* were significantly higher than those of F2 wild-type XX individuals. The expression level of *cyp19a1a* was significantly lower in F2 heterozygous mutant male individuals than those in F2 wild-type XX individuals. The expression levels of *hsd17b1* and *foxl2* tended to be lower in F2 heterozygous mutant male individuals than those in F2 wild-type XX individuals. All F2 homozygous mutant XX individuals had small ovaries, the oocytes did not develop beyond the primary growth phase. No individuals with testes were observed. The expression levels of *cyp11a1*, *cyp17a1,* and *cyp19a1a* were significantly lower in genetic XX F2 homozygous mutant individuals than those in F2 wild-type XX individuals. The expression levels of *hsd3b*, *hsd17b1*, *foxl2*, *ad4bp*, and *lhr* tended to be lower in genetic XX F2 homozygous mutant individuals compared to wild-type XX individuals. The mRNA expression levels of *gsdf* and *dmrt1* in genetic XX F2 homozygous mutant individuals were not significantly different from those of wild-type XX individuals in F2. These results suggest that FSH signaling plays a role in regulating the expression of *cyp11a1*, *cyp17a1,* and *cyp19a1a*, but its effect on ovarian differentiation is not strong and it may only play a supporting role.

In summary, in XY Nile tilapia, FSH signaling may be involved in androgen synthesis by affecting the expression of *cyp11a1*, *hsd3b*, and *cyp17a1* to induce testis development during morphological sex differentiation from 25 dah. Thus, loss of *fshr* in XX cannot cause sex reversal

while only genetic XX heterozygous *fshr* mutant Nile tilapia changed sex and became male. In XX Nile tilapia, FSH signaling is not only induce the expression of *cyp19a1a* with *foxl2* and *ad4bp*/*sf1*, but also induces the expression of *cyp11a1*, *hsd3b*, *cyp17a1* to contribute E2 production to be involved in ovarian differentiation and ovarian development in XX Nile tilapia. Moreover, FSH signaling is essential in folliculogenesis and the loss of *fshr* resulted in a complete failure of follicle development. There was no genetic XX male homozygous *fshr* mutant Nile tilapia, while homozygous *fshr* mutants in genetic XX medaka and female zebrafish can achieve sex reversal and fertility with alternative mechanisms between FSH and LH signaling. Therefore, FSH signaling is involved in ovarian differentiation through affecting *cyp11a1*, *hsd3b*, *cyp17a1*, and *cyp19a1a* expression levels. When the expression level of *fshr* is enough, undifferentiated gonads in genetic XX can develop into ovaries. When the expression level of *fshr* is not enough, undifferentiated gonads in genetic XX cannot develop into ovaries and sex reversal will happen due to lower E2 during molecular sex differentiation period leading to higher expression levels of *dmrt1* and *gsdf*. FSH signaling regulates ovarian differentiation and follicle development in Nile tilapia, mainly by elevation of estrogen levels.

In conclusion, FSH signaling facilitates E2 production by inducing the expression of steroidogenic enzymes although the effect is not strong. FSH signaling induces transcription of *cyp19a1a* at a higher level in the presence of *ad4bp*/*sf1* and *foxl2*. Under weak FSH stimulation, a part of XX tilapia fails to develop an ovary, therefore, it differentiates to the testis. However, no FSH signaling leads the XX gonads into the very small ovary probably because testicular development does not proceed because of very low steroidogenic activity. Taking all together, FSH has supporting roles for the initiation of ovarian differentiation through boosting E2 production. This study revealed the roles and involvement of FSH on the Nile tilapia gonadal sex differentiation and contributes to understanding the mechanism of sex differentiation in fish.