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The Novel Mutant *scl* of the Medaka Fish, *Oryzias latipes*, Shows No Secondary Sex Characters

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A new mutant that has neither male nor female secondary sex characters was found in the medaka, *Oryzias latipes*. Both XX and XY mature mutants had gonads with many spermatozoa, but spawning did not occur when the mutants were paired with normal males or normal females. F_1 progeny were successfully obtained by artificial insemination using unfertilized eggs from wild-type females and spermatozoa of the XY mutant. The mutant phenotype did not occur in the F_1 progeny from this cross. Incrossing among the F_1 progeny produced 17 mutant offspring out of 68 progeny (25%), demonstrating that the mutant phenotype is caused by a single recessive mutation. This mutant was named *scl* (*sex character-less*). Because papillary processes, a male secondary sex character, were induced in the XY mutants by androgen administration, it seems that the androgen receptor is functioning normally. We found a loss-of-function type mutation in the *P450c17* gene of the mutant; this gene encodes a steroidogenic enzyme required for the production of estrogen and androgen. The *scl* phenotype was completely linked to the mutant genotype of *P450c17*, strongly suggesting that mutation at the *P450c17* locus is responsible for the *scl* mutant phenotype.

Key words: medaka, *P450c17*, *scl*, secondary sex characters, sex steroid

INTTRODUCTION

In vertebrates, gonadal sex is determined by major sex factors contained on the sex chromosomes or by environmental factors (e.g., temperature and social interaction). Following gonadal sex determination, steroid hormones secreted from the gonads determine the sex of gonadal accessory organs. Secondary sex characters are similarly induced by sex steroid hormones.

The medaka, *Oryzias latipes*, has been used as a model organism for examining mechanisms of sex determination and sex differentiation. This species has a firm XX/XY sex-determining system (Aida, 1921), with *DMY* on the Y chromosome found to be the sex-determining gene (Matsuda et al., 2002, 2007; Nanda et al., 2002). In XY fish, *DMY* can induce testicular development, whereas ovarian development occurs in the absence of *DMY*.

The medaka has clear sexual dimorphism (Fig. 1A–D). The shape of the dorsal and anal fins and the presence of papillary processes on the anal fins are conspicuous male-specific secondary sex characters. The dorsal fin of the male has a saw-toothed distal edge, and the edge between the hindmost ray and other rays has a deep notch, not observed in females. The papillary processes on the anal fin rays are present only in males. In females, on the other hand, the small anal fin has a right-triangle shape. The urinogenital papilla (UGP) is a female-specific secondary sex character (reviewed by Egami, 1975). Formation of the papillary processes can be induced by androgen (Okada and Yamashita, 1944), and the UGP by estrogen (Yamamoto and Suzuki, 1955).

Here, we found XY fish of the Hd-rR.Y^{HNI}(R1) strain (Matsuda et al., 2002) of medaka lacking both male and female secondary sex characters. Genetic analyses revealed that this trait was heritable. In this paper, we describe the phenotypic and genetic traits of this mutant and suggest a primary candidate gene for the mutation. We carried out histological observation of gonads, genetic analysis using artificial insemination, and induction of formation of papillary processes by using an artificial androgen, methyltestosterone. We found that the phenotype was associated with a deficiency of sex steroids caused by a recessive mutation at the *scl* (sex character less) locus.

MATERIALS AND METHODS

Animals

All fish used in this study originated from stocks kept at the Faculty of Science, Niigata University, Japan. They were reared at 27°C±2°C, under a photoperiod of 14 h light and 10 h dark. From

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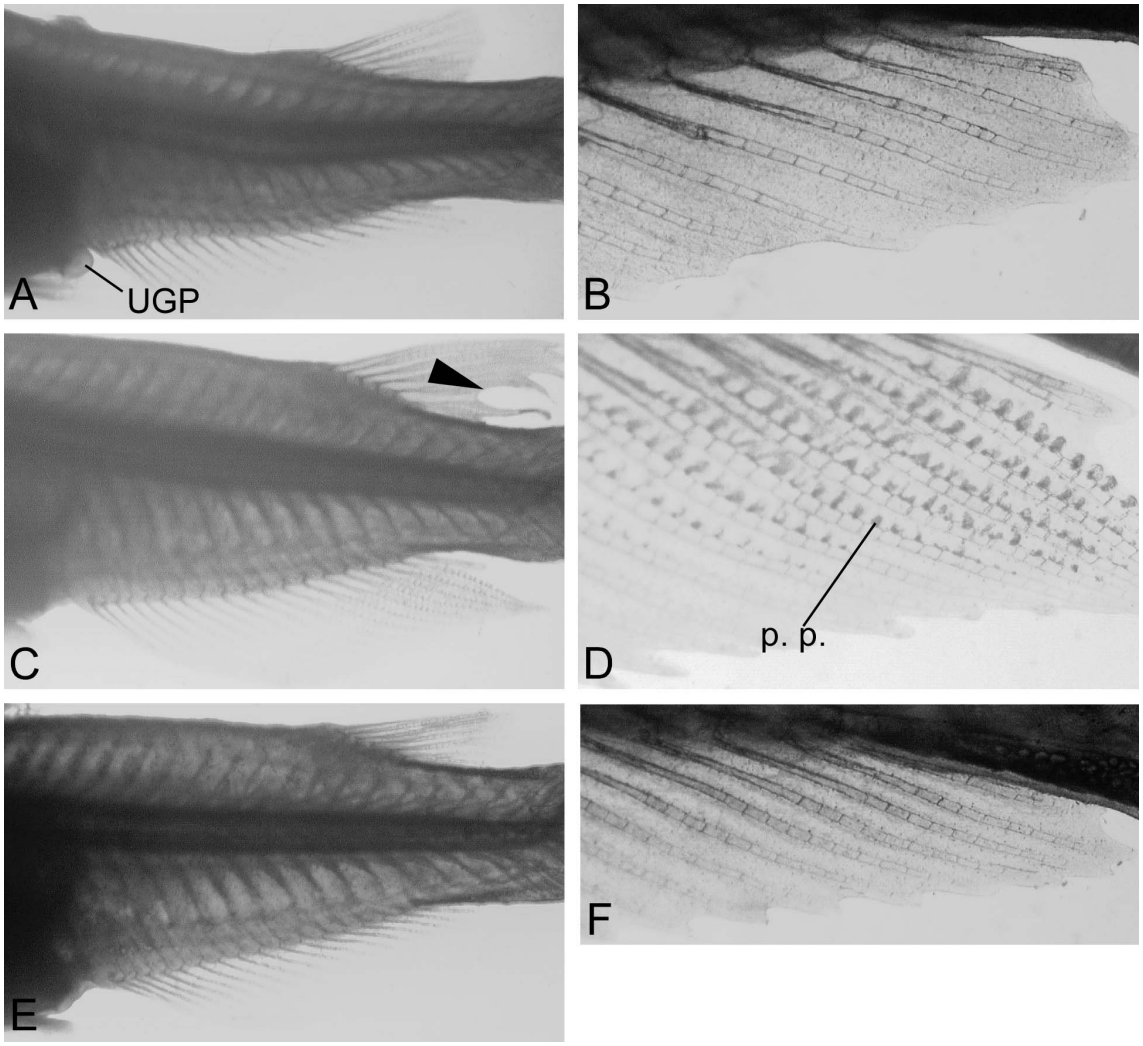

Fig. 1. Medaka fish, *Oryzias latipes*: **(A, B)** normal XX female; **(C, D)** normal XY male; **(E, F)** XY fish with no secondary sex characters. All fish were derived from the Hd-rR.Y^{HNI}(R1) strain. UGP, urinogenital papilla; p.p., papillary process; the arrowhead points to a deep notch between the hindmost ray and the other rays in the dorsal fin of male fish. The abnormal XY fish (E, F) had neither a UGP nor papillary processes, and had female-like fins.

two inbred strains of medaka, Hd-rR and HNI (Hyodo-Taguchi and Sakaizumi, 1993), we established a Y-congenic strain, Hd-rR.Y^{HNI}. XY fish of the Hd-rR.Y^{HNI} strain have a genetic background derived from the Hd-rR strain, and a Y chromosome that includes the sex-determining gene from the HNI strain (Matsuda et al., 1998). Next, we established a recombinant strain, Hd-rR.Y^{HNI}(R1), in which recombination occurred between the X and Y chromosomes (Matsuda et al., 2002). Genotypic sex (XY or XX) of the fish used in this study was determined by establishing the presence or absence of the *DMY* gene by PCR on DNA extracted from a caudal fin clip (Shinomiya et al., 1999; 2004).

Histological observation

Gonads of mature fish at about six months after hatching were dissected and fixed in Bouin's solution. Specimens were embedded in paraffin, sectioned serially at 5-μm thickness, and stained with hematoxylin and eosin for observation under a light microscope.

Treatment with methyltestosterone

Fish were reared in aged tap water containing methyltestosterone (MT; Sigma Chemical Co., St. Louis, MO) at a concentration of 10 μg/L. The MT solution was changed every three days. At water changing, the fish were anesthetized with ethylene glycol monophenyl ether (0.025%), and the number of papillary processes on the anal fin rays was counted under a dissection microscope.

Determination of *P450c17* genotypes

Genotypes of the *P450c17* gene, which is responsible for both androgen and estrogen synthesis, were determined by PCR analysis. The intron and exon structure of *P450c17* was estimated on the basis of the cDNA sequence from ovarian follicles of the orange-red variety of medaka (DDBJ Accession No. D87121) and the genomic sequence of the Hd-rR inbred strain (Medaka Genome Project: http://dolphin.lab.nig.ac.jp/medaka/index.php, version 1.0, scaffold 557) (Fig. 2). *P450c17* PCR primers were C17-14F (5'-ACA GAA GTA CGG ACA GAC CT-3'), C17-14R (5'-AAC AGA GCG AGC AGA TGA CG-3'), and C17-24F (5'-TGT TTG GAG AGG GTT CTG-3'). The thermal cycling conditions were 5 min at 94°C for 1 cycle; 30 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C for 30 cycles; and 5 min at 72°C for 1 cycle. After the PCR reaction, PCR products were electrophoresed on 1% agarose gel.

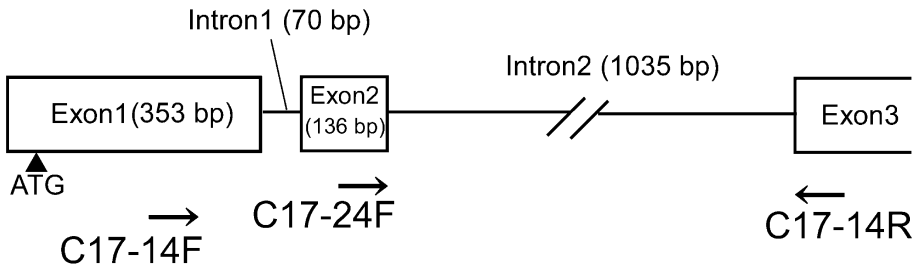

Fig. 2. The 5-prime end genomic region of the *P450c17* gene in the Hd-rR inbred strain. Arrows show the positions of the *P450c17* primers used for amplification. Boxes indicate exons; lines, introns. The nucleotide sequences of the primers are given in the text.

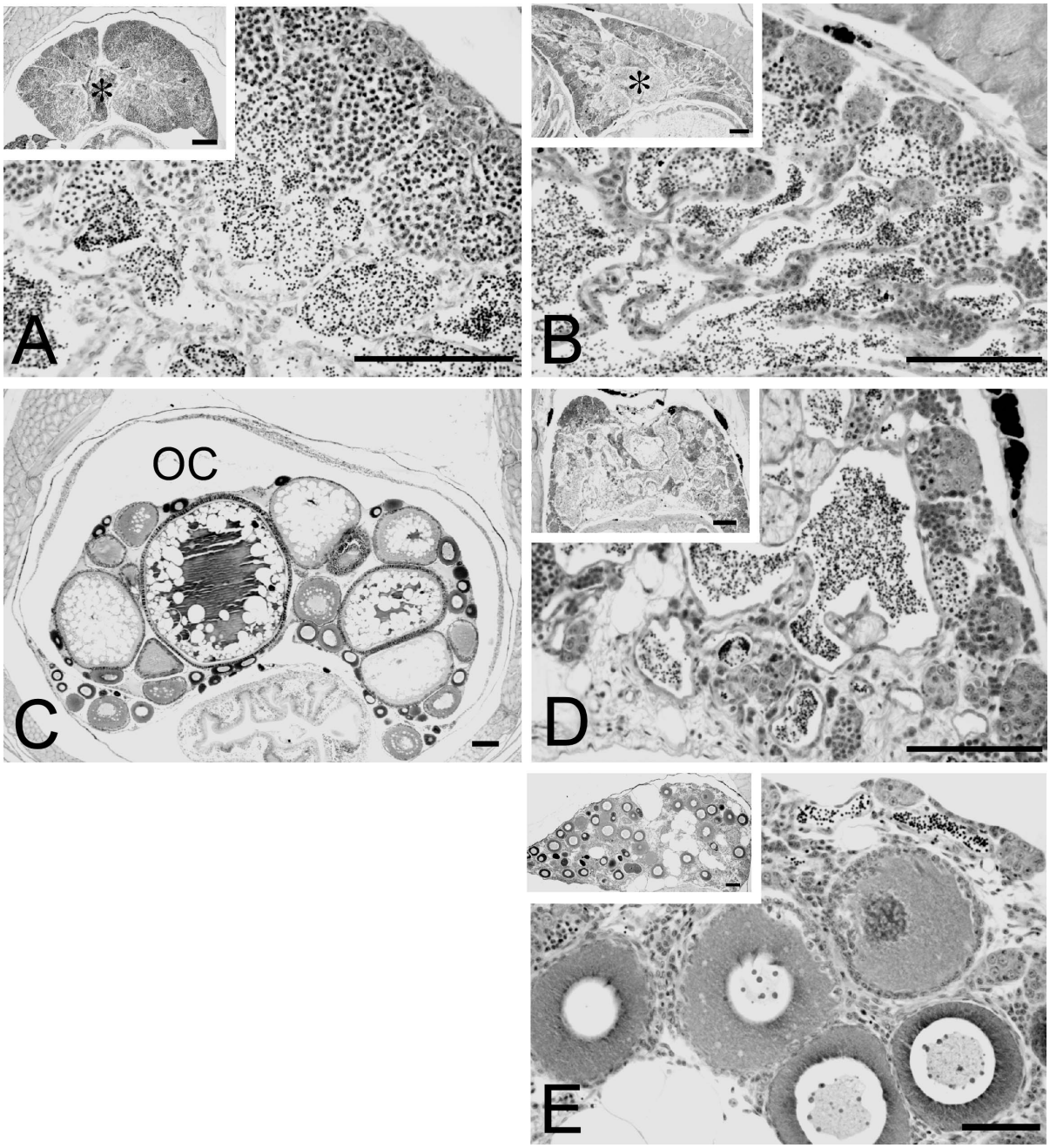

Fig. 3. Cross-section photomicrographs of normal and *scl* gonads. **(A)** Normal testis; **(B)** testis of an *scl* XY; **(C)** normal ovary; **(D)** spermatogenesis observed in an *scl* XX gonad at six months after hatching; **(E)** an *scl* XX gonad at six months after hatching, showing oocytes and spermatozoa. Asterisk, efferent duct in a testis; OC, ovarian cavity. Scale bars except E, 100 μm; E, 50 μm.

RESULTS

Phenotype of XY fish with no secondary sex characters

We found XY fish with female-like fins and no UGP in the Hd-rR.Y^{HNI}(R1) strain (Fig. 1E, F). When XY fish with no secondary sex characters were paired with either normal females or normal males, spawning did not occur. We performed a histological examination of the gonads from 11 mature XY fish with no secondary sex characters at six months after hatching. All 11 XY fish had testes, and spermatogenesis appeared to have been proceeding normally (Fig. 3A, B). That is, these XY fish were neither intersex, nor neuter, nor sex-reversed female, but males without secondary sex characters.

Induction of papillary processes by exogenous MT

Since male secondary sex characters of the medaka have been reported to be induced by the administration of androgens (Okada and Yamashita, 1944), the lack of male secondary sex characters in the fish in this study was thought to be caused by either deficiency of androgen or low sensitivity to androgen. To examine the sensitivity of the fish to androgen, we treated XY fish with no secondary sex characters and normal Hd-rR females with MT. In addition, because the presence of the Y chromosome might affect the development of papillary processes, we also treated sex-reversed XY females of the Hd-rR.Y^{HNI}(R1) strain, which were induced by the treatment of embryos with estradiol-17β (Iwamatsu, 1999). We used three individuals in each group.

In all groups, the papillary processes began to appear between three and six days after the beginning of treatment, and then increased in number (Figs. 4, 5). The time-course of change in the number of papillary processes did not differ among the three groups.

Genetic features of fish with no secondary sex characters

Because the XY fish with no secondary sex characters had mature spermatozoa but did not reproduce naturally, we performed artificial insemination (Iwamatsu, 1973), using a testis from an XY fish to obtain offspring from these fish. The testis was dissected, and mature ova from the Hd-rR strain were artificially inseminated in a saline solution (111.2 mM NaCl, 5.4 mM KCl, 1.1 mM $CaCl_2$, 0.6 mM $MgSO_4$, pH adjusted to 7.3 with $NaHCO_3$; Iwamatsu, 1973). We obtained 16 adult F_1 fish, all of which showed secondary sex characters. We then crossed the F_1 females with the F_1 males to obtain 68 F_2 fish, and obtained XY fish without male secondary sex characters and XX fish without a UGP (Table 1). The ratio of fish with no sex characters to normal fish in the F_2 was just one-third (17:51), and there was no significant difference in this ratio between XX (8:27) and XY (9:24) fish (χ^2 test, $P<0.05$), indicating that the phenotype of no secondary sex characters is caused by a single recessive mutation on an autosome. Therefore, we named the mutant *scl* (sex character less).

We performed a histological examination of gonads from five XX fish with no UGP at about six months after hatching. Spermatogenesis had occurred in the gonads of all five fish; however, oocytes at the diplotene stage were also present in two of them. In the testes of all 11 *scl* XY fish, cysts of type B spermatogonia, spermatocytes, and spermatids were aligned in seminiferous tubules toward the central region. In contrast, in gonads of the XX fish with no UGP, these cysts were in disorderly alignment in comparison with those in the testes of normal XY and *scl*-XY fish, and neither an ovarian cavity nor efferent duct was identified. That is, the structure of these XX gonads appeared to be different from those of both normal ovaries and testes (Fig. 3A, C, D, E).

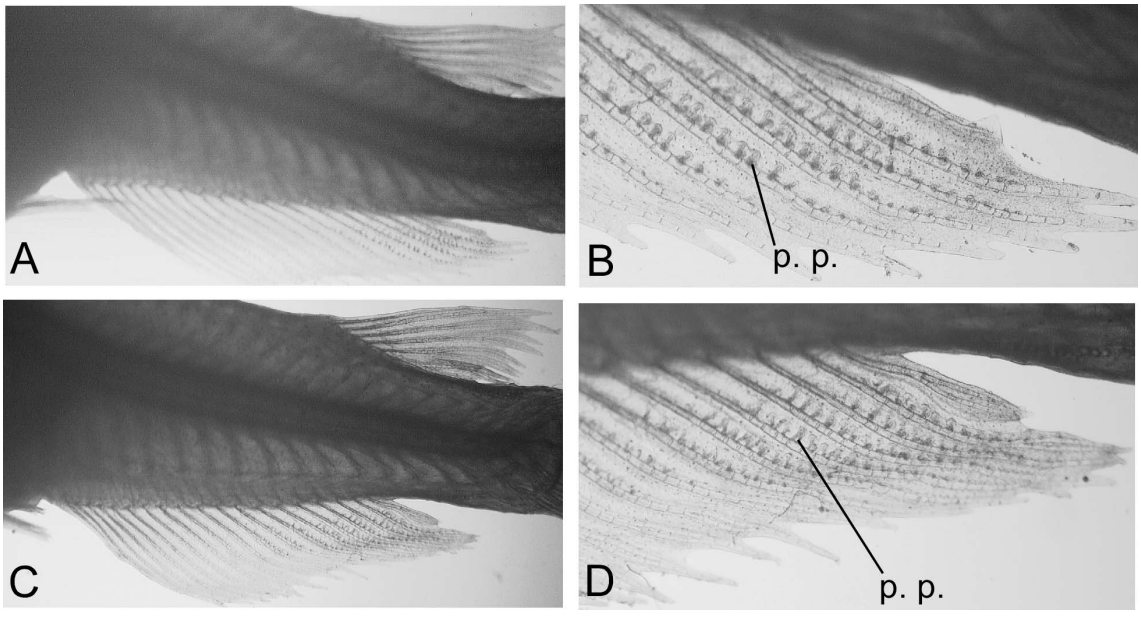

Fig. 4. Photographs of methyltestosterone (MT)-treated fish after 21 days of treatment. **(A, B)** An MT-treated XY female; **(C, D)** an MT-treated XY fish with no secondary sex characters. p.p., papillary processes.

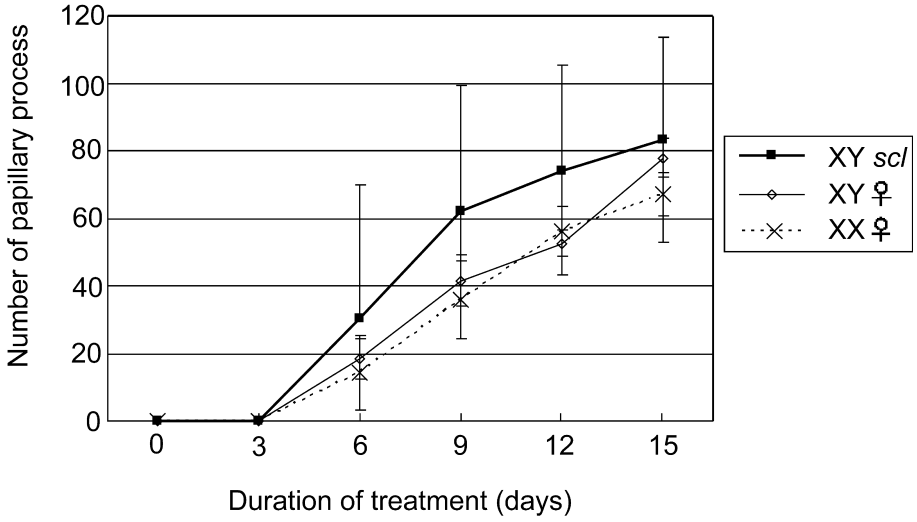

Fig. 5. Time-course of changes in the number of papillary processes on the anal fin rays of normal XX females, sex-reversed XY females, and XY fish with no secondary sex characters treated with methyltestosterone (10 μg/ml). Vertical bars indicate SD around the mean (n=3).

Table 1. Number and genetic and phenotypic sex in F_1 and F_2 offspring from an XY *scl* mutant and Hd-rR female cross.

	XX female	XY male	XX No secondary sex characters (XX *scl*)	XY No secondary sex characters (XY *scl*)	Total
F_1	6	10	0	0	16
F_2	27	24	8	9	68

Identification of a candidate gene for *scl*

The induction of papillary processes by MT in the *scl* XY mutant suggests that a lack of androgens might be responsible for the *scl* phenotype. In addition, the *scl* XX mutants had no UGP. Therefore, estrogen might also be lacking in the *scl* mutants. The fact that both male and female secondary sex characters were absent in the *scl* XX and XY mutants indicates that the *scl* phenotype might be induced by the deficiency of an enzyme related to an early steroid hormone-producing pathway.

Of the genes that encode for enzymes related to the early steroidogenic pathway in the medaka, only the *P450c17* gene had been cloned when we began this study. *P450c17* encodes an enzyme with multiple catalyzing functions at a branching point of the biosynthesis pathway of cortisol and sex steroids, including androgens and estrogens. Thus, we believed that *P450c17* could be a candidate *scl* mutant gene. We examined the *P450c17* genotypes of wild-type, *scl*, and F_1 fish. Using a combination of C17-24F and C17-14R primers, a 1.2-kilobase pair (kb) band was detected in all genotypes. On the other hand, with a combination of C17-14F and C17-14R primers, a 1.5-kb band was observed only in the wild type and the F_1, but not in the *scl* mutants (Fig. 6). In other words, the C17-24F/C17-14R primer set could amplify both the wild-type and mutant alleles, but the C17-14F/C17-14R primer set could not amplify the mutant allele. Thus, the *scl* mutation was thought to be located between primer sites C17-14F and C17-24F.

Next, we carried out linkage analysis between the *P450c17* genotype and *scl* phenotype. All 30 *scl* mutants lacked the 1.5-kb band, demonstrating that the *scl* pheno-

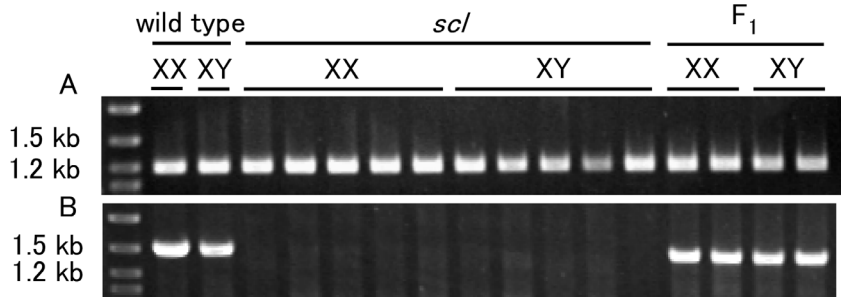

Fig. 6. PCR products of *P450c17* in wild-type, *scl*, and F_1 fish obtained using **(A)** primer set C17-24F/C17-14R and **(B)** primer set C17-14F/C17-14R. Using primer set C17-24F/C17-14R, a 1.2-kb band was observed in all genotypes. However, with primer set C17-14F/C17-14R, a 1.5-kb band was present in wild-type and F_1 fish, but not in *scl* mutant fish.

type was completely linked to the mutant genotype of *P450c17*. Analysis of medaka genome data (Medaka Genome Project: http://dolphin.lab.nig.ac.jp/medaka/index.php, version 1.0, scaffold 557) showed that the *P450c17* gene is located in autosomal linkage group 15, consistent with the results of our genetic cross, i.e., that the *scl* phenotype is caused by a single recessive mutation on an autosome.

DISCUSSION

Expression mechanism of *scl* phenotypes

Secondary sex characters are known to be induced by sex steroid hormones secreted from gonads in vertebrates, including the medaka. Here, in the *scl* mutants, neither male nor female secondary sex characters were seen. Moreover, the induction of papillary processes in the *scl* mutant treated with MT suggests that the processes of expression of male secondary sex characters after binding of androgen to the receptor are normal. Therefore, the ability to synthesize androgen seems defective in the *scl* mutant. Furthermore, since a UGP, which is a female secondary sex character, does not appear in the *scl* mutant, the ability to synthesize estrogen also seems defective. These results strongly suggest that the *scl* phenotype is caused by a lack of sex steroids—both androgens and estrogens. In fact, concentrations of testosterone and 17β-estradiol did not reach detectable levels in the *scl* mutants (Suzuki et al., in press). Another mutant that lacks male secondary sex characters and has testes in XY individuals is the testicular feminization mutant (*Tfm*) in mammals. However, *Tfm* exhibits complete androgen insensitivity caused by a mutation of the androgen receptor (Lyon and Hawkes, 1970). Accordingly, we suggest that *scl* is a novel sex differentiation mutant in vertebrates.

The sex steroidogenic pathway and associated enzymes have been clarified (reviewed by Devlin and Nagahama, 2002), and *P450c17*, a sex steroid synthesis-related gene, has been cloned in the medaka (DDBJ Accession No. D87121). Our results suggest that the *P450c17* gene of the *scl* mutants is distinguishable from that of normal fish. Using the C17-14F and C17-24F primer set, a 1.5-kb band was amplified in normal and F_1 fish, but not in the *scl* mutants. We then confirmed that the DNA sequence of the Hd-rR inbred strain was the same as that of the *scl* mutant at the C17-14F primer site (data not shown). These results indicate that a very large DNA fragment is inserted in the region between the C17-14F and C17-24F primer sites. Therefore, *scl* may be a loss-of-function *P450c17* mutant caused by the insertion.

P450c17 deficiency in humans results in impaired production of cortisol and sex steroids, leading to overproduction of mineralocorticoids, as well as hypertension, pseudohermaphroditism, and a delay in sexual maturation (Kater and Biglieri, 1994; Yanase, 1995; New, 2003). In the *scl* mutant, the loss-of-function *P450c17* mutation appears to cause a loss of sex steroids, apparently resulting in the complete absence of both male and female secondary sex characters. In addition, XY fish with the *scl* mutation do not show the pseudohermaphroditism evident in humans with the *P450c17* mutation.

Mutation of *P450c17* appears to cause impaired production of cortisol, which is vital to the existence of vertebrates, including fish. Why, then, is the *scl* mutant alive in the absence of *P450c17* function? Recently, Zhou et al. (2007a and 2007b) identified a novel type of *P450c17* (*P450c17-II*) in several fishes, including the medaka. P450c17-II in the medaka and tilapia possesses only 17α-hydroxylase activity, without any 17,20 lyase activity, unlike conventional P450c17 (P450c17-I), which has both of these activities. In the head kidney, *P450c17-II* was strongly expressed, whereas no signal for *P450c17-I* expression was detected. Our data suggest that *scl* is a loss-of-function mutant of *P450c17-I*. Taken together, these results indicate that because of the presence of a normal *P450c17-II*, which is responsible for the production of cortisol, the *scl* mutant is viable despite its deficiency in the *P450c17-I* gene.

Role of androgens in medaka spermatogenesis

The *scl* XY fish had well-developed testes with many spermatozoa, similarly to normal male fish. Artificial insemination using a testis from an *scl* XY fish showed that the spermatozoa of the *scl* XY fish were normal. Androgens are believed to be necessary in mammals for expression of the normal male phenotype, including the initiation and maintenance of spermatogenesis (reviewed by Collins et al., 2003; Holdcraft and Broun, 2004). In *Tfm* mutants and androgen receptor knockout mice, spermatogenesis is arrested in pachytene spermatocytes (Lyon and Hawkes, 1970; reviewed by Yeh et al., 2002). A deficiency in P450c17 causes infertility in male mice, and many sperm of these mice are morphologically abnormal (Liu et al., 2005). In teleost fish, androgens are also believed to be important for spermatogenesis. When 11-ketotestosterone, an androgen of fish, was added to testicular organ culture, spermatogenesis, from the proliferation of spermatogonia to spermiogenesis, was induced in the Japanese eel, *Anguilla japonica* (Miura et al., 1991). The action of 11-ketotestosterone in the promotion of spermatogenesis has also been recognized in goldfish (Kobayashi et al., 1991) and *Hucho perryi* (Amer et al., 2001). However, our results from the *scl* mutant suggest that spermatogenesis in medaka can occur without androgens. Saiki et al. (1997) showed that the spermatocytes of the medaka develop into fertile sperm in cell culture without contact with somatic cells. The report is consistent with normal spermatogenesis in XY *scl* mutants. Therefore, the *scl* mutant may provide novel insight into the relationship between spermatogenesis and androgens in vertebrates.

We note that a progestin, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), also plays an important role not only in final maturation but also in the initiation of meiosis in Japanese eel spermatogenesis (Miura et al., 2006). However, relationship between DHP and spermatogenesis in the *scl* mutants is unclear.

Spermatogenesis in the *scl* XX mutant

Mature *scl* XX fish had a testis instead of ovaries, and spermatogenesis seemed to occur normally, although oocytes at the diplotene stage were observed in the gonads of two *scl* XX fish. Several studies in teleost fish have suggested that estrogens are involved in ovarian differentiation and development (reviewed by Fostier et al., 1983). Suzuki et al. (2004) reported that in the medaka, aromatase inhibitor (AI) treatment suppressed ovarian cavity formation,

and spermatogenesis was occasionally induced in the ovaries of AI-treated adult fish. However, AI treatment did not affect early oogenesis. These results suggest that endogenous estrogen is not essential for sex determination nor early oogenesis; we thus assume that early-stage oogenesis occurs normally in *scl*-XX fish, but that the ovarian cavity does not develop in the absence of estrogen. Subsequently, spermatogenesis may occur in *scl* XX gonads after they reach maturity and ultimately only spermatogenesis may be observed, but not oogenesis. In fact, in *scl* XX gonads, early oogenesis is not affected, but ectopic spermatogenesis occurs in gonads at the adult stage (from 70 days after hatching) (Suzuki et al., in press). At that time, the cysts may be aligned in a disorderly fashion, unlike those in the testes of normal males.

Estrogen receptor α and β knockout ($\alpha\beta$ERKO) mice show normal ovarian development at an early stage; however, the ovaries of adult $\alpha\beta$ERKO XX individuals exhibit transdifferentiation of follicles to structures resembling seminiferous tubules of the testes after puberty (Couse et al., 1999; Dupont et al., 2000). These reports suggest that in mammals, estrogen is required for the maintenance of ovaries, but not for early ovarian development. Since the gonadal development in $\alpha\beta$ERKO XX mice is similar to that of the *scl* XX medaka, it appears that estrogen is required in vertebrates for maintenance of the ovary, but not for sex determination nor early oogenesis. However, the mechanism of the sex reversal in the XX *scl* mutant is unknown, and it is unclear whether steroids other than androgens and estrogens (for example, DHP) are involved. Thus, the mechanism of the sex reversal needs further investigation.

We found a viable *P450c17* loss-of-function mutant in medaka. Although *P450c17* has been isolated in many vertebrates, including several teleost fish (for example, rainbow trout [Sakai et al., 1992], Japanese eel [Kazeto et al., 2000] and zebra fish [Wang and Ge, 2004]), a *P450c17* loss-of-function mutant had not yet been found in vertebrates. Thus, *scl* may serve as an in-vivo animal model for analyzing the role of sex steroids in sex determination and gametogenesis.

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