ZOOLOGICAL SCIENCE 20: 43–47 (2003)

[SHORT COMMUNICATION]

No Effects of Estrogen Receptor Overexpression on Gonadal Sex Differentiation and Reversal in Medaka Fish

Toshiyuki Kawamura, Seiichi Omura, Shuichi Sakai, and Ichiro Yamashita*

Center for Gene Science, Hiroshima University, Kagamiyama 1-4-2, Higashi-hiroshima 739-8527, Japan

ABSTRACT—In order to elucidate a possible role of estrogen receptor in the gonadal sex differentiation and the sex reversal with sex steroids, we examined for the formation of testis or ovary in transgenic medaka fish overexpressing the medaka estrogen receptor under the constitutive medaka β-actin promoter. The transgenic fish underwent the genetically determined gonadal differentiation and showed the same sex-reversal rates as those of wild-type non-transgenic fish after treatments with estrogen and androgen. These results present invaluable data to reconsider the role of estrogen receptor in the gonadal sex determination.

Key words: transgenic medaka fish, sex differentiation, sex reversal, estrogen, estrogen receptor

INTRODUCTION

The fate of primordial gonad to testis or ovary is determined during the distinct stage of development. Although the testis-determining gene, *Sry*, and downstream genes for sexual differentiation are discovered in mammals (Koopman *et al.*, 2001), mechanisms for gonadal sex determination are poorly understood in lower vertebrates except the recent discovery of medaka fish male-determining gene, *DMY* (Matsuda *et al.*, 2002). The environmental contamination of estrogenic chemicals has been a serious concern in the industrial nations. Estrogenic compounds such as a natural estrogen (17β-estradiol, E2), a synthetic estrogen (17α-ethinylestradiol) used as oral contraceptives, and xeno-estrogens (such as bisphenol A and 4-tert-pentylphenol) are detected in sewage effluents and pollute aquatic environments and wild life (Aherne and Briggs, 1989; Naylor, 1992; Colborn *et al.*, 1993; Lee and Peart, 1998; Belfroid *et al.*, 1999; Korner *et al.*, 2000). These chemicals can cause reproductive abnormalities in wild animals and, possibly, in man (Colborn *et al.*, 1993). In the laboratory experiment, estrogen affects the testicular development and ultimately reverses it to ovary formation, if applied early enough during the development of lower vertebrates such as bird (Scheib, 1983), reptile (Dorizzi *et al.*, 1991), amphibia (Hayes, 1998), and fish (Yamamoto, 1969; Gimeno *et al.*, 1996; Kawahara and Yamashita, 2000). In chickens (Scheib, 1983) and turtles (Dorizzi *et al.*, 1991), estrogens are synthesized by morphologically undifferentiated female gonads, but at reduced levels in male gonads. It is generally believed from these circumstantial evidences that estrogen is a natural inducer of ovary formation.

However, there are several pharmacological studies using anti-estrogens (that bind to estrogen receptor [ER] and compete with estrogens), which do not support the sex steroid hypothesis. Treatment with anti-estrogens does not disrupt ovarian development but disturb it only slightly showing partial musculinization (some testicular appearance of female gonads), whereas the treatment completely inhibits the male to female gonadal sex reversal caused by exogenous estrogens (Scheib, 1983; Dorizzi *et al.*, 1991; Kawahara and Yamashita, 2000). Furthermore, it remains to be seen whether exogenous estrogens can cause the sex reversal at the equivalent concentrations found in undifferentiated female embryos. Treatment with aromatase inhibitors (that block the synthesis of estrogen from androgen, a male sex steroid) causes females to develop testes (Elbrecht and Smith, 1992; Richard-Mercier *et al.*, 1995). However, this sex reversal may be interpreted as that the accumulated androgen elicited the male gonadal differentiation but not as widely believed that the absence of estrogen caused the alternative gonadal development, because of the well-identified positive role of androgen in the testicular development (Yamamoto, 1969). In conclusion, exogenous

* Corresponding author: Tel. +81-824-24-6271;
FAX. +81-824-22-7184.
E-mail: iyama@hiroshima-u.ac.jp

estrogen induces ovarian development in genetic male possibly after binding ER, but, there is no convincing evidence supporting a key role of estrogen/ER in the fate determination to ovary in genetic female although estrogen level is higher in female primordial gonad than in male.

In contrast, histological and ultrastructural studies in some fishes including medaka reveal that steroid hormone biosynthesis and steroid-producing cells appear after the completion of gonadal sex differentiation (Iwasaki, 1973; Takahashi and Iwasaki, 1973; Kagawa and Takano, 1979; Schreibman *et al.*, 1982; van den Hurk *et al.*, 1982; Kanamori *et al.*, 1985). Furthermore, pharmacological studies in medaka support the absence of sex steroids during the sex differentiation and provide sufficient evidence for an estrogen-independent mechanism for ovarian development (Kawahara and Yamashita, 2000).

Numerous studies have been done using a lot of animals from fish to birds to clarify the possible role of estrogen and ER in the female sex determination (Clinton, 1998; Hayes, 1998; Jeyasuria and Place, 1998; Nakamura *et al.*, 1998; Patino, 1997; Pieau *et al.*, 1998). However, at present, the results obtained from these studies can not be explained solely by the action of estrogen-activated ER. Our working model is as follows: (1) in medaka fish, female sex determination is governed by an unknown factor that is not related to estrogenic actions but shares with ER the ability to activate downstream genes for female sex development, because ER is expressed in both sexes (but at very low levels during the sex-determining period) (Kawahara *et al.*, 2000), but inactive because of the lack of estrogen; (2) we cannot exclude the possibility that ER is activated only in female by the estrogen-independent mechanism as proposed in other biological systems (Power *et al.*, 1991; Smith *et al.*, 1993; Kato *et al.*, 1995; Bunone *et al.*, 1996; Das *et al.*, 1997; Zwijsen *et al.*, 1998); and (3) in chickens and turtles, there are at least two pathways for ovarian development: one involves the estrogen-activated ER and another is independent of estrogen.

To genetically elucidate the possible role of ER in the sex determination of medaka fish, we constructed transgenic (Tg) medaka fish overexpressing medaka ER, and examined for the sexual differentiation and the sex reversal by the treatment with estrogen and androgen. The results were such that overexpression of ER did not affect the sexual development in both male and female or the sex-reversal rates after the treatment with estrogen and androgen. These negative results are, of course, not conclusive, but suggestive of the estrogen/ER-independent female sex determination.

MATERIALS AND METHODS

Fish and embryo culture

We used the d-rR strain of medaka fish, *Oryzias latipes*. This strain is very useful to uncover mechanisms for gonadal sex determination and sex reversal, because the genotype of sex can be judged by body color with more than 99% reliability with orange-red male (X^rY^R) and white female (X^rX^r) (Yamamoto, 1969). The Tg fish overexpressing the medaka ER in the entire region of the body was established in this genetic background (Kawamura *et al.*, 2002). Fish was maintained at 25–26°C under artificial photo-period of 14L:10D, and fed by powdered Tetramin (Tetra). Eggs were collected within 10 h postfertilization (hpf) (Kawamura and Yamashita, 2002), rinsed with tap water, and immersed in Yamamoto's salt solution (Yamamoto, 1969).

Sex reversal

Sex-reversal experiments were done as follows. Eggs (10 hpf) from mating between hemizygous Tg and wild-type fish were incubated under the same condition as above in Yamamoto's solution containing 17β-estradiol (E2). The hatching fry were then transferred to plastic aquaria and reared to adult by normal diet for 5 months. E2 was dissolved in dimethyl sulfoxide. The stock solution was diluted over 1,000-fold with Yamamoto's solution. In other experiments, newly hatched fries in Yamamoto's solution from mating between hemizygous Tg and wild-type fish were fed to adult with diet containing E2 or methyltestosterone (MT). The genotype of sex was inferred by body color with orange-red male (XY) and white female (XX). Adult fish were dissected for sexing gonads under a dissecting microscope. DNA was extracted from caudal fins of individual fish and examined for the presence of the transgene by PCR and Southern blot analysis as described (Kawamura *et al.*, 2002).

RESULTS

The hemizygous Tg fish of "A"- and "C"- lines developed normal gonads as determined genetically (testis and ovary for XY and XX, respectively) and mated with opposite sex partners of wild-type, indicating that overexpression of ER does not affect the sexual differentiation. This is as expected because estrogen is not synthesized during the sex determination period in medaka (Kawahara and Yamashita, 2000), thus ER is considered to be inactive.

If exogenous estrogen induced the sex reversal from the genetic male to female through binding to and activating ER, the Tg fish of genetic male would develop ovary or ovotestis after treatment with lower concentrations of estrogen than the lowest effective to wild-type. For this purpose, two methods were applied as follows: (1) the 10-hpf embryos were immersed in Yamamoto's solution containing 0.2 or 2.0 μg/l of E2, which is 50 or 5 times lower concentration than the lowest effective (Iwamatsu, 1999), and fed to adult with normal diet after hatching; and (2) the newly hatched fry in the absence of E2 were fed to adult with diet containing 5, 10, or 20 μg of E2 per gram of diet. The E2 dosage of 20 μg/g of diet is sufficient for complete sex-reversal, and the dosages of 5 and 10 are less effective or result in no sex-reversal depending on each experiment (Yamamoto, 1969; Kawahara and Yamashita, 2000). In two methods, the sex-reversal rates from male to female were not enhanced in the hemizygous Tg fish of "A"- and "C"-lines (Table 1). These results indicate that overexpression of ER does not affect the estrogen-induced sex reversal.

Sex reversal with steroid hormones has been considered as a consequence of competition between a genetically determined gonadal fate and an antagonizing activity of sex steroids (Yamamoto, 1969). If ER were involved in the female sex differentiation after activation by an E2-inde-

Table 1. Sex reversal of the Tg fish treated with estrogen and androgen.

Fish	Treatment		Orange-red (XY)				White (XX)			
			T	OT	O	SR(%)	T	OT	O	SR(%)
Wild	E2 (μg/l)	0.2	12	0	0	0	0	0	14	0
		2.0	26	0	0	0	0	0	26	0
A-line		0.2	19	0	0	0	0	0	23	0
		2.0	17	0	0	0	0	0	10	0
C-line		0.2	12	0	0	0	0	0	11	0
Wild	E2 (μg/g diet)	0	100	0	0	0	0	0	100	0
		5	47	1	0	2	0	0	32	0
		10	7	23	9	82	0	0	19	0
		20	0	0	106	100	0	0	105	0
A-line		0	50	0	0	0	0	0	50	0
		5	8	0	0	0	0	0	4	0
		10	10	0	0	0	0	0	7	0
		20	0	0	8	100	0	0	13	0
C-line		0	50	0	0	0	0	0	50	0
		5	6	0	3	33	0	0	5	0
		10	1	0	4	80	0	0	12	0
		20	0	0	7	100	0	0	13	0
Wild	MT(μg/g diet)	10	22	0	0	0	7	0	16	30
		20	22	0	0	0	15	0	4	79
		30	25	0	0	0	30	0	0	100
A-line		30	16	0	0	0	17	0	0	100
C-line		30	4	0	0	0	10	0	0	100

Eggs and fish (wild-type and "A"- and "C"-line Tg) were treated with E2 or MT at the indicated concentrations. Adult fish were dissected, and their gonads were classified as ovary (O), ovotestis (OT), and testis (T). Sex reversal (SR) rates were determined as percentage of XY fish carrying ovary or ovotestis in XY population and of XX fish carrying testis in XX population.

pendent mechanism, overexpression of ER would increase the amount of active ER, which enhances the activity of female primordial gonad to differentiate into ovary and competes with the female-to-male reversal activity of androgen, and would result in the decrease in the rates of female-to-male reversal after the oral administration of MT. Firstly, we examined MT-induced sex reversal in the wild-type fish. The sex-reversal rate increased progressively with increasing dosages of MT with the lowest for complete sex reversal of 30 μg of MT/g of diet (Table 1), as expected from the previous report (Yamamoto, 1969). The newly hatched fries from mating between the "A"- or "C"-line Tg and wild-type fish were fed to adult with diet containing 30 μg of MT/g of diet. Both Tg fish were also completely sex-reversed by the treatment with MT (Table 1), indicating that overexpression of ER does not affect the androgen-induced sex reversal.

DISCUSSION

It is as expected that the ER-overexpressing Tg fish underwent normal gonadal sex differentiation, because estrogen is not present during the sex-determining period in medaka fish (Kawahara and Yamashita, 2000). However, it was quite unexpected that the estrogen-induced sex reversal rates from male to female was not enhanced by overproduction of ER in the Tg fish, because the estrogen-induced sex reversal in wild-type fish is prevented by the anti-estrogen, tamoxifen, thus considered to be dependent on ER (Kawahara and Yamashita, 2000).

The sex-reversal experiments in the Tg fish suggest two possibilities: one is that ER is not involved in the sex reversal; and another is that expression levels of ER are not rate-limiting in the sex reversal, for example, overproduced ER induces similar expression levels of target gene(s) to those in wild type. Recently, several studies report that estrogen binds to and activates proteins such as androgen receptor (Kousteni *et al.*, 2001), maxi-K channels (Valverde *et al.*, 1999), and γ-adrenergic receptor (Nadal *et al.*, 2000) and that tamoxifen also binds to multiple targets (Williams *et al.*, 1996; Kedjouar *et al.*, 1999), providing proofs against specific actions of estrogen and anti-estrogen on ER. It is unlikely that, in the Tg fish, ER is not produced at increased levels in a target tissue for sex reversal (probably in a primordial gonad), because ER was in fact abundantly expressed from the β-actin promoter in the entire region of

the Tg fish as well as β-actin that is known to be abundantly expressed in all tissues including gonads (Kawamura *et al.*, 2002). We are not in favor of the possibility that the ER used for construction of the Tg fish is different from one involved in sex differentiation, because we could not detect any ER homologs (other than the ER cloned previously) in RNA samples from embryos and fries by RT-PCR with complementary primers (Kawahara *et al.*, 2000) and several combinations of degenerate primers (data not shown). These results are not conclusive but consistent with the possibility that ER is not involved in the estrogen-induced sex reversal.

We also examined the female-to-male sex reversal of the Tg fish after oral administration of androgen. We anticipated that overexpression of ER would increase the amount of active ER and compete with the female-to-male sex-reversal activity of androgen. However, the overexpression did not affect the rates of female-to-male sex reversal in two Tg fish lines. These results do not support the possibility that ER is involved in the ovarian development after activation by the E2-independent mechanism.

The present study using the Tg fish does not provide convincing results, but suggests that the role of ER in the sex determination should be open to reconsideration. In this context, it was recently reported in mice that ER is required for the maintenance of adult ovary but not for the gonadal sex determination before birth: mice lacking ERs α and β exhibit normal reproductive tract development but adult ovaries transdifferentiate to structures resembling seminiferous tubules of the testis (Couse *et al.*, 1999). There remains the possibility that ER is not responsible for the female sex determination in lower vertebrates as in mammals including human and mouse.

ACKNOWLEDGEMENTS

We are grateful to T. Kawahara for Discussion.

REFERENCES

Aherne GW, Briggs R (1989) The relevance of the presence of certain synthetic steroids in the aquatic environment. J Pharm Pharmacol 41: 735–736

Belfroid AC, Van der Horst A, Vethaak AD, Schafer AJ, Rijs GB, Wegener J, Cofino WP (1999) Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci Total Environ 225: 101–108

Bunone G, Briand PA, Miksicek RJ, Picard D (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15: 2174–2183

Clinton M (1998) Sex determination and gonadal development: a bird's eye view. J Exp Zool 281: 457–465

Colborn T, vom Saal FS, Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101: 378–384

Couse JF, Curtis Hewitt S, Bunch DO, Sar M, Walker VR, Davis BJ, Korach KS (1999) Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. Science 286: 2328–2331

Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB (1997) Estrogenic responses in estrogen receptor-α deficient mice reveal a distinct estrogen signaling pathway. Proc Natl Acad Sci USA 94: 12786–12791

Dorizzi M, Mignot T-M, Guichard A, Desvages G, Pieau C (1991) Involvement of oestrogens in sexual differentiation of gonads as a function of temperature in turtles. Differentiation 47: 9–17

Elbrecht A, Smith RG (1992) Aromatase enzyme activity and sex determination in chickens. Science 255: 467–470

Gimeno S, Gerritsen A, Bowmer T, Komen H (1996) Feminization of male carp. Nature 384: 221–222

Hayes TB (1998) Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. J Exp Zool 281: 373–399

Iwamatsu T (1999) Convenient method for sex reversal in a freshwater teleost, the medaka. J Exp Zool 283: 210–214

Iwasaki Y (1973) Histochemical detection of Δ^5-3β-hydroxysteroid dehydrogenase in the ovary of medaka, *Oryzias latipes*, during annual reproductive cycle. Bull Fac Fish Hokkaido Univ 23: 177–184

Jeyasuria P, Place AR (1998) Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: a role for P450 aromatase (CYP19). J Exp Zool 281: 428–449

Kagawa H, Takano K (1979) Ultrastructure and histochemistry of granulosa cells of pre- and post-ovulatory follicles in the ovary of the medaka, *Oryzias latipes*. Bull Fac Fish Hokkaido Univ 30: 191–204

Kanamori A, Nagahama Y, Egami N (1985) Development of the tissue architecture in the gonads of the medaka *Oryzias latipes*. Zool Sci 2: 695–706

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270: 1491–1494

Kawahara T, Okada H, Yamashita I (2000) Cloning and expression of genomic and complementary DNAs encoding an estrogen receptor in the medaka fish, *Oryzias latipes*. Zool Sci 17: 643–649

Kawahara T, Yamashita I (2000) Estrogen-independent ovary formation in the medaka fish, *Oryzias latipes*. Zool Sci 17: 65–68

Kawamura T, Sakai S, Omura S, Hori-e R, Kawahara T, Kinoshita M, Yamashita I (2002) Estrogen inhibits development of yolk veins and causes blood clotting in transgenic medaka fish overexpressing estrogen receptor. Zool Sci 19: 1355–1361

Kawamura T, Yamashita I (2002) Aryl hydrocarbon receptor is required for prevention of blood clotting and for the development of vasculature and bone in the embryos of medaka fish, *Oryzias latipes*. Zool Sci 19: 309–319

Kedjouar B, Daunes S, Vilner BJ, Bowen WD, Klaebe A, Faye J-C, Poirot M (1999) Structural similitudes between cytotoxic antiestrogen-binding site (AEBS) ligands and cytotoxic sigma receptor ligands. Evidence for a relationship between cytotoxicity and affinity for AEBS or sigma-2 receptor but not for sigma-1 receptor. Biochem Pharmacol 58: 1927–1939

Koopman P, Bullejos M, Bowles J (2001) Regulation of male sexual development by *Sry* and *Sox9*. J Exp Zool 290: 463–474

Korner W, Bolz U, Sussmuth W, Hiller G, Schuller W, Hanf V, Hagenmaier H (2000) Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 40: 1131–1142

Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC (2001) Non-genotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104: 719–730

Lee HB, Peart TE (1998) Determination of 17 beta-estradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. J AOAC Int 81: 1209–1216

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417: 559–563

Nadal A, Ropero AB, Laribi O, Maillet M, Fuentes E, Soria B (2000) Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor α and estrogen receptor β. Proc Natl Acad Sci USA 97: 11603–11608

Nakamura M, Kobayashi T, Chang XT, Nagahama Y (1998) Gonadal sex differentiation in teleost fish. J Exp Zool 281: 362–372

Naylor CG (1992) Environmental fate of alkylphenol ethoxylate. Soap Cosmet Chem Special 68: 27–32

Patino R (1997) Modifications of the reproductive system of fishes by means of exogenous chemicals. Prog Fish Cult 59: 118–128

Pieau C, Dorrizzi M, Richard-Mercier N, Desvages G (1998) Sexual differentiation of gonads as a function of temperature in the turtle *Emys orbicularis*: endocrine function, intersexuality and growth. J Exp Zool 281: 400–408

Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW (1991) Dopaminergic and ligand-independent activation of steroid hormone receptors. Science 254: 1636–1639

Richard-Mercier N, Dorizzi M, Desvages G, Girondot M, Pieau C (1995) Endocrine sex reversal of gonads by the aromatase inhibitor Letrozole (CGS 20267) in *Emys orbicularis*, a turtle with temperature-dependent sex determination. Gen Comp Endocrinol 100: 314–326

Scheib D (1983) Effects and role of estrogens in avian gonadal differentiation. Differentiation 23 (Suppl): S87–S92

Schreibman MP, Berkowitz EJ, van den Hurk R (1982) Histology and histochemistry of the testis and ovary of the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. Cell Tissue Res 224: 81–87

Smith CL, Conneely OM, O'Malley BW (1993) Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc Natl Acad Sci USA 90: 6120–6124

Takahashi H, Iwasaki Y (1973) The occurrence of histochemical activity of 3β-hydroxysteroid dehydrogenase in the developing testes of *Poecilia reticulata*. Dev Growth Differ 15: 241–253

Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R (1999) Acute activation of maxi-K channels (hSlo) by estradiol binding to the β subunit. Science 285: 1929–1931

van den Hurk R, Lambert JGD, Peute J (1982) Steroidgenesis in the gonads of rainbow trout fry (*Salmo gairdneri*) before and after the onset of gonadal sex differentiation. Reprod Nutr Dev 22: 413–425

Williams JP, Blair HC, McKenna MA, Jordan SE, McDonald JM (1996) Regulation of avian osteoclastic H^+-ATPase and bone resorption by tamoxifen and calmodulin antagonists. J Biol Chem 271: 12488–12495

Yamamoto T (1969) Sex differentiation In "Fish Physiology 3" Eds by WS Hoar, DJ Randall, Academic Press, New York, pp 117–175

Zwijsen RML, Buckle RS, Hijmans EM, Loomans CJM, Bernards R (1998) Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. Genes Dev 12: 3488-3498

(Received July 24, 2002 / Accepted October 7, 2002)