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Mini Review

Sex Reversal Induced by Steroid Hormones in *Glandirana rugosa* Frogs

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Abstract

In general, sex is determined at fertilization of zygotes by sex chromosome composition; this is known as genotypic sex determination in many vertebrate species. Interestingly, steroid hormones can reverse sex of many species in fish, amphibians and reptiles; androgens induce the female-to-male sex reversal, whereas estrogens cause the male-to-female one. For such sex reversal, a functioning sex-determining gene on the sex chromosome is not required. However, little is known about the mechanisms involved in the sex-reversal at histological and molecular levels. To clarify the mechanism of sex reversal, it is very important to detect the first signs of histological changes in the sex-reversing gonads. For this purpose, we have determined a threshold dosage of steroid hormones to induce sex reversal. When tadpoles of *Glandirana* (G.) *rugosa* are reared in water containing a threshold dosage of steroid hormones, genetic females and males form a mixture of testis and ovary, the so-called ovotestis during the transit period of sex reversal. This enables us to understand what is happening in the sex-reversing gonads of amphibians. The first sign of histological changes in the male-to-female sex-reversing gonads occurs in the medulla and later in the cortex. This direction is opposite to that observed during female-to-male sex reversal in this frog. Thus, this frog is an ideal experimental animal to clarify the mechanism of sex reversal induced by steroid hormones in vertebrate species.

INTRODUCTION

The sex of many vertebrate species is generally determined by a key-gene on the sex chromosome or a sex-determining gene. Since the discovery of the *SRY* on the Y chromosome in humans [1,2], many efforts have been made by researchers to understand the mechanism of the sex determination in mammals. In mice, *Sry* acts synergistically with steroidogenic factor 1 (SF1), an orphan nuclear receptor, through an enhancer of *Sox9* to promote Sertoli cell differentiation [3]. Are steroid hormones involved in sex determination in mammals? It is known that androgenic effects are mediated by tissue-specific transcriptional control of target genes via the nuclear androgen receptor (AR) [4]. In *AR*-knockout mice, males have a female-like appearance and body weight, but female-to-male sex reversal does not occur [5,6]. Thus, it is unlikely that androgens participate in sex determination at least in mice.

In reptiles, the sex of many species is determined by the ambient temperature during egg incubation [7]. Recently, Ge et al. [8] have shown that the histone H3 lysine 27 (H3K27) demethylase KDM6B exhibits temperature-dependent sexually dimorphic expression in early *Trachemys scripta elegans* embryos before the gonad is distinct and that KDM6B directly promotes the transcription of the male sex-determining gene *Dmrt1* by eliminating the trimethylation of H3K27 near its promoter. The effects of androgens and estrogens on eggs of *Emys orbicularis* [9], and *E. orbicularis* and *Testugo graeca* [10,11], respectively have been examined also. According to these reports, estrogens induce various degrees of gonadal feminization at male-producing temperature, which depends on the embryonic stages and on the dose of estrogens. However, no difference is observed in structures between estrogen- and temperature-induced ovaries [12]. By contrast, androgens do not reverse the sex from female to male at the female-producing temperature [12]. Thus, the mechanism underlined for the temperature-dependent gonadal formation does not differ from that for the steroid hormone-dependent gonadal formation in reptiles.

In amphibians, the Japanese pond frog *G. rugosa* is known to be the only vertebrate species carrying the two sex-determining systems or the XX/XY and ZZ/ZW systems within one species [13]. The *AR* is located on the sex (X, Y, W and Z) chromosomes, reportedly on the inverted region of the Y and W chromosomes [14]. The *AR* on the W chromosome (*W-AR*) is barely expressed in this frog, whereas the *Z-AR* is well expressed [15]. In addition, indifferent male gonads of *G. rugosa* synthesize more androgens than females [16]. These results suggest that the *AR* could be a candidate for the male sex-determinant in this frog. In fact, incomplete female-to-male sex-reversal occurs in *Z-AR* transgenic female frogs [17]. Complete female-to-male sex-reversal takes place in the *AR*-transgenic ZW female frogs when a low dosage of T is supplied in the rearing water of tadpoles [18]. However, no sex reversal is observed in *AR*-knockdown ZW females when the gonads are treated with dosages of T high enough to induce complete female-to-male sex reversal in wild type frogs [18]. Thus, AR with its androgen ligand undoubtfully functions as the male sex-determinant in *G. rugosa*. This is the first report showing that androgen and its receptor determine the sex of a vertebrate species.

Steroid hormones strongly influence gonadal differentiation

in many species of amphibians [19,20] just as they do in fish [21], reptiles [22] and birds [23]. In *G. rugosa*, steroidogenic genes are expressed in indifferent gonads of male and female tadpoles before sex determination [24]. The *CYP19* expression is significantly higher in the indifferent female gonads of *G. rugosa* than in those of males [24]. Steroidogenic enzymes such as CYP17, 3βHSD and 17βHSD are present and active in indifferent gonads during sex differentiation in *G. rugosa* [16]. Indifferent female gonads can convert progesterone into androstenedione more efficiently than indifferent male gonad of *G. rugosa* [16]. *CYP17* is expressed at high levels in the indifferent male gonads. Its expression becomes much stronger in the female ovary of *G. rugosa* when female tadpoles are reared in water containing T for sex reversal [25]. Thus, this frog is an ideal animal to study the role of steroid hormones in gonadal sex determination and differentiation.

What is happening in the gonad of vertebrates in the presence of steroid hormones? In some species of amphibians such as the *Ranidae* and *Hylidae*, female-to-male sex reversal occurs in response to T [25,26]. However, the mechanism of this sex reversal still remains unclear at both the histological and molecular levels. To clarify the mechanism of sex reversal, it is very important to find the first signs of histological changes in the gonad during sex reversal in amphibians. For this purpose, it is necessary to determine a threshold dosage of steroid hormones and correlated the histological changes with the dosage of steroid hormones, because expectedly *G. rugosa* females transform the ovary to the testis at different degrees depending on the sensitivity of individuals to the drug.

At a threshold dosage of T, female froglets form different types of masculinized gonads with histological changes reflecting various levels of transformation [27]. Histological analysis reveals that at the threshold dosage of T the oocytes disappear in the cortex of the ovary and, later, in the medulla. In parallel, small germ cells specific to male gonads begin to proliferate in the cortex and then spread toward the medulla. Positive signals of CYP17 involved in testis formation are localized in the cortex of the ovary at the initial stage of masculinization and they increase toward the medulla as masculinization proceeds [27]. Thus, the internal structure in masculinizing gonads changes from the cortex toward the medulla. In these gonads, the expression levels of *Dmrt1* and *Cyp17a* necessary for testis formation are increased, whereas *Pat1a* and *CYP19* for ovary formation are decreased.

On the other hand, estradiol-17β (E2) can induce male-to-female sex reversal in fish [28], amphibians [29] and reptiles [30]. However, the first signs in histological changes in the gonads are not known during this sex reversal. For this purpose, we need to determine a threshold dosage of E2 and correlate the histological changes with the dosage of E2. When male tadpoles are reared in water containing a threshold dosage of E2, male froglets form phenotypically four types of the gonads; the ovary, testis and ovotestis [31]. First, the gonad testis filled with small germ cells. The basement membrane of the gonad is the same as that of the wild-type of testis. Second and third, the gonads are the ovotestes. In the former, small germ cells specific to the testis have disappeared from the medulla and the basement membrane has been disrupted. Immuno-positive signals of Pat1a specific

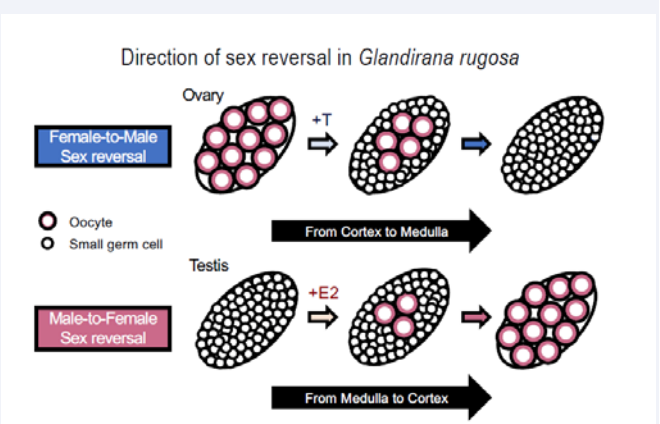

Figure 1 Direction of histological changes in the gonad during sex reversal in *G. rugosa*.

to oocytes are undetectable in the gonad. CYP17 signals have disappeared from the medulla of the gonad. In the latter, small germ cells are observed in only the cortex. Testis structure is completely disrupted, followed by emergence of many oocytes in the gonad. The basement membrane has been rearranged in the feminizing gonad. CYP17-signals are observed in the cortex of the gonad. Fourth, the gonad is the ovary filled with many oocytes. The basement membrane has been reconstructed just like that of the ovary. The expression of *Dmrt1*, *AMHRII* and *Sox9* is significantly decreased in the feminizing gonads of *G. rugosa* [31]. Thus, the treatment of tadpoles with a threshold dosage of E2 would be very useful to detect primary changes in the histology of the gonad during male-to-female sex reversal in *G. rugosa*. Most interestingly, the structural changes in the gonad during female-to-male sex reversal proceed outward from the cortex, whereas those during male-to-female sex-reversal are directed inward from the medulla (Figure 1). Further work must be performed to understand why the opposite directions are observed during male-to-female sex reversal and vice versa.

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