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*Original Article*

# Sex reversal of genetic females (XX) induced by the transplantation of XY somatic cells in the medaka, *Oryzias latipes*

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**ABSTRACT In order to investigate the function of gonadal somatic cells in the sex differentiation of germ cells, we produced chimera fish containing both male (XY) and female (XX) cells by means of cell transplantation between blastula embryos in the medaka, *Oryzias latipes*. Sexually mature chimera fish were obtained from all combinations of recipient and donor genotypes. Most chimeras developed according to the genetic sex of the recipients, whose cells are thought to be dominant in the gonads of chimeras. However, among XX/XY (recipient/donor) chimeras, we obtained three males that differentiated into the donor's sex. Genotyping of their progeny and of strain-specific DNA fragments in their testes showed that, although two of them produced progeny from only XX spermatogenic cells, their testes all contained XY cells. That is, in the two XX/XY chimeras, germ cells consisted of XX cells but testicular somatic cells contained both XX and XY cells, suggesting that the XY somatic cells induced sex reversal of the XX germ cells and the XX somatic cells. The histological examination of developing gonads of XX/XY chimera fry showed that XY donor cells affect the early sex differentiation of germ cells. These results suggest that XY somatic cells start to differentiate into male cells depending on their sex chromosome composition, and that, in the environment produced by XY somatic cells in the medaka, germ cells differentiate into male cells regardless of their sex chromosome composition.**

KEY WORDS: *sex differentiation, testis, germ cell, chimera, medaka*

## Introduction

The distinction between females and males is a common polymorphism seen in vertebrates. Their phenotypes depend upon their gonadal sex, that is, ovary or testis. It is generally accepted that the undifferentiated gonad is sexually bipotential and can differentiate into either ovary or testis. During the gonadal sex differentiation, primordial germ cells differentiate into oogonia or spermatogonia, whereas the germ cell supporting cells also differentiate into female- or male-type cells, namely, granulosa or Sertoli cells. Germ cells and their supporting cells in a given gonad differentiate into the same sex, so that either type of cell must have a primary effect on sex determination, which is dominant over that of the other type.

In mammals, a single gene on the Y chromosome, *Sry* (*sex-determining region Y*), determines the sex of the organism by initiating the development of a testis instead of an ovary (Koopman *et al*., 1991). Because pre-Sertoli cells are the first male-specific cells to differentiate in the fetal testis, and because they differentiate predominantly from the XY component in XX/XY chimeric mice (Palmer and Burgoyne, 1991; Patek *et al*., 1991), it is thought that the supporting cell lineage in the XY gonad is the cell type which expresses *Sry* and plays a key role in gonadal sex determination. On the other hand, germ cells in mammals are considered not to be critical for primary sex determination because gonadal sex differentiation occurs even in the absence of germ cells (Marchant, 1975; Marchant-Larios and Centeno, 1981; McCoshen, 1982). In addition, XY germ cells that mismigrate to the outside of the testis can differentiate into oocytes (Upadhyay and Zamboni, 1982; Zamboni and Upadhyay, 1983), while XX germ cells can differentiate into prospermatogonia in testes (Palmer and Burgoyne, 1991). These observations strongly suggest that germ cells basically tend to differentiate into oocytes regardless of their genetic sex, and that the existence of pre-Sertoli cells is necessary for the

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differentiation of germ cells into male cells. Thus, sex determination in mammals is mediated *via* the supporting cell lineage in the fetal gonad, while sex differentiation of germ cells depends upon the sex of the supporting cells.

In the teleost fish medaka, *Oryzias latipes*, an XX-XY mechanism of sex determination like that in mammals has been demonstrated (Aida, 1921; Yamamoto, 1955, 1958). However, the order of events observed in the early sex differentiation in the medaka is different from that in mammals. The difference between males and females is first observed in germ cells in terms of higher proliferative activity and earlier occurrence of meiotic cells in females (Satoh and Egami, 1972; Hamaguchi, 1982). No morphological differences between the supporting cells of males and females are observed until 10 days after hatching, when the male-specific structure among supporting cells first appears as an acinous structure, the precursor of the testicular seminiferous tubule (Kanamori *et al*., 1985). In females, ovarian follicles constitute the first female-specific structure and can be identified around diplotene oocytes about 20 days after hatching. To investigate the role of germ cells in early sex differentiation of supporting cells in the medaka, we studied gonadal differentiation after the number of germ cells was reduced with an alkylating agent (Shinomiya *et al*., 2001). The results indicated that testis-specific structures, corresponding to the precursors of seminiferous tubules, were generated by the supporting cells in the gonadal regions without germ cells only in genetic males (XY). This suggested that, although sex differentiation in supporting cells becomes manifest later than in germ cells in this species, XY supporting cells initiate sex differentiation independently of the sex of the germ cell. In addition, ovarian follicles in XX individuals were not observed in gonadal regions without germ cells, which suggests that the differentiation of granulosa cells depends on the developing oocytes. The question remains, however, whether sex differentiation in primordial germ cells into male cells (spermatogonia) is controlled by the surrounding somatic cells.

In the study presented here, we produced chimeric medaka containing both male (XY) and female (XX) cells, and investigated the sex of germ cells and somatic cells in these chimeras at the early sex differentiation stage and the adult stage. Our findings demonstrate that XY somatic donor cells induce the sex reversal of XX germ cells and XX somatic cells and that XY donor cells affect the early sex differentiation of germ cells. In conclusion, the results presented here strongly suggest that XY somatic cells perform the primary role in sex differentiation and cause germ cells to divert into male-type cells in the medaka.

## Results

The design of the experiment is outlined in Fig. 1. A total of 199 transplants were performed on 16 different days, and 124 of these transplants survived and developed apparently normally throughout the hatching stage. Forty-nine chimera fish grew up and matured at about three months of age, almost the same as for normal fish.

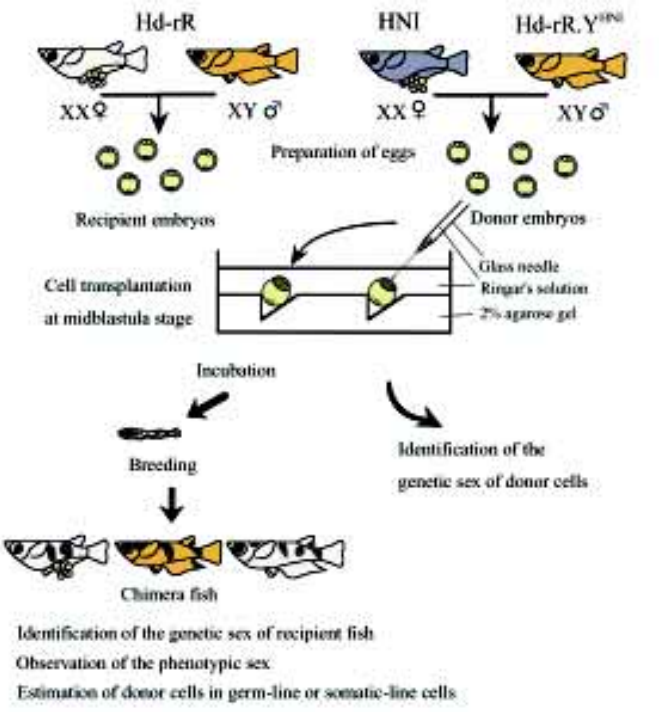


**Fig. 1. Schematic summary of the experiment** *(see text for details).*

### *Identification of Donor Cells in Somatic Tissues of Chimera Fish*

The melanization of the melanophore in the medaka is controlled by the gene on the *b* locus. HNI contains a *B* allele, and Hd-rR and Hd-rR.$Y^{HNI}$ contain a *b* allele. The recipient strain (Hd-rR) does not contain any melanized melanophores, while the donor strain (F1 hybrid of HNI and Hd-rR.$Y^{HNI}$ strains) contains wild-type melanophores. Melanized melanophores, considered to be donor cells, were detected in all the chimera fish examined. In addition, we investigated whether the donor-specific DNA fragment (N-band in Fig. 2) was detectable in the PCR products of the DNA extracted from two parts of the muscle tissues of chimeras (six XY/XX and ten XX/XY chimeras), and this was found to be the case in all chimeras (Fig. 2, lane 6-21). These results demonstrated the presence of chimerism in the somatic tissues. The distribution pattern of melanized melanophores in individuals (Fig. 3) and the quantity of amplified donor-specific bands compared with that of bands of the recipient strain in muscles as determined by means of PCR (Fig. 2) varied among these chimeras, indicating differences in the contributions of donor cells in each individual.

TABLE 1

**PHENOTYPIC SEX OF CHIMERA FISH**

| Genotype [a] | N | Female | (%) | Male | (%) |
|---|---|---|---|---|---|
| XY/XY | 9 | 0 | (0) | 9 | (100) |
| XY/XX | 12 | 0 | (0) | 12 | (100) |
| XX/XY | 16 | 13 | (81) | 3 | (19) |
| XX/XX | 12 | 12 | (100) | 0 | (0) |

[a]Genetic sex of recipient/donor

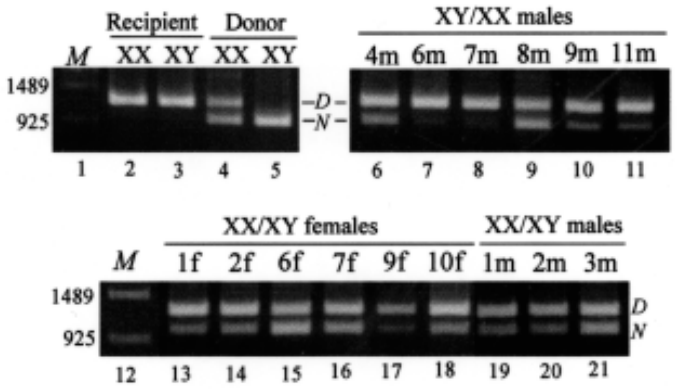


**Fig. 2. PCR detection of *SL1* fragments originating from the donor or the recipient strain in muscles from the anterior trunk of XY/XX (recipient/donor) and XX/XY chimeras.** *All samples show the donor strain specific fragments (N band) as well as the fragments of the recipient strain (D band).* **Lanes: (1,12)** *DNA size markers;* **(2,3)** *Recipient XX female and XY male, Hd-rR;* **(4,5)** *Donor XX female and XY male, (HNI X Hd-rR.* $Y^{HNI}$*)*$F_1$ *hybrid;* **(6-11)** *XY/XX males;* **(13-18)** *XX/XY females;* **(19-21)** *XX/XY males. The sample numbers correspond to the numbers in Table 2.*

### *Phenotypic Sex of Chimeras*

The phenotypic sex of nine XY/XY (recipient/donor), twelve XY/XX, sixteen XX/XY, and twelve XX/XX chimeras is shown in Table 1. In chimeras with XY recipients, all individuals grew into males in accordance with the genetic sex of the recipients, irrespective of the genetic sex of the donors. All XX/XX and thirteen out of sixteen (81%) XX/XY chimeras developed into females in accordance with their recipient's sex, whereas three (19%) XX/XY chimeras differentiated into their donor's, that is, male sex.

Gonads from two XY/XY males, four XY/XX males, eight XX/XY females, three XX/XY males, and three XX/XX females were histologically examined. In all cases, secondary sex characters coincided with the gonadal sex, and the histology of their testes and ovaries was completely normal even in the three XX/XY males that had taken on the donor's sex (Fig. 4).

### *Germline Chimeras*

These chimeras were mated with adult fish of the Hd-rR strain to determine whether they would give rise to any melanized offspring derived from the transplanted donor cells. A total of 27 chimeras were mated, and batches of spawned eggs from each pair were collected between days 29 and 112 of the experiment. About 5 to 10 fertilized eggs were obtained per batch, and 63 to 333 offspring from 16 to 69 batches from each mating were examined. The frequency of melanized offspring and the extent of the contribution of donor cells to germline cells in chimeras were estimated (Table 2).

Among the XX/XY (recipient/donor) male fish that differentiated into the donor's sex, one of the three chimeras produced melanized offspring derived from XY cells (3m in Table 2). This indicates that XY germ cells from the donor were present in its testes. The fact that two XX/XY males yielded no melanized offspring (1m and 2m in Table 2) suggests that they had only XX spermatogenic cells from recipients cells.

Two of the three XY/XY males, four of the seven XY/XX males, three of the seven XX/XY females, and three of the seven XX/XX females also yielded melanized progeny. Thus, 14 (52%) of the chimeras examined were found to be germline chimeras, with the contributions of donor cells to germline cells higher in the male chimeras (XY/XY: 75-95%; XY/XX: 28-73%; XX/XY: 42%) than those in females (XX/XX: 7-42%; XX/XY: 4-6%). The reason for these findings is not clear, but it is possible that differences in germ cell proliferation kinetics between donor and recipient strains in the early developmental stages result in different contributions by the donor to the germline in male and female chimeras.

### *Chimerism in XX/XY Testes*

In order to ascertain the chimerism of XX and XY cells in the XX/XY (recipient/donor) testes, we examined the DNA extracts from three different regions of serial histological sections of each testis. PCR was performed to identify the genotype of *SL1* in these samples and demonstrated the presence of a donor-specific as

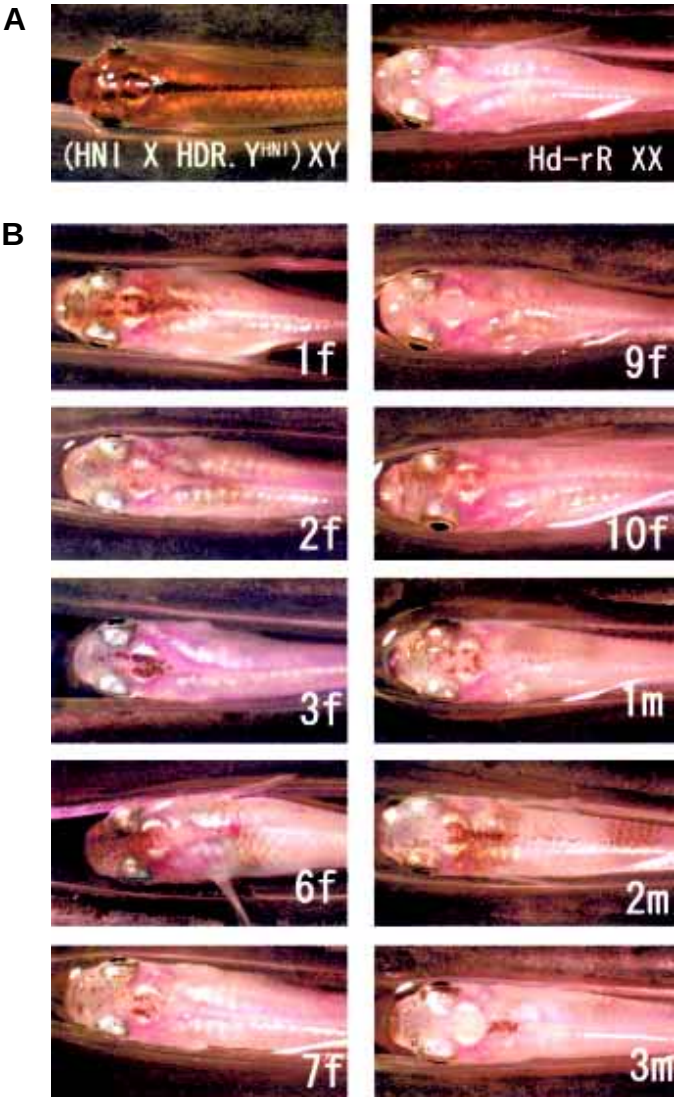


**Fig. 3. Body color of a donor, a recipient, and chimera fish.** *The distribution patterns and degrees of melanophore melanization vary in both females and males.* **(A)** *XY fish of the donor strain (left) and XX fish of the recipient strain (right).* **(B)** *XX/XY (recipient/donor) chimeras. Females: 1f, 2f, 3f, 6f, 7f, 9f, and 10f; Males: 1m, 2m, and 3m. The numbers of the chimeras correspond to the numbers in Table 2.*

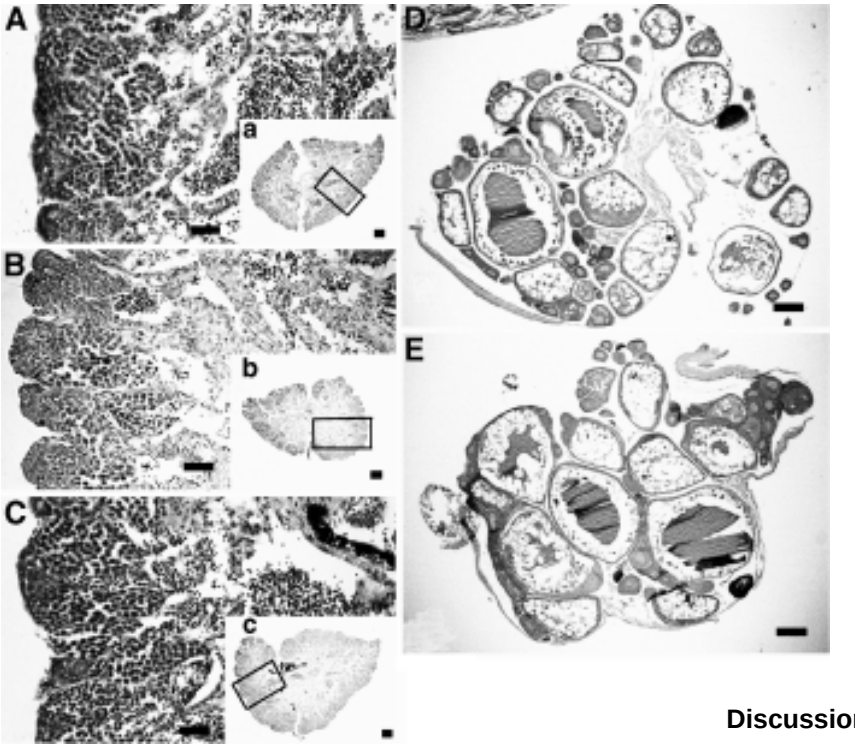


**Fig. 4. Light micrographs of the gonads in homo- and hetero-sex chimeras.** *Each gonad is histologically normal.* **(A, a)** *Testis of XY/XY (recipient/donor) male.* **(B, b)** *Testis of XY/XX male.* **(C, c)** *Testis of XX/XY male.* **(D)** *Ovary of XX/XX female.* **(E)** *Ovary of XX/XY female. A, B and C show higher magnifications of the areas outlined in black in a, b and c respectively. Scale bars, 20 μm (A,B,C); 50 μm (a,b,c), and 200 μm (D,E).*

well as of a band of the recipient strain in all of them (Fig. 5). This indicates that donor XYcells were distributed over a wide range in these testes including the two testes that did not produce any melanized offspring (1m and 2m in Table 2).

### *Early Stage of Sex Differentiation of XX/XY Chimeras*

Gonads in the XX/XY (recipient/donor) chimeras were histologically examined 10 days after hatching to investigate the effect of XY cells on germ cells in the early stage of sex differentiation. Ten days after hatching, ovaries had become morphologically distinguishable from testes in terms of the larger number of germ cells and the presence of meiotic oocytes in this species (Satoh and Egami, 1972; Hamaguchi, 1982). Serial sections of the gonads were examined, and the numbers of oogonia or spermatogonia and meiotic oocytes were counted in 16 XY individuals, 18 XX individuals, and 37 XX/XY chimeras. The total numbers of their germ cells are plotted in Fig. 6.

There was an obvious difference in these numbers between XY and XX individuals, with those for XY ranging from 46 to 136, and those for XX from 340 to 1228. All of the XX individuals possessed a large number of oocytes, with a minimum of 137. Among XX/XY chimeras, most specimens showed a large number of germ cells, from 297 to 1374, with numerous oocytes, which were regarded as representing normal ovaries. However, two specimens with as few germ cells as those of XY were identified in this group. These two fry possessed only 95 and 106 germ cells and no oocytes, so that these gonads could be regarded as developing testes. In addition to these two, three XX/XY chimeras had gonads of a type intermediate between developing testes and ovaries, and their total number of germ cells (151, 156, and 185) was below the range of normal XX. All of these gonads possessed oocytes, but only 15, 26, and 74, and thus markedly fewer than those in normal XX.

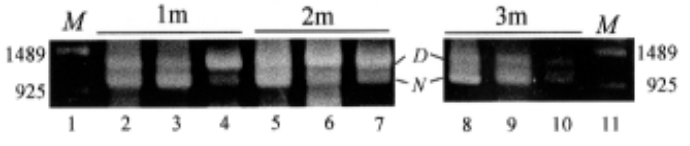


**Fig. 5. PCR detection of *SL1* fragments originating from the donor or the recipient strain in testes of XX/XY (recipient/donor) males.** *All samples indicate the donor strain specific fragments (N band) as well as the fragments of the recipient strain (D band). Lanes 1 and 11, DNA size markers; lanes 2-10, XX/XY males, 1m (2-4), 2m (5-7), 3m (8-10). The sample numbers correspond to the numbers in Table 2.*

## Discussion

In the study presented here, chimera fish were obtained from all combinations of recipient and donor genotypes. All homo-sex chimeras differentiated into males or females in accordance with their genetic sex. In hetero-sex chimeras, most fish developed in accordance with the genetic sex of the recipient, whose cells were dominant in each chimera. Donor cells in these chimeras, the minor component, differentiated into the sex opposite to that of their own genetic sex. However, we also obtained three chimeras among the XX/XY (recipient/donor) chimeras that differentiated according to their donor's, that is, male sex. In these males, XY cells from the donor developed into male cells in accordance with their genetic

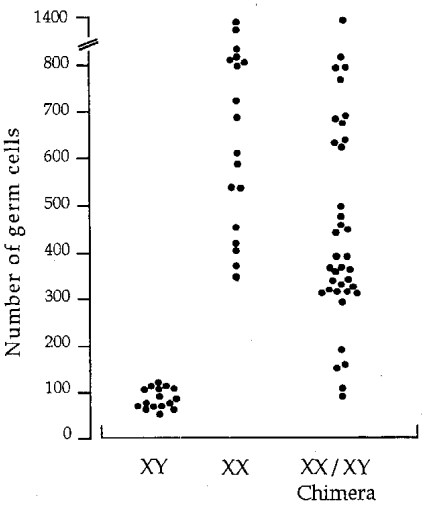


**Fig. 6. Total numbers of germ cells in XY and XX individuals, and XX/XY (recipient/donor) chimeras 10 days after hatching.** *See text for details.*

sex, and XX cells from the recipient trans-differentiated according to the sex of the donor cells.

### *Chimerism of Germ Cells and Somatic Cells in XX/XY Testes*

In various fish species, the presence of a small number of primordial germ cells (PGCs) in early development has been demonstrated (reviewed by Braat *et al*., 1999). In zebrafish, only four cells were found as precursor of PGCs at the blastula stage when the *vas* gene was used as the molecular marker of germline cells (Yoon *et al*., 1997). At the late gastrula stage of the medaka, 10 to 25 PGCs could be detected with whole-mount *in situ* hybridization using the *vas* probe (Shinomiya *et al*., 2000), but these PGCs were scattered in the posterior shield and the zygotic expression of the *vas* gene was detected at the early gastrula stage by means of RT-PCR (Shinomiya *et al*., unpublished data), suggesting that PGCs in the medaka must be traced back to a few cells as in the zebrafish. Thus, PGCs in fish originate from a very small population in the early embryonic stages, that is, it appears that the germline precursors are not always present in transplanted donor cells. In other words, it is possible that some chimeras do not have germ cells from transplanted donor cells.

In the our study we examined the progeny of chimera fish, obtained from the eggs from 16 to 69 batches, in order to evaluate the contribution of donor cells to the germline. In two of the three XX/XY (recipient/donor) males, no melanized offspring originating from XY donor cells appeared in as many as 248 or 268 specimens from 47 or 56 batches of eggs collected over 71- or 104-day periods. Although these results did not constitute direct proof that all germ cells were XX cells in these two XX/XY testes, our results strongly suggest that no or an extremely small number of XY germ cells were present in their testes as spermatogenic cells. Genotyping of the histological sections of these testes also demonstrated that these testes contained XY cells over a wide range. These results lead to the conclusion that spermatogenic germ cells in these testes were XX cells, and testicular somatic cells consisted of XX and XY cells. Our findings thus suggest that XY somatic cells in the gonad perform a critical role in the differentiation of XX/XY gonads into testes because they induce sex reversal of both XX somatic cells and XX germ cells of the recipient.

Thirteen XX/XY chimeras became females regardless of the presence of XY cells. Because the chimerism in the somatic tissues of the chimeras varied, it is likely that, in these females, the number of XY somatic cells compared to that of XX somatic cells in the undifferentiated gonad was not enough to induce sex differentiation into testis. In three of these XX/XY females (1f, and 6f and 7f in Table 2), the contribution of donor XY cells to germ-line cells was observed, which agrees with our conclusion that XY germ cells do not play a critical role in the sex reversing process of XX cells in XX/XY males.

### *Early Sex Differentiation in XX/XY Gonads*

We observed gonads of XX/XY (recipient/donor) fry 10 days after hatching. Two fry were estimated to have testes on the basis of the small number of germ cells and absence of meiotic oocytes. In addition, the number of germ cells was remarkably smaller than in normal ovaries in three fry with a small number of oocytes. Considering that all chimera fish examined had donor cells in their somatic tissues, it can be concluded that these XX/XY fry possessed XY cells, at least in their somatic cells. These XX/XY fry must also have possessed XX germ cells because all chimera fish in our study produced offspring from germline cells of the recipient strain. Therefore, it can be safely said that XX germ cells in these XX/XY fry had already deviated from the female pathway and had started male development under the influence of XY cells at a stage earlier than 10 days after hatching. In other words, XY somatic cells in these chimeras began to differentiate into male cells earlier than germ cells and generated some conditions that repressed the proliferating activity of XX germ cells, so that these cells developed into the male type.

### *Primary Role of Somatic Cells in the Sex Differentiation in the Medaka*

It has been established that germ-cell supporting cells play a crucial role in gonadal sex differentiation in mammals. Cell-autonomous expression of *Sry* triggers the differentiation of the supporting cells into Sertoli rather than granulosa cells (reviewed by Swain and Lovell-Badge, 1999; Capel, 2000). Germ cells in mammals are thought to differentiate according to the sex of their supporting cells (reviewed by McLaren, 1991, 1995). A similar view prevails regarding gonadal sex determination and differentiation for amphibians and birds as a result of experimental observations of the transplan-

TABLE 2

**FREQUENCY OF MELANIZED PROGENY FROM MATING OF CHIMERAS WITH FISH OF Hd-rR STRAIN**

| | | | Number of offspring | | | |
|---|---|---|---|---|---|---|
| **Chimera[a]** | **Phenotype[b]** | | **Total** | **Melanized[c]** | **Contribution[d,e]** | |
| XY/XY | 15m | M | 225 | 0(0%) | 0% | |
| | 17m | M | 128 | 61(48%) | 95% | |
| | 18m | M | 83 | 31(37%) | 75% | |
| XY/XX | 4m | M | 135 | 19(14%) | 28% | |
| | 6m | M | 149 | 0(0%) | 0% | |
| | 7m | M | 184 | 0(0%) | 0% | |
| | 8m | M | 126 | 46(37%) | 73% | |
| | 9m | M | 105 | 18(17%) | 34% | |
| | 11m | M | 146 | 30(21%) | 41% | |
| | 12m | M | 63 | 0(0%) | 0% | |
| XX/XY | 1f | F | 249 | 6(2%) | 5% | |
| | 2f | F | 231 | 0(0%) | 0% | |
| | 3f | F | 98 | (0%) | 0% | |
| | 6f | F | 241 | 7(3%) | 6% | |
| | 7f | F | 261 | 5(2%) | 4% | |
| | 9f | F | 380 | (01%) | 0% | |
| | 10f | F | 207 | 0(0%) | 0% | |
| | 1m | M | 248 | 0(0%) | 0% | |
| | 2m | M | 268 | 0(0%) | 0% | |
| | 3m | M | 333 | 70(21%) | 42% | |
| XX/XX | 14f | F | 209 | 0(0%) | 0% | |
| | 15f | F | 191 | 0(0%) | 0% | |
| | 16f | F | 221 | 13(6%) | 12% | |
| | 18f | F | 266 | 9(3%) | 7% | |
| | 19f | F | 118 | 0(0%) | 0% | |
| | 20f | F | 229 | 48(21%) | 42% | |
| | 21f | F | 137 | 19(14%) | 28% | |

[a]The genetic sex of the chimeras (recipient/donor); [b]M: male, F: female; [c]The melanized offspring were counted 3-4 days after fertilization.; [d]The donor cell contribution to germline cells is twice the extentof the melanized offspring of cach chimera.; [e]White/black columns indicate the recipient/donor cell contributionto germ-line cells of the chimera.

tation of germ-cell containing grafts into an embryo with the opposite sex in *Xenopus* (Blacklar, 1965) and *Pleurodeles waltl* (Dournon *et al*., 2001), and cell transplantation between different-sex embryos in chick (Kagami *et al*., 1995; 1997). These results showed that the sex chromosome composition of germ cells has no effect on the sex determination process, and that the sex of germ cells is determined by their surrounding environment.

In the medaka, on the other hand, it has been inferred that germ cells can have a primary influence on gonadal sex differentiation from the fact that sexual differences during normal development are first observed in germ cells but not in somatic cells at the time of hatching (Satoh and Egami, 1972; Hamaguchi, 1982). The morphological differences between male and female in the gonadal somatic cells of this fish can be identified about 10 days after hatching when the male-specific structure among supporting cells first appears as an acinous structure, the precursor of testicular seminiferous tubules (Kanamori *et al*., 1985). Recently, we have demonstrated that acinous structures can be generated from supporting cells without contact with germ cells only in genetic males (XY) (Shinomiya *et al*., 2001). This suggests that XY supporting cells can initiate sex differentiation into the male type regardless of the presence of coexisting germ cells. However, it was not known whether the sex differentiation of primordial germ cells into male cells is determined by the surrounding somatic cells in this species.

In this study, we investigated the sex differentiation of germ cells and somatic cells in chimeras having both XX and XY cells during the sex differentiation stage and the adult stage. The results suggest that the primary role in gonadal sex differentiation in the medaka is performed not by germ cells but by gonadal somatic cells, and that XY somatic cells can induce germ cells to follow the male pathway. On the basis of our findings, we propose the following mechanisms of early sex differentiation in the normal development of the medaka. The autonomous differentiation of XY somatic cells into male cells starts according to their sex chromosome composition earlier than that of germ cells. XY somatic cells produce an environment conducive to the differentiation of germ cells into male cells regardless of their own sex chromosome composition. These cells would then act on germ cells by inhibiting their proliferation, and this constitutes the first indication of sex differentiation of somatic cells in the medaka. The morphological differentiation into males first appears in the supporting cell lineage among gonadal somatic cells, so that these cells are thought to play the most important role in sexual differentiation in the medaka as they do in mammals. Recently, a candidate for the sex-determining gene in the medaka was identified, and the expression of this gene was detected by *in situ* hybridization not in germ cells but in somatic cells at the early sex differentiation stage (Matsuda *et al*., 2002). These results support our conclusion. Further work is required to identify the factor(s) from XY somatic cells which induce germ cells to follow the male-differentiating pathway.

## Materials and Methods

### *Medaka Strains*

Two inbred strains, the HNI and the Hd-rR strains (Hyodo-Taguchi and Sakaizumi, 1993), of the medaka, *Oryzias latipes*, were used. In addition, we also used a congenic strain, Hd-rR.$Y^{HNI}$, which was established from Hd-rR females and an HNI male (Matsuda *et al*., 1997). Most of the genome of the congenic strain is constructed from that of the Hd-rR strain, but it also contains the region, which includes the sex-determining region on the Y chromosome derived from the HNI. For the transplantation experiment, the Hd-rR strain was used as the recipient, and the $F_1$ hybrid from the mating of the HNI females with the Hd-rR.$Y^{HNI}$ males as the donor (Fig. 1). By using these strains, we could identify the genetic sex of donors and recipients with sex-linked gene markers, namely *SL1* (Shinomiya *et al*., 1998), a sex-chromosome-specific DNA sequence, and the allele *R* of the *r* locus located on the Y chromosome (Aida, 1921).

Fish were maintained in aquaria under an artificial light phase of 14L: 10D at 27 ± 2°C.

### *Preparation of Chimera Fish*

Spontaneously spawned eggs were collected and dechorionated with a hatching enzyme solution (Wakamatsu *et al*., 1993). Embryos were incubated in a balanced salt solution for medaka eggs, BSS (Iwamatsu, 1983), with 100 U/ml penicillin and 100 μg/ml streptomycin (BSS+PS). Cell transplantation was performed on embryos during the blastula stage (about 1000-2000 cells) (Fig. 1). Donor and recipient embryos were placed on V-shaped grooves of 2% agarose gel in BSS+PS in 6-cm plastic dishes covered with BSS+PS. Microinjection was performed with a micromanipulator attached to a stereomicroscope. Transplantation needles were made from 1-mm glass capillaries, which were pulled with a horizontal pipette puller and clipped using a fine forceps with a stereomicroscope to create a the sharp tip with an approximately 30 μm diameter. The needle was filled with distilled water and connected to the micromanipulator. Following the drawing of light mineral oil up to 10-15 mm from the top of the needle, BSS+PS was drawn into the needle. Approximately 100-200 cells from a donor embryo were then loaded into the needle by suction and transplanted into a recipient embryo. The transplants were incubated in BSS with 2 ppm methylene blue at 26 ±0.5°C until hatching. Hatched fry were grown under a same condition of adult fish, and the phenotypic sex of these specimens was determined by their secondary sex characteristics.

The genetic sex of chimeras was shown as “the genotype of recipient/ that of donor”.

### *Polymerase Chain Reaction (PCR)*

The DNA templates for PCR were prepared from donor embryos after cell transplantation, from muscles in chimera fish with phenol-chloroform extraction, and from paraffin sections of testicular tissue with a DNA Isolator PS Kit (Nippon Gene, Osaka, Japan). Two parts of muscle tissues (anterior and tail parts of the trunk) were prepared in each chimera.

The *SL1* fragments were amplified with the following PCR primers,
pHO5.5-Fc
5’-CCTGTGGTCTGTAAAGCTGCTTCAAATTGCTCAGCTTCG-3’ and
pHO5.5-RVc
5’-AACTTTTAGGCCATGCTATAAAAAATGTCTAGTTGATTGGCT-3’
for donor embryos, or pHO5.5-Fc and HNI-RVa
5’-AAGTCTGATACTACAAACAAGCAAAAATGCCTTAGAAAACT-3’
for muscles and testes of chimeras. The primers pHO5.5-Fc and HO5.5-RVc were designed in the region of consensus alignments among strains used in this study, while the primer HNI-RVa was designed according to the alignment of the HNI strain in order to amplify the fragments from the HNI strain more effectively than those from Hd-rR. The reaction mixture for amplification by PCR contained *Ex Taq* Buffer, 0.2 mM dNTPs, 0.2 μM each of the primers, 0.6 units of *Ex Taq* polymerase (Takara Shuzo Co., Kyoto, Japan) and template DNA. The reaction procedure for donor embryos was performed at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, at 55°C for 1 min, at 72°C for 1 min, and finally an additional 72°C for 5 min. For muscles the procedure was performed at 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec, at 62°C for 1 min, at 72°C for 1 min, and finally an additional 72°C for 2 min. For testes the first stage was 94°C for 2 min, followed by 55 cycles at 94°C for 30 sec, at 66°C for 1 min, at 72°C for 1 min, and finally an additional 72°C for 2 min. The reaction mixture was then analyzed by electrophoresis in a 1% agarose gel.

### *Progeny Test*

Mature chimera fish were mated to adult fish of the Hd-rR strain, and the melanized and non-melanized offspring were counted at 3 or 4 days after fertilization. Because the genotype of the *b* locus in the donor fish was the

hetero type of the wild type *B* and the mutant type *b*, the extent of the contribution of donor cells to germline cells in chimeras was calculated as twice of incidence of melanized offspring.

***Histological Observation of Gonads***

Gonads were dissected from each of the mature chimera fish, and fixed in 4% paraformaldehyde, embedded in paraffin, sectioned serially at 5 μm thickness, stained with hematoxylin and eosin, and prepared for histological observation with a light microscope.

Fry of XY and XX individuals as well as XX/XY (recipient/donor) chimeras 10 days after hatching were fixed *in toto* in Bouin's solution, and serial sections were prepared as described above. The total numbers of spermatogonia or oogonia and oocytes in each specimen were counted by careful examination on serial sections under a light microscope. For part of this experiment, embryos reproduced from XX females and XX males or YY males, which were the result of artificial sex reversals in the d-rR strain (Yamamoto, 1955; 1958), were used as recipients or donors of chimeras.

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