Potency of testicular somatic environment to support spermatogenesis in XX/Sry transgenic male mice

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The sex-determining region of Chr Y (Sry) gene is sufficient to induce testis formation and the subsequent male development of internal and external genitalia in chromosomally female mice and humans. In XX sex-reversed males, such as XX/Sry-transgenic (XX/Sry) mice, however, testicular germ cells always disappear soon after birth because of germ cell-autonomous defects. Therefore, it remains unclear whether or not Sry alone is sufficient to induce a fully functional testicular soma capable of supporting complete spermatogenesis in the XX body. Here, we demonstrate that the testicular somatic environment of XX/Sry males is defective in supporting the later phases of spermatogenesis. Spermatogonial transplantation analyses using XX/Sry male mice revealed that donor XY spermatogonia are capable of proliferating, of entering meiosis and of differentiating to the round-spermatid stage. XY-donor-derived round spermatids, however, were frequently detached from the XX/Sry seminiferous epithelia and underwent cell death, resulting in severe deficiency of elongated spermatid stages. By contrast, immature XY seminiferous tubule segments transplanted under XX/Sry testis capsules clearly displayed proper differentiation into elongated spermatids in the transplanted XY-donor tubules. Microarray analysis of seminiferous tubules isolated from XX/Sry testes confirmed the missing expression of several Y-linked genes and the alterations in the expression profile of genes associated with spermiogenesis. Therefore, our findings indicate dysfunction of the somatic tubule components, probably Sertoli cells, of XX/Sry testes, highlighting the idea that Sry alone is insufficient to induce a fully functional Sertoli cell in XX mice.

KEY WORDS: Sry, Sertoli cell, Transplantation, Spermatogenesis, Spermiogenesis, Mouse

INTRODUCTION

In many non-mammalian vertebrate species with a genetic sex-determination system, it has been shown that experimental and spontaneous sex-reversed XX or ZW males show complete spermatogenesis and the production of functional sperm (Yamamoto, 1955; Elbrecht and Smith, 1992; Hayes, 1998; Geffen and Evans, 2000; Nanda et al., 2003). In mammals, Sry is essential in pre-Sertoli cells for initiating male sex differentiation (Sinclair et al., 1990; Koopman et al., 1991). Sry alone is sufficient to promote testis formation and the subsequent male development of internal and external genitalia in chromosomally female mice (Koopman et al., 1991). However, XX sex-reversed males such as XX/Sry-transgenic (XX/Sry), XXSxr and XXXY mice, are always infertile because of the loss of spermatogonial germ cells soon after birth (Cattanach et al., 1971; Lue et al., 2001). In both XX and XXSxr testes, XY or XSexr O germ cells occasionally survive to take part in spermatogenesis because of the loss of the second X chromosome in a progenitor cell (Lyon et al., 1981; Mroz et al., 1999; Hall et al., 2006). Such defects arising from a double X dosage are also sufficient to explain the germ cell-autonomous demise of XX spermatogonia in XX<->XY chimeric testes (Palmer and Bugoyne, 1991). Because Y-linked genes in spermatogenic cells are essential for spermatogenesis (Levy and Bugoyne, 1986; Mazeyrat et al., 2001; Toure et al., 2004), the germ cell demise in XX male is due to germ cell-autonomous defects caused by both the extra X- and the missing Y-chromosome. Therefore, it still remains unclear whether or not XX sex-reversed males such as XX/Sry mice have a fully functional testicular somatic environment capable of supporting complete spermatogenesis in mammals.

In this report, in order to elucidate the potency of the XX/Sry testicular somatic environment, we examined the differentiation ability of donor XY spermatogonia in recipient XX/Sry testes, compared to germ cell-deficient W/W* testes, which were used as the XY control.

MATERIALS AND METHODS

Animals

For XY-donor spermatogenic cells, we used wild-type C57BL/6 [B6] mice, ROSA26 mice (B6×129 genetic background, Jackson Laboratories), Green mice [B6-Tg(CAG-EGFP), SLC, Japan], and Steel/Steelidakis (Sl/St*) mice (WB×B6; SLC). For recipient testes, the sex-reversed transgenic mouse line (B6/Hsp-Sry lines carrying the autosomally-located Sry transgene driven by a basal weak Hsp70.3 promoter) (Kidokoro et al., 2005) and the germ cell-deficient W/W*-mutant line (WB×B6; SLC) were used in this study. The Hsp-Sry line displays XX testes at embryonic stages because of transgenic Sry expression in embryonic gonads at the sex-determining periods. Because XY Hsp-Sry males display normal spermatogenesis and fertility even after 1 year of age, the integration position and transgene misexpression elicit no appreciable defect in spermatogenesis in these mice.

Transplantation of XY cells prepared from the immature testes

For spermatogonial transplantation, cell suspensions (including spermatogonial cells) were prepared from 10-day old testes of ROSA, Green, wild-type B6 or Sl/St* males. They were then transplanted into the testes of 8-week-old recipient XX/Sry and XY W/W* mice as described previously (Brinster and Zimmermann, 1994; Ogawa et al., 2000). At 2.5-3 months after transplantation, the recipient testes were dissected and processed for histological and histochemical analyses. Some recipient XX/Sry testes injected with enhanced green fluorescent protein (EGFP)-positive spermatogonial-cell suspensions (from Green mice) were also

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dissected 2.5 months after transplantation. Spermatogenic tubules with EGFP-positive spermatogenic colonies were collected from these mice under the epifluorescence stereomicroscope and were further used as donors for the second transplantation experiment of seminiferous tubules, as follows.

The seminiferous tubule transplantation was carried out as described previously (Tanemura et al., 1996). Spermatogenic tubules of the immature B6 testes (2-3 weeks of age) or fluorescent-positive seminiferous tubules of the primary-recipient XX/Sry testes were cut into small segments (1 cm in length), washed in DMEM medium to remove interstitial tissue and then transplanted under the testicular capsules of the recipient males. The recipient testes were examined histologically at 4 weeks after transplantation.

**Histology and immunohistochemistry**

The transplanted testes were fixed in Bouin’s solution or 4% paraformaldehyde solution and were then routinely embedded in paraffin. Paraffin sections (4 μm) were subjected to conventional histological and immunohistochemical staining. For quantitative analysis of the incidence ratio of each advanced spermatid stage, we used periodic acid-Schiff (PAS)-stained transverse sections (three sections per testis) of the testes which had a higher contribution of donor XY germ cells (XX/Sry: six testes; XY W/Wv: four testes). All seminiferous tubules in the three sections were classified by direct microscopic observation into tubules lacking donor germ cells, tubules with spermatocytes, or tubules with round (steps 1~7) or early (step 8~10)/late (steps 11~16) elongated spermatids. The incidence ratio of each spermatid stage represented the mean percentage of the relative tubule number ±/− standard error (s.e.m.; number of tubules with spermatocytes was set at 100%).

For immunohistochemical staining, two consecutive sections were separately incubated with anti-MVH (2 ng/ml (Ttoyoka et al., 2001)) antibody, anti-EGFP (1/3000 dilution; Molecular probes, OR) antibody, or anti-HSC70T (1/1000 dilution (Tsunekawa et al., 1999)) antibody at 4°C for 12 hours. After washing in TBS, the reaction was visualized with biotin-labeled secondary antibody provided by Dr Hirokazu Fujiimoto and anti-EGFP (1/3000 dilution; Molecular probes, OR) antibody at 4°C for 12 hours. After washing in TBS, the reaction was visualized with biotin-labeled secondary antibody provided by Dr Toshiaki Noce, antigen (2 ng/ml (Toyoko et al., 2001)) antibody in combination with Elite ABC kit (Vector Laboratories, CA).

For transmission electron microscopy, the transplanted testes were fixed in 4% glutaraldehyde at 4°C for 12 hours. After post-fixation with 1% OsO4, the seminiferous tubules were dehydrated and embedded in Araldite M. Ultrathin sections were observed under a JEOL 1010 transmission electron microscope at 80 kV (JEOL, Japan).

For LacZ staining, the transplanted testes were fixed with 1% PFA-0.2% glutaraldehyde-0.02% NP40-PBS at 4°C for 4 hours and were then subjected to whole-mount X-gal staining (Kanai-Azuuma et al., 2002). Paraffin sections of the stained testes were prepared for histological analysis.

**RNA extraction, microarray and RT-PCR analyses**

Whole testes and seminiferous tubules of 8-week-old XX/Sry and W/Wv males were used for microarray expression analysis using the Affymetrix GeneChip system (Affymetrix, CA). After total RNA was extracted using a RNeasy Mini kit (Qiagen, MD), double-stranded cDNA and biotin-labeled cRNA were synthesized using One-Cycle cDNA Synthesis and IVT Labeling kits (Affymetrix, respectively). Fragmented biotin-labeled cRNA (20 μg) was hybridized to the Affymetrix Mouse Expression Array MOE 430A for 16 hours at 45°C. The chips were washed, stained, scanned and then analyzed using Microarray Suite version 5.0 (Affymetrix), in accordance with the manufacturer’s standard protocols. Differential expression was defined as a difference of twofold or more in both whole-testis and seminiferous tubule samples between two recipient males. The microarray data have been deposited in the Gene Expression Omnibus of NCBI (accession number: GSE5319).

For reverse transcriptase (RT)-PCR analysis, the RNA of seminiferous tubules was treated with DNase I and was then reverse-transcribed using an oligo(dT) primer with a Superscript III cDNA synthesis kit (Invitrogen, CA). PCR was performed with 27-30 cycle amplifications at 94°C for 40 seconds, 55°C for 1 minute and 72°C for 1 minute by using the appropriate primer sets (see Table 1).

**RESULTS AND DISCUSSION**

Before transplantation, recipient testes displayed no spermatogenic cells beyond the pre-leptotene spermatogonial stage in both XX/Sry and XY W/Wv-mutant mice (Fig. 1A,B). First, we transplanted XY-donor testicular suspensions prepared from LacZ-positive ROSA26 pups into the seminiferous tubules of recipient testes and then examined their colonization patterns in the testes at 3 months after transplantation. LacZ staining revealed that XY spermatogonia were able to colonize the seminiferous tubules of recipient XX/Sry testes (Fig. 1C,D), in which only spermatogenic cells were positive (Fig. 1E,F). Histological analyses at 3 months after transplantation of

<table>
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<th>Gene</th>
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wild-type (B6) testicular cells revealed no difference in the frequency of testes with settled donor germ cells between XX/Sry and XY W/W\* males (number of testes with donor germ cells per total testes injected: 22/44 testes [50.0%] in XX/Sry males vs. 5/10 testes [50.0%] in XY W/W\* males). In both XY W/W\* and XX/Sry testes, spermatocytes and round spermatids were frequently observed inside the tubules (Fig. 1G,H). In order to exclude a possible contribution of donor-derived somatic cells in XX/Sry testes, we also transplanted a cell suspension prepared from XY Sl/Sld testes (normal spermatogonia, but defective Sertoli cells) (Zsebo et al., 1990; Ogawa et al., 2000), and obtained the same results as those observed in the transplantation of wild-type donor cells (Fig. 1I,J). Therefore, it was concluded that the XX sex-reversed male body is capable of supporting the settlement, proliferation and complete meiosis of XY germ cells even in the absence of other Y-linked genes except for Sry. Because several lines of XY-female mutant mice are able to produce litters (Lovell-Badge and Robertson, 1990; Capel et al., 1993), these findings indicate that, however, the incidence of early-elongated spermatids was approximately 75% and 50%, respectively. In XX/Sry testes, the incidence of round spermatids was approximately 65%, which did not significantly differ from that of W/W\* testes. In XX/Sry testes, the incidence of early-elongated spermatids was significantly (P<0.01) reduced (only 5.9%), with no elongated spermatids detected after step 11. These data suggest that the XX/Sry testicular somatic environment is defective in the maintenance of round spermatids and their differentiation into spermatozoa.  

Next, in order to evaluate the potency of the interstitial environment of XX/Sry testes, we prepared seminiferous tubule segments from immature wild-type testes at 2-3 weeks of age. At this stage, the testes consist mainly of spermatogonia and Sertoli cells (Fig. 2A-D). In the XY W/W\* testes, HSC70T-positive cells with a sperm tail were frequently observed in most seminiferous tubules containing MVH-positive cells (Fig. 2B,D). By contrast, XX/Sry testes showed a drastic reduction of HSC70T-positive cells in MVH-positive seminiferous tubules (Fig. 2A,C). Periodic acid-Schiff (PAS) staining also demonstrated that round spermatids were sometimes detached from the seminiferous epithelia and were located in the lumen of XX/Sry testes (Fig. 2E). They frequently exhibited apoptotic-like cell death, with typical crescent-like condensation of the chromatin (Fig. 2F,I). Some large, multi-nucleated giant cells of spermatids could also be observed in XX/Sry testes (Fig. 2G,J). By contrast, no anomalies were detectable in the XY W/W\* testes, in which round and elongated spermatids displayed normal morphology similar to that seen in intact XY testes (Fig. 2H,K). The incidence ratio of each advanced spermatid stage was estimated as the number of seminiferous tubules with round or early/late elongated spermatids relative to the total number of tubules with spermatocytes (Fig. 2L). In XY W/W\* testes, the incidence ratio of round and elongated spermatids was approximately 75% and 50%, respectively. In XX/Sry testes, the incidence of round spermatids was approximately 65%, which did not significantly differ from that of W/W\* testes. In XX/Sry testes, however, the incidence of early-elongated spermatids was significantly (P<0.01) reduced (only 5.9%), with no elongated spermatids detected after step 11. These data suggest that the XX/Sry testicular somatic environment is defective in the maintenance of round spermatids and their differentiation into spermatozoa.

Fig. 1. Spermatogonial transplantation into testicular seminiferous tubules of XX/Sry and XY W/W\* (control) males. (A,B) HE staining of the intact XX/Sry and XY W/W\* testes at 8 weeks of age, showing lack of germ cells in most tubules of both testes. (C) The recipient testes were injected with spermatogonial-cell suspension prepared from immature ROSA26 (C-F), wild-type (G,H) and Sl/Sld (I,J) testes, and were then histologically examined at 3 months after transplantation. (C-F) LacZ staining of the transplanted testes, visualizing ROSA26-derived XY germ cells in XX/Sry (C,E) and XY W/W\* (D,F) testes. Asterisks indicate LacZ-negative Sertoli cells located at the basal region of the seminiferous tubules. Insets show higher-magnification images of boxed area. (G-I) HE staining of XX/Sry (G,I) and XY W/W\* (H,J) testes injected with the wild-type-derived (G,H) or Sl/Sld-derived (I,J) testicular cell suspension. Higher-magnification images of recipient tubules with donor germ cells are shown on the right. Scale bars: 50 μm in A,G,I and inset of E; 200 μm in E.
survived inside the recipient XX/Sry testes (Fig. 3A-D). These data indicate that the interstitial environment of XX/Sry testes is capable of supporting normal spermiogenesis, which in turn suggests the defective environment inside the seminiferous tubules of the XX/Sry testis. Moreover, we prepared the seminiferous tubules composed of XX/Sry somatic and EGFP-positive XY spermatogenic colonies from the recipient XX/Sry testes 2.5 months after spermatogonial transplantation (Fig. 3E,F). The EGFP-positive tubule segments were further transplanted under the testis capsules of the secondary recipient XY W/Wv males. At 4 weeks after tubule transplantation, it was shown that no restoration of spermiogenesis in EGFP-positive XY spermatogenic colonies was detected in all XX/Sry tubule segments that survived in the testicular interstitium of the secondary XY W/Wv recipients (n=4; Fig. 3G-K). These findings clearly indicate dysfunction of the somatic tubule component, probably Sertoli cells, in XX/Sry testes.

The extra X- and/or missing Y-chromosome in XX/Sry testes could be the primary cause of their defective spermiogenesis. In XXY mice, spermatagonia are eliminated within the first few days of postnatal life (Mroz et al., 1999; Lue et al., 2001). However, rare breakthrough patches of spermatogenesis, composed of XY germ cells, are observed in the testes of adult mice (Hall et al., 2006). Some men with non-mosaic Klinefelter syndrome are also able to produce functional sperm from 46,XY spermatogonial cells, because live-births involving 47,XXXXY fathers are almost always chromosomally normal, with as many 46,XY as 46,XX children (Lanfranco et al., 2004). These reports, therefore, indicate that the XXY testicular somatic environment is capable of supporting complete spermatogenesis of XY spermatogonia. This, in turn, suggests that the primary cause of the defective XX/Sry somatic environment is likely to be attributable to the absence of other Y-linked genes rather than to the presence of the extra X chromosome. In order to understand the molecular basis of defective XX/Sry testes, we performed microarray analyses of isolated seminiferous tubules from XX/Sry and W/Wv testes. Despite their histological similarity, the present microarray screens identified 48 downregulated and 93 up-regulated genes in both seminiferous tubule fractions and whole testis of the XX/Sry testes compared with those of W/Wv testes (Fig. 4, and see Tables 1, 2 in the supplementary material). Among the downregulated genes, the expression of five Y-linked genes – Ddx3y (previously known as Dby), Utb, Eif2s3y, Jarid1d (previously known as Smcy), and Ube1yl – was missing in the XX/Sry testes. Because these genes are all mapped in the Sry-deletion-interval region (the essential region for germ cell development after the early postnatal period) of the mouse Y chromosome (Burgoine, 1998; Mazeyrat et al., 2001), they may be the Y-linked candidate genes for spermiogenic failure in XX/Sry Sertoli cells.

Interestingly, we also found several autosomal genes whose expression was reduced in the seminiferous tubules of XX/Sry testes (Fig. 4), including some genes involved in ion channel and/or transport molecules (Clca1, Kcnd14, Slc6a4) and cytoskeletal and/or cell-junction components (Dst, Tuba3, Alcam, Jam2). Ion transport regulations in Sertoli cells are important for the secretion
of a potassium- and chloride-rich fluid in spermiogenesis (Hinton and Setchell, 1993; Pace et al., 2000). JAM2, an immunoglobulin-superfamily protein mediating homophilic and heterophilic interactions, is expressed on the Sertoli cell surface facing round and elongated spermatids (Gliki et al., 2004). JAM3, a partner molecule for trans-interactions with JAM2, is essential for the differentiation of round spermatids into spermatozoa (Gliki et al., 2004), suggesting a possible function of JAM2 in spermiogenesis.

By contrast, of the 93 genes up-regulated in XX/Sry tubules, six members of the kallikrein gene family [Klk1b16 (previously known as Klk16), Klk6, Klk1b27 (previously known as Klk27), Klk1b24 (previously known as Klk24), Klk1b22 (previously known as Klk22), Klk9 and Klk1b21 (previously known as Klk22)] were found within the top 20 up-regulated genes in XX/Sry tubules. The kallikrein genes encode the tissue-specific protease required to liberate kinins, small peptide hormones involved in multiple physiological processes. Kinin (bradykinin) receptors are highly expressed in spermatocytes and round spermatids, indicating a possible function of the kallikrein-kinin system in the local regulation of later spermatogenesis [see Monsees et al. (Monsees et al., 2002) and references therein]. Therefore, it is likely that such misregulation of several spermiogenesis-regulatory genes in XX/Sry tubules reflects the inability of these tubules to support the maintenance of round spermatids and their differentiation into spermatozoa. Further spermatogonial transplantation and microarray analyses using XXY, XX, Sxrb, XO/Sry and X SxrbO testes would be required to resolve whether these transcriptional changes in XX/Sry tubules are a consequence of the Y-gene deficiency or of the double X dosage.

In conclusion, we show here that XX/Sry testicular soma is not capable of supporting the differentiation and morphogenesis of haploid germ cells into spermatozoa. This is probably due to the dysfunction of the XX/Sry Sertoli cells, highlighting the idea that Sry alone is insufficient to induce a fully functional Sertoli cell in XX mice.
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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/3/449/DC1

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