博士（水産科学）　闫　　红 伟

学 位 論 文 題 名

Studies on involvement of gonadotropic hormones and their receptors on sex differentiation in Nile tilapia, *Oreochromis niloticus*

（ナイルティラピアにおける生殖腺刺激ホルモンの性分化への関与に関する研究）

学位論文内容の要旨

Mechanisms of sex determination and differentiation in vertebrates have been studied for many years, and gonadal sex differentiation in vertebrates is controlled by strict regulation of specific gene expressions that initiate and direct its developmental pathway. However, the gene regulation and network involving differentiation remains largely unknown in vertebrates including teleost. Nile tilapia is considered as not only an economically important fish, but also a useful teleost model for studying mechanism of sex determination and differentiation. Therefore, studies for sex determination and differentiation have been well conducted in this species. In Nile tilapia, sex-specific expression of forkhead box L2 (*foxl2*) and *cyp19a1a* (Encoding aromatase which convert testosterone into estradiol-17β) in XX gonads, and *doublesex and mab-3 related transcription factor 1* (*dmrt1*) and *gonadal soma-derived growth factor* (*gsdf*) in XY gonads during early gonadal differentiation 5-6 days after hatching (dah) is critical for differentiation of the gonads into either ovaries or testes. In addition, recent studies have demonstrated that the endogenous estradiol-17β (E_2) is a key steroid hormone, and aromatase (cyp19a1a) is a key enzyme which determines ovarian differentiation in tilapia, as well as in reptiles, and birds. However, the gene cascade leading to the female specific expression of *cyp19a1a*, and the subsequent ovarian differentiation has been poorly investigated in fish, including the tilapia. The gonadotropic hormones (GTHs), which belong to the glycoprotein hormone family and are secreted from the pituitary gland, are key hormones in regulating gametogenesis. GTHs include the follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which consist of an identical glycoprotein α-subunit (CGA) and a hormone-specific β-subunit (FSHB and LHB). Gonadotropins act on the gonadal tissue *via* their cognate receptors, designated FSH receptor (FSHR) and

LH/choriogonadotropin receptor (LHCGR). Although the primary factor that stimulates *cyp19a1a* gene expression and enzyme activity is FSH in mammals, whether the GTHs (particularly FSH) are involved in regulation of *cyp19a1a* gene expression and play a role in early sexual determination and differentiation has been unclear in fishes yet. Therefore, the present study aimed to address the involvement and roles of GTHs on sex determination and differentiation in Nile tilapia.

In Chapter 2, as a first step of this study, the precise timing of expression of GTHs and their receptors in genetic all-female (XX) and genetic all-male (XY) tilapia during early sexual differentiation were investigated by quantitative RT-PCR (qRT-PCR) and immunohistochemical analysis. Expression of *cga* mRNA, *fshb* mRNA and FSH protein in the pituitary was detected on the first sampling day (3 dah) to 25 dah in both XX and XY tilapia larvae without sexual dimorphism, and the levels increased gradually after 25 dah in the pituitary. *Fshra* mRNA was expressed as early as 5 dah and was present at significantly higher levels in the XX gonads than in the XY gonads in 6-25 dah. Moreover, *lhb* mRNA was not detected until 25 dah in the pituitaries of both sexes, and sexual dimorphism in *lhcgrbb* mRNA levels appeared later (10–25 dah) than that of *fshra* in the gonads. These results indicate that up-regulation of *fshra* might be necessary to induce female-specific *cyp19a1a* expression, which in turn, initiates E_2 production and subsequent ovarian differentiation in female. In contrast, LH and LHCGRBB seems play limited or no role in regulating gonadal differentiation of the Nile tilapia.

Detailed information about the influences of masculinization or feminization process, especially the influences of absence or presence of E_2 on the expression profiles of GTHRs can be a potent help for understanding of the relationship between FSH signaling and ovarian differentiation. Therefore, in Chapter 3, 100% sex-reversal XX males populations were induced by AI (letrozole, aromatase inhibitor, 200 mg/kg food) or MT (17α-methyltestosterone, 10 mg/kg food) with 4 times treatments per day during 9-20 dah, and 100% sex-reversal XY females populations were obtained by E_2 (900 ng/ml) exposure during 4-10 dah, respectively. Using those sex-reversal populations, changes in expression profiles of GTHRs in gonads during sex reversal processes induced by AI, MT, and E_2 were examined by qRT-PCR. The expressions of both *fshra* mRNA and *lhcgrbb* mRNA were suppressed by AI or MT treatment with XX fry. However, they showed different expression patterns during masculinization. By using MT and AI treatment, the expression levels of *fshra* mRNA was down-regulated just after the beginning of the treatments. These results suggested that

the suppression of E_2 synthesis may induce the down-regulation of *fshra* mRNA expression during masculinization process. This may indicate that *fshra* expression may lie under the E_2 signal. Moreover, it is also suggested that there might be a positive feedback between FSH signaling and E_2, and *fshra* might be involved in the positive regulation of the expression of *cyp19a1a* and E_2 production in Nile tilapia. However, following feminization by E_2 treatment, compared with the control XX gonads, the high expression levels of *fshra* mRNA was not observed in E_2-treated XY gonads before 15 dah. The results seem contradict the initial assumption based on the MT and AI masculinization experiments. This difference may attribute to the rather unusual physiological conditions of this sex inversion treatment. The endogenous estrogen may still not be sufficient to induce high *fshra* expression before the E_2 treatment (4-10 dah) terminated. After 15 dah, endogenous estrogen synthesis may provide sufficient E_2 which favor the up-regulation of the expression of *fshra*, therefore, the female expression pattern of *fshra* was observed. After MT treatment, the expression of *lhcgrbb* mRNA during 10-25 dah was repressed rapidly. However, in different from *fshra*, the suppression of *lhcgrbb* expression was not observed before 20 dah in AI-treated gonads, which occurred in an obviously delayed pattern compared with that in the MT-treated gonads. Moreover, during E_2 induced feminization process, the expression of *lhcgrbb* did not show the induced female expression pattern in the E_2-treated XY gonads before 15 dah. At 25 and 40 dah, *lhcgrbb* mRNA showed similar expression patterns in the induced-masculinized gonads and the control XY male gonads, while showed the similar expression patterns in induced-feminized gonads and the control XX female gonads. It is implied that the expression of *lhcgrbb* may not be effected by E_2, and LH and LHCGRBB seems not to play a role in the sex differentiation in Nile tilapia.

In Chapter 4, in order to examine whether disruption of FSH signaling has influence on the expression of *cyp19a1a*, E_2 production and ovarian differentiation, morpholino (MO)-knockdown of *fshra* and *fshb* gene expression was carried out. *fshra* and *fshb* morpholino antisense oligonucleotides were single- or co-microinjected into tilapia 1-2 cell stage embryos, then the changes in the expression of cyp19a1a and gsdf were examined using immunohistochemical analysis with tilapia-specific antibodies. At 40 or 50 dah, whether the female gonads undergoing masculinization process were determined by histological observation of the gonads. In addition, to verify the effect of the MO antisense oligo, the expression of the FSH protein in the *fshb* MO

injected fry were examined. The immunohistochemical analysis demonstrated that no expression of FSH protein was observed in all of the abnormal larvae or most of the normal larvae at 4 and 7 dah in the *fshb*-MO injected group, which indicated that microinjection of the MO successfully caused the depletion of the target FSH protein. However, it is impossible to check the effective of the *fshra* MO microinjection due to the low expression level of *fshra* mRNA in the gonads as well as the anti-tilapia fshra antibody is unavailable as shown previously. The results with MO knockdown showed specific effects on tilapia embryonic development. However, none of the defects seemed related to reproduction, indicating FSH signaling may involve in non-reproductive function during early embryonic development. Moreover, similar with the control XX female tilapia, the XX female tilapia injected or co-injected with MO showed normal cyp19a1a expression without increasing gsdf expression in the gonad at 10 and 25 dah. At 40 or 50 dah, morphological observation reveals that normal ovarian differentiation was developed in the gonads of XX female tilapia after FSH signaling depletion. It is indicated that FSH signaling may locate just downstream of E_2 signal and suppression of FSH signaling do not interfere the normal ovarian differentiation. Additionally, due to FSH protein can be detected in the *fshb*-MO injected larvae at 10 dah, it is also possible that the uncompleted knockdown of FSH signaling after 10 dah could not influence the normal ovarian differentiation.

Based on these findings in the present study, LH and LHCGRBB seems not play a role in the sex differentiation in Nile tilapia. On the other hand, it is suggested that FSH signaling plays some roles in the ovarian differentiation. While the roles and functions of FSH signaling on sex differentiation have not been addressed in this study, yet it is indicated that FSH signaling may not involved in the regulation of cyp19a1a expression and E_2 signal directly. Rather, FSH signaling may work for ovarian differentiation after receiving E_2 signaling which begins after 5 dah. Although further studies are necessary to characterize the clear roles of FSH signaling on ovarian differentiation, this study reveals a novel aspect on a mechanism of gonadal sex differentiation and provides useful knowledge for assessing and controlling sex of fish which is important for aquaculture industry.

学位論文審査の要旨

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Studies on involvement of gonadotropic hormones and their receptors on sex differentiation in Nile tilapia, *Oreochromis niloticus*

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ナイルティラピアは、ティラピア類の中でも最も大型になる上、肉質、味共に優れていることから世界中で大規模に養殖されている。また、ナイルティラピアは雄の方が雌よりも成長が速く、より大型化するため、安定的全雄生産法の開発が望まれている。しかし、性統御を行なう上ではまず、その性分化機構を理解する必要がある。ナイルティラピアでは、遺伝的雌の未分化生殖腺において孵化後 5 日目以降アロマターゼ遺伝子が発現することによって雌性ホルモンであるエストラジオール-17β（E2）が産生されて卵巣分化の方向が決定される。この時、アロマターゼ遺伝子の発現は雌特異的に発現する転写因子、foxl2によって発現誘導される。しかし、一般にアロマターゼ遺伝子の発現誘導は生殖腺刺激ホルモン（GTH）によって制御されていることが広く知られており、卵巣分化のメカニズムにおいても、その関与を無視することはできない。本研究では、ナイルティラピアの性分化における GTH の役割を調べ、以下の評価すべき成果を得た。

（1）遺伝的雌（XX）および雄（XY）の性分化に伴う脳下垂体中の GTH 発現量の変化、生殖腺の GTH 受容体 mRNA 量の変化を詳細に調べ、生殖腺の性分化への関連を調べた。

GTH には黄体形成ホルモン（LH）と濾胞刺激ホルモン（FSH）の 2 種類存在し、それぞれに特異的なβ-サブユニット mRNA 量の脳下垂体中の変化を調べた。その結果、LH mRNA は孵化後 15 日目までは検出されず、25 日目以降に雌雄差なく発現上昇すること、一方、FSH mRNA は最初のサンプリング日である孵化後 3 日目から検出され、孵化後 40 日目までは雌雄差なくその発現量は上昇することが示された。

FSH のタンパクとしての発現を調べるために、ティラピア FSH β抗血清を作製し、タンパク発現の雌雄における変化を調べた。その結果、孵化後1日目では陽性細胞は検出されず、3日目から脳下垂体前葉主部において雌雄共に陽性細胞

が観察され、その数は成長に伴い増加したが、雌雄差は全く認められなかった。

GTH の受け手である FSH 受容体（FSHR）および LH 受容体（LHR）mRNA の未分化生殖腺における発現変化を調べた。FSHR は孵化後 5 日目から 25 日目まで雌で雄よりも高く発現していた。LHR は孵化後 10 日目以降 25 日目まで雌で雄よりも高く発現していた。

以上の結果から、脳下垂体における LH 発現は性分化が決定される期間には認められないことから、LH は性分化には関与していないと結論された。一方、FSH は早くから発現しているものの、雌雄差はなかった。しかし、未分化生殖腺における FSHR は分子的性分化期の雌で有意に高く発現していることから、FSH シグナルが卵巣分化に関与することが強く示唆された。

（2）E2産生とFSHRの発現の関係を明らかにするために、XX仔魚にアロマターゼ阻害剤またはメチルテストステロン（MT）を孵化後9-20日の間処理し、それぞれ完全な雄化誘導を行ない、生殖腺におけるFSHR mRNA量の発現変化を調べた。処理開始時には雌特異的な高い発現を示したFSHRは、いずれの処理においても処理開始から速やかに発現量が低下し、対照群の雄と同等のレベルにまで低下した。また、XY仔魚にE2を孵化後4-10日の間処理し、完全な雌化を誘導し、生殖腺におけるFSHR mRNA量の発現変化を調べた。処理開始時には雄特異的な低い発現を示したFSHRは、E2処理期間中は低値を維持したが、処理終了後の孵化後15日目以降から発現上昇し、雌と同等のレベルにまで高まった。

以上の結果から、XXを雄化誘導すると、正常雄同様にFSHRの発現は低下すること、XYを雌化誘導すると、発現上昇は遅れるものの正常雌同様にFSHR発現は上昇することが明らかになった。

（3）分子的性分化開始時において、FSHシグナルがアロマターゼ遺伝子発現の誘導に関わっているのか否か、また、卵巣分化に必須であるか否かを調べるために、FSHおよびFSHRのノックダウン実験を行なった。その結果、孵化後7日までFSHおよびFSHR発現を抑制しても、10日目以降アロマターゼは発現し、卵巣分化は開始されることがわかった。従って、FSHシグナルはアロマターゼ遺伝子の発現に主体的には関与せず、アロマターゼ遺伝子の上流ではなく下流で働いていると示唆された。

申請者による以上の研究成果は、魚類の性分化機構において初めてE2シグナル以降の経路を明らかにし、基礎生物学および水産科学上重要な成果と評価でき、審査員一同は、申請者が博士（水産科学）の学位を授与される資格のあるものと判定した。