The behavior of the X- and Y-chromosomes in the oocyte during meiotic prophase in the B6.Y^{TIR} sex-reversed mouse ovary

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Abstract

Sexual differentiation of the germ cells follows gonadal differentiation, which is determined by the presence or the absence of the Y-chromosome. Consequently, oogenesis and spermatogenesis take place in the germ cells with XX and XY sex chromosomal compositions respectively. It is unclear how sexual dimorphic regulation of meiosis is associated with the sex-chromosomal composition. In the present study, we examined the behavior of the sex chromosomes in the oocytes of the B6.Y^{TIR} sex-reversed female mouse, in comparison with XO and XX females. As the sex chromosomes fail to pair in both XY and XO oocytes during meiotic prophase, we anticipated that the pairing failure may lead to excessive oocyte loss. However, the total number of germ cells, identified by immunolabeling of germ cell nuclear antigen 1 (GCNA1), did not differ between XY and XX ovaries or XO and XX ovaries up to the day of delivery. The progression of meiotic prophase, assessed by immunolabeling of synaptonemal complex components, was also similar between the two genotypes of ovaries. These observations suggest that the failure in sex-chromosome pairing is not sufficient to cause oocyte loss. On the other hand, labeling of phosphorylated histone γ H2AX, known to be associated with asynapsis and transcriptional repression, was seen over the X-chromosome but not over the Y-chromosome in the majority of XY oocytes at the pachytene stage. For comparison, γ H2AX labeling was seen only in the minority of XX oocytes at the same stage. We speculate that the transcriptional activity of sex chromosomes in the XY oocyte may be incompatible with ooplasmic maturation.

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Introduction

In normal mammalian development, the presence or absence of a Y-chromosome dictates the differentiation of a gonadal primordium into a testis or an ovary. Consequently, the primordial germ cells that have migrated into the gonadal primordium undergo sexual differentiation according to the gonadal sex. Thus, XY germ cells initiate spermatogenesis in the testis and XX germ cells differentiate into oocytes in the ovary. This hierarchical relation between gonadal and germ cell differentiation obscures the role of sex chromosomes in gametogenesis.

The ultimate goal of gametogenesis is to provide haploid genome to the zygote. However, the behavior and transcriptional activity of sex chromosomes in the germ line are very different between the two sexes. In the female germ line, the second X-chromosome is reactivated prior to the onset of meiosis and remains active throughout oogenesis (Monk & McLaren 1981). On the other hand, in the male germ line, both the X- and Y-chromosomes are placed in a compartment, named XY or sex body, and transcriptionally repressed during meiotic prophase (Turner *et al.* 2000). The loss of

X-encoded gene products is compensated by either activation of autosomal homologs (e.g., *Pgk2*) or stabilization of gene products (e.g., *Hprt*; Handel & Eppig 1998). It has been suggested that the repression of sex chromosomes is necessary for preventing meiotic recombination between them outside the pseudoautosomal region. In addition, mouse mutants for various genes involved in meiotic progression show sexual dimorphism; while male mutants lose almost all spermatocytes during meiotic prophase, the corresponding female mutants only partially lose their oocytes and often maintain fertility (Morelli & Cohen 2005).

Sex-reversed mice provide the opportunities to explore the role of sex chromosomes in gametogenesis. For examples, the XX male is infertile because the second X-chromosome is incompatible with the survival of spermatogonia. By contrast, XY females have variable fertility dependent on the cause of sex reversal or the genetic background. When the Y-chromosomes of certain local variations of *Mus musculus domesticus* (DOM) are placed onto a C57BL/6J (B6) genetic background, the XY progeny (named B6.Y^{DOM}, B6.Y^{POS}, or B6.Y^{TIR}) develops only

ovaries or ovotestes in fetal life (Eicher *et al.* 1982, 1996, Nagamine *et al.* 1987). Subsequently, about a half of the XY progeny develop bilateral ovaries and female phenotype but fail to produce offspring (Eicher *et al.* 1982, Taketo-Hosotani *et al.* 1989). Our laboratory has been particularly interested in the cause of this female infertility.

We have previously demonstrated that the ovaries of the B6.Y^{TIR} female contain all of the follicular stages except for the preovulatory stage, and consequently discharge very few eggs following gonadotropin treatment (Taketo-Hosotani *et al.* 1989). The few ovulated eggs can be fertilized either *in situ* or *in vitro* but they cease development at the 1- or 2-cell stage (Merchant-Larios *et al.* 1994). A larger number of eggs can be obtained from B6.Y^{TIR} females by collecting fully grown oocytes and maturing them in culture (Amleh *et al.* 1996, Villemure *et al.* 2007). These oocytes, although they mature at a normal rate, again fail to develop after fertilization. To determine whether the developmental incompetence of the oocytes can be attributed to their XY chromosomal composition or to their surrounding XY somatic cells, we constructed female chimeras composed of B6.Y^{TIR} and XX cells (Amleh & Taketo 1998). All female chimeras turned out to be fertile and produced progeny exclusively derived from XX oocytes. We concluded that the XY chromosomal composition in the oocyte is responsible for the failure in postfertilization development. Furthermore, to determine whether the failure in embryonic development can be attributed to the nucleus or cytoplasm of the oocyte, we transferred the nuclei of XY oocytes into enucleated XX oocytes, both at the GV stage. The reconstituted oocytes went through meiotic divisions and developed into healthy adults (Obata *et al.* unpublished data). These results suggest that the cytoplasm of the XY oocyte becomes defective during the growth phase (or earlier) and influences the developmental competence of the oocyte during maturation.

In the present study, we examined the progression of meiotic prophase in the B6.Y^{TIR} ovary in comparison with its XX control. We were particularly interested in the behavior of X- and Y-chromosomes in the oocyte and its association with meiotic progression and oocyte loss. We also examined some aspects in the XO ovary for comparison. It is well known that all germ cells (oogonia) enter meiosis and go through meiotic prophase, in which homologous chromosomes pair and recombine, in fetal and neonatal mouse ovaries. However, it is less understood how the pairing between homologous chromosomes influences meiotic progression and oocyte loss. Our results indicate that the X–Y pairing failure in the oocyte is not sufficient for activation of a pachytene checkpoint mechanism or for inducing oocyte elimination. On the other hand, the transcriptional activity of sex chromosomes in the XY oocyte appeared to be different from that in the XX or XO oocyte and may be responsible for the cytoplasmic defect leading to the impairment in postfertilization development.

Results

Distribution of germ cells in ovarian sections

Comparison of XY ovaries with their XX controls

Distribution of germ cells was compared in the histological sections of ovaries isolated from XY females and their XX littermates by immunohistochemical staining of germ cell nuclear antigen 1 (GCNA1; Figs 1 and 2). At 14.5 and 15.5 days postcoitum (dpc), numerous GCNA1-positive cells were seen in the entire ovary of both genotypes (Fig. 1A–D). The intensity of GCNA1 labeling in the XY ovary was considerably weaker than that in the control XX ovary. At 17.5 dpc, the intensity of GCNA1 labeling was comparable between XX and XY ovaries (Fig. 1E and F). However, while germ cells were distributed in the entire region of the XX ovary, fewer germ cells were seen in the central region of the XY ovary. At 19.5 dpc, total number of germ cells was reduced in both genotypes of ovaries (Fig. 1G and H). The germ cells were particularly scarce in the central region of the XY ovary. At 21.5 dpc, many GCNA1-positive cells were seen in the entire region, although more concentrated in the peripheral region, of the XX ovary (Fig. 1I). On the other hand, fewer cells with weaker GCNA1-labeling were seen in the XY ovary (Fig. 1J). At 24.5 dpc, very few GCNA1-positive cells were seen in both genotypes of ovaries (Fig. 2A and B).

To test whether the observation of fewer GCNA1-positive cells in the ovary at 24.5 dpc was due to lack of germ cells or GCNA1 labeling, we used another marker for germ cells, mouse vasa homolog (MVH), for immunohistochemical staining (Fig. 2C and D). Numerous oocytes were seen in the entire region of the XX ovary. The oocytes in the central region were very large and showed a donut-shaped staining due to the absence in nuclei. By contrast, no MVH-labeling was seen in the XY ovary. To further confirm whether the absence of GCNA1- and MVH-labeling in the XY ovary was due to lack of germ cells, we used hematoxylin and eosin (HE) staining (Fig. 2E and F). Oocytes of various sizes were seen in the entire region of the XX ovary, corresponding to the MVH labeling. By contrast, oocytes were absent in the central region but present in the peripheral region of the XY ovary. However, it was difficult to clearly identify small oocytes in the peripheral area in both genotypes of ovaries at this stage. We then stained the XX and XY ovaries at 28 days after birth with HE (Fig. 2G and H). Many oocytes at various follicular stages were seen in both genotypes of ovaries.

Comparison of XO ovaries with their XX controls

Distribution of germ cells was also compared in the histological sections of ovaries isolated from XO females (carrying the maternal X chromosome) and their XX littermates (carrying the X^{Pa} mutation; Fig. 3). A similar distribution was observed in the two genotypes of ovaries at three developmental stages examined. At 15.5 dpc, numerous GCNA1-positive cells were seen in the entire

Figure 1 Immunohistochemical staining of GCNA1 in ovarian sections from XY and XX females at 14.5–21.5 dpc. The bar indicates 200 μ m at the same magnification in A–F and in G–J. (A–D) Ovaries at 14.5 and 15.5 dpc. Numerous germ cells are distributed throughout the ovary. GCNA1 staining in the XY ovary is weaker than that in the XX ovary. No other difference between XY and XX ovaries is recognized. (E and F) Ovaries at 17.5 dpc. The intensity of GCNA1 staining is comparable between XY and XX ovaries. Germ cells are distributed in the entire region of the XX ovary, whereas considerably fewer germ cells are seen in the central region of the XY ovary. (G and H) Ovaries at 19.5 dpc (the day of delivery). Fewer germ cells are seen compared with earlier stages (E and F) in both XY and XX ovaries. In particular, germ cells are scarce in the central region of the XY ovary. (I and J) Ovaries at 21.5 dpc. Germ cells are seen in the entire region of the XX ovary although a larger number is seen in the peripheral region. By contrast, fewer germ cells are seen, mainly in the peripheral region of the XY ovary, with weaker GCNA1-staining.

region of the ovary. At 20.5 dpc, the number of GCNA1-positive cells considerably reduced in the central region. At 24.5 dpc, the distribution of MVH-positive cells in the ovary was similar to that of GCNA1 at 20.5 dpc. However, MVH staining occupied a larger area due to the large cytoplasm of oocytes in the central region.

Figure 2 Detection of germ cells in ovarian sections from XY and XX females at (A–F) 24.5 dpc and (G and H) 28 days after birth. The bar indicates 200 μ m, at the same magnification in A–F and in G–H. (A and B) Immunohistochemical staining of GCNA1. A few germ cells with faint GCNA1 staining are seen in the peripheral region of the XX ovary. GCNA1 staining is almost absent in the XY ovary. The intense staining in clusters is likely non-specific binding of the secondary antibody to the blood cells. (C and D) Immunohistochemical staining of MVH. Numerous cells with cytoplasmic staining of MVH are seen in the XX ovary. Note the large size of oocytes in the central region. By contrast, no such cytoplasmic staining is seen in the XY ovary. The shadow was given to show the structure of the section. (E and F) HE staining. Numerous oocytes of large sizes are seen in the central region in the XX ovary. Oocytes in the peripheral region are harder to recognize with this staining. In the XY ovary, small oocytes are seen in the peripheral region (arrows) but no large oocytes are seen in the central region. (G and H) HE staining. Numerous oocytes at varying follicular stages are seen in the entire region of both XX and XY ovaries.

Changes in germ cell populations

The total number of GCNA1-positive cells in spread chromatin preparation was compared between XY and XX ovaries or between XO and XX ovaries from 15.5 through 20.5 or 22.5 dpc (Fig. 4). In XY and XX ovaries, the number of GCNA1-positive cells continuously decreased from 16.5 to 19.5 dpc and then leveled at 20.5 dpc. No significant difference was found between

Figure 3 Immunohistochemical staining of germ cells in ovarian sections from XO and XX females. The bar indicates 200 μ m at the same magnification in A–D and E–F. (A and B) Ovaries at 15.5 dpc. Immunohistochemical staining of GCNA1. Numerous germ cells are seen throughout the ovary. No distinct difference is found between XO and XX ovaries. (C and D) Ovaries at 20.5 dpc. Immunohistochemical staining of GCNA1. Fewer germ cells are seen, particularly in the central region, when compared with the earlier developmental stage. No prominent difference is found between XO and XX ovaries. (E and F) Ovaries at 24.5 dpc. Immunohistochemical staining of MVH. Numerous germ cells are seen throughout the ovary. Note large size of oocytes in the central region. Similar distribution of germ cells is seen between XO and XX ovaries.

the two genotypes of ovaries up to 19.5 dpc, whereas significantly fewer GCNA1-positive cells were found in XY ovaries at 20.5 dpc ($P < 0.05$). In XO and XX ovaries, the number of GCNA1-positive cells decreased rapidly from 15.5 to 16.5 dpc and much more slowly afterwards. No significant difference was found between the two genotypes of ovaries at all stages examined.

Progression of meiotic prophase

Progression of meiotic prophase was assessed in spread chromatin preparations from XY and XX ovaries by immunocytochemical labeling of synaptonemal complex components (SC). It was anticipated that SC labeling would be negative in the oogonium and show characteristic patterns of meiotic prophase substages in the oocyte (Supplementary Fig. 1, which can be viewed online at www.reproduction-online.org/supplemental/). Typically, SC labeling was seen along the axial element of sister chromatids at the leptotene stage, thicker along the partially paired cores of homologous chromosomes at the zygotene stage, and evenly intense along the entire paired cores at the pachytene stage. In early diplotene stage, the residual SC labeling was seen in Y- or loop-shaped

Figure 4 Changes in GCNA1-positive cell populations in (A) XY versus XX ovaries and (B) XO versus XX ovaries from 15.5 through 20.5 or 22.5 dpc. Each value indicates the mean $\pm$ S.E.M. The number of females examined at each point is shown in parentheses. *Significant difference between the females of the two genotypes ($P < 0.05$).

separating chromosomes. In addition, we often observed the cells without clear structures or with fragmented short SC labeling, presumably representing degenerating oocytes.

The pattern of meiotic progression was similar between XY and XX ovaries up to 20.5 dpc (Fig. 5A). At 15.5 dpc, 20% of GCNA1-positive cells were in the leptotene stage, while the rest were SC negative. At 16.5 dpc, 60% of GCNA1-positive cells were in meiotic prophase, at either the leptotene or zygotene stage. At 17.5 dpc, over 90% of GCNA1-positive cells were in meiotic prophase and 40% were in the pachytene stage. At 19.5 and 20.5 dpc, the proportion of GCNA1-positive cells at either the zygotene or pachytene stage was $< 50\%$, whereas the proportion of those without clear identity or SC negative increased. At 21.5 dpc and later, the number of GCNA1-positive cells was limited in XY ovaries. Nonetheless, the proportions of GCNA1-positive cells at various cell cycle stages were similar to those in XX ovaries except that a larger proportion was seen at the zygotene stage, while a smaller proportion was seen at the diplotene stage in XY ovaries at 21.5 dpc ($P < 0.05$).

Progression of meiotic prophase was also assessed in XO and XX ovaries (Fig. 5B). Similar results were obtained in both genotypes of ovaries. At 15.5 dpc, 60–70% of GCNA1-positive cells were in the meiotic prophase, at either the leptotene or zygotene stage, while the rest were SC negative. At 16.5 dpc, a small proportion of

Figure 5 Meiotic progression in (A) XY versus XX and (B) XO versus XX females. The mean proportions of GCNA1-positive cells at various cell-cycle stages are shown. The number of ovaries examined is indicated on the top of each column. *Significant difference between the females of the two genotypes ($P < 0.05$). L, leptotene; Z, zygotene; P, pachytene; D, diplotene; ?, unclear identity; CN, SC negative.

GCNA1-positive cells were in the pachytene stage in both genotypes of ovaries. While a smaller proportion was seen at the leptotene stage, a larger proportion was SC negative in XO ovaries ($P < 0.05$). At 18.5 dpc, 50 and 15% of GCNA1-positive cells were seen at the zygotene and pachytene stages respectively in both genotypes of ovaries. At 20.5 dpc, the number of GCNA1-positive cells at the zygotene stage decreased, while those without clear identity increased. At 22.5 dpc, most GCNA1-positive cells were either SC negative or without clear identity. Although the proportion of SC-negative cells was significantly larger in XO ovaries ($P < 0.05$), the proportion of GCNA1-positive cells with unclear identity was not different.

Pairing between homologous chromosomes

Pairing between homologous chromosomes was examined in spread chromatin preparations from XY and XX ovaries at 19.5–20.5 dpc by immunocytochemical labeling of SC and centromeres (Supplementary Fig. 2, which can be viewed online at www.reproduction-online.org/supplemental/). The labeling of centromeres with the CREST autoimmune serum helped distinguish paired and unpaired chromosomes. All oocytes from XX ovaries contained 20 discrete paired cores, which were maximally condensed at the mid-pachytene stage (Supplementary Fig. 2A and B, which can be viewed online at www.reproduction-online.org/supplemental/). On the other hand, many oocytes from XY ovaries

contained 19 paired cores and separate X- and Y-chromosomes (Supplementary Fig. 2C and D). The single X-chromosome was distinguished by its thinner SC staining and a smaller centromere. The Y-chromosome was distinguished by short SC staining and a smaller centromere.

Pairing between homologous chromosomes was also compared between XO and XX ovaries at 18.5 dpc. The homologous pairing was successful with 20 discrete paired cores including the X–X pair in the most oocytes from XX females (Supplementary Fig. 3A and B). In the oocytes from XO females, pairing between 19 homologous autosomes was complete, whereas the single X-chromosome was seen in varying configurations (Supplementary Fig. 3C and D). In most oocytes, the single X-chromosome was recognized by a smaller centromere relative to the paired centromeres of autosomes. In addition, the single X-chromosome was often seen with short or broken SC stretch (Supplementary Fig. 3C). A short and thick SC stretch in a ring structure was also seen (Supplementary Fig. 3D).

γ H2AX labeling of sex chromosomes

The association of unpaired X- and Y-chromosomes with phosphorylated histone H2AX (γ H2AX) was examined by immunocytochemical labeling of SC, centromeres, and γ H2AX in spread chromatin preparations. Although both SC and centromeres were labeled with red fluorescence, the distinct round shape of centromeres helped identify the end of chromatins, particularly the short Y-chromosome. As previously reported (Mahadevaiah *et al.* 2001), γ H2AX labeling was seen over the spermatocyte nucleus at the leptotene and zygotene stages (Fig. 6A). In pachytene spermatocytes, intense γ H2AX labeling was seen only in association with the sex body (Fig. 6B). In most XX oocytes, γ H2AX labeling was observed at the zygotene and early pachytene stages (Fig. 6C) and absent at the mid-pachytene stage (Fig. 6D). However, intense labeling was occasionally observed in one distinct region of some oocytes at the pachytene stage (Fig. 6E). In these oocytes, γ H2AX labeling was often observed in association with asynapsed cores. Such cases were found in 10% of the oocytes at 17.5 dpc ($n = 2$, total 146 examined) and in 11 ± 2 (S.E.M.) % at 18.5 dpc ($n = 3$, total 239 examined). In XY pachytene oocytes, only those in which the Y-chromosome was recognizable were analyzed. The X-chromosome was identified by small centromere and thinner SC staining. Although many pachytene oocytes were present in the XY ovary at 17.5 dpc, most showed intense γ H2AX labeling over the nucleus and were not used for analysis (Fig. 6F). At 18.5 dpc ($n = 4$, total 202 examined), 16 ± 2 % showed γ H2AX labeling over both X- and Y-chromosomes (Fig. 6G and H). In many oocytes, the sex chromosomes were close together but the Y-chromosome was clearly separate and yet labeled for γ H2AX. By contrast, 72 ± 5 % showed

Figure 6 Immunocytochemical labeling of γ H2AX (green), SC, and centromeres (both red) in the germ cells during meiotic prophase. The bar indicates 200 μ m. (A) Spermatocyte at the leptotene stage. About 40 centromeres are scattered in the nucleus, which is homogeneously labeled for γ H2AX. (B) Spermatocyte at the pachytene stage. Discrete paired cores between homologous chromosomes with a centromere at each end are seen, while γ H2AX labeling is restricted to the XY body (arrowhead). (C) XX oocyte at the early pachytene stage. γ H2AX labeling is seen over the nucleus, which contains discrete paired cores with centromeres. (D) XX oocyte at the mid pachytene stage. Discrete paired cores with centromeres are seen, while γ H2AX labeling is absent. (E) XX oocyte at the pachytene stage. Discrete paired cores with centromeres are seen, while intense γ H2AX labeling is restricted to a region. On the right, the red labeling alone is shown in black and white. The γ H2AX-positive region overlaps with a pair of asynapsed SC stretches. (F) XY oocyte at the early pachytene stage. Discrete paired cores between homologous chromosomes with centromeres are seen, while γ H2AX labeling is seen over the nucleus including the short Y-chromosome (arrowhead). (G and H) XY oocyte at the pachytene stage. γ H2AX labeling is seen over both the X-chromosome (double arrowheads) and the Y-chromosomes (arrowhead). On the right, the red labeling alone in H is shown in black and white. The γ H2AX-positive regions overlap with a thinner SC stretch, most likely the single X-chromosome, and the short Y-chromosome. (I and J) XY oocyte at the pachytene stage. γ H2AX labeling is restricted to one region, presumably the X-chromosome, but not the Y-chromosome (arrowhead). On the right of each, the red labeling alone is shown in black and white. γ H2AX-positive regions overlap with thinner SC stretches, most likely single X-chromosomes. (K) XY oocyte at the pachytene stage. γ H2AX labeling is absent over either the X-chromosome or the Y-chromosome (arrowhead). (L) XY oocyte with fragmentation of SC-positive stretches. γ H2AX labeling is seen over the nucleus. (M and N) XO oocyte at the pachytene stage. γ H2AX labeling is restricted to one region (arrowhead). On the right of each, the red labeling alone is shown in black and white. The γ H2AX-positive regions overlap with thin SC stretches, most likely single X-chromosomes. (O) XO oocyte at the pachytene stage. γ H2AX labeling is absent although thinner SC stretch (arrowhead) is seen tangled with autosome pairs. On the right, the red labeling alone is shown in black and white.

γ H2AX labeling only over the X-chromosome and not the Y-chromosome (Fig. 6I and J). The remaining $12 \pm 4\%$ had no γ H2AX labeling (Fig. 6K). The oocytes with abnormal features, such as fragmented paired cores, were either γ H2AX-positive or γ H2AX-negative in either genotype of ovaries (Fig. 6L). Similar results were obtained at 19.5 and 20.5 dpc although the total number of pachytene oocytes available for the analysis decreased with age.

γ H2AX labeling was similarly examined in the spread chromatin preparations from XO and XX ovaries at 18.5 dpc. In the XO oocytes at the pachytene stage, $73 \pm 3\%$ ($n=6$, total 458 examined) showed γ H2AX labeling in one distinct region, whereas the rest showed no labeling except for small patches (Fig. 6M–O). In the XX oocytes from the control females, $12 \pm 2\%$ ($n=6$, total 475 examined) showed γ H2AX labeling in one distinct region. γ H2AX labeling was often but not always associated with asynapsed cores in the XX oocyte (not shown).

Detection of Y-encoded gene transcripts by RT-PCR

The presence or the absence of transcripts of two Y-encoded genes, *Ubely1* and *Rbmy1a1*, in individual gonads was examined by RT-PCR. At 12.5 dpc, all gonads of the B6 strain were sexually differentiated and easily recognized under the dissecting microscope with transmitted light. On the other hand, most gonads of the B6.Y^{TIR} strain were sexually undifferentiated at this stage. At 13.5 dpc and later, sexual differentiation of B6.Y^{TIR} gonads was easily recognized under the dissecting microscope with transmitted light and categorized into either ovotestes or ovaries. In all cases, the presence of the Y-chromosome was confirmed by PCR amplification of the zinc finger protein on the Y (*Zfy*) sequence. At 12.5 dpc, both *Ubely1* and *Rbmy1a1* transcripts were detectable in B6.XY testes and all B6.Y^{TIR} gonads examined but not in B6.XX ovaries (Fig. 7A). At 17.5 dpc, *Ubely1* transcripts were

detectable in all B6.Y^{TIR} gonads examined, either ovaries or ovotestes, as well as B6.XY testes, but not B6.XX ovaries (Fig. 7B). *Rbmy1a1* transcripts were detectable in B6.Y^{TIR} ovotestes and B6.XY testes but not in B6.Y^{TIR} or B6.XX ovaries.

The relative transcript levels against β -actin were compared among B6.Y^{TIR} and B6.XY gonads from 11.5 through 17.5 dpc (Fig. 8). *Ubely1* transcript levels increased from 11.5 to 13.5 dpc regardless of the gonadal phenotype, and then remained high in B6.Y^{TIR} ovaries but declined in both B6.Y^{TIR} ovotestes and B6.XY testes by 17.5 dpc. By contrast, *Rbmy1a1* transcript levels gradually increased in all gonads from 11.5 to 13.5 dpc, and remained high in B6.Y^{TIR} ovotestes and B6.XY testes but declined to an undetectable level in B6.Y^{TIR} ovaries by 17.5 dpc. The difference of *Ubely1* and *Rbmy1a1* transcript levels between B6.Y^{TIR} ovaries and ovotestes (or B6.XY testes) was statistically significant by ANOVA at $P < 0.05$ and $P < 0.001$ respectively.

Contents of GCNA1 and MVH proteins in XY ovaries

Since immunohistochemical staining of both GCNA1 and MVH in the XY ovary was considerably weaker than that in the XX ovary at 14.5–15.5 dpc or 21.5 dpc and later, we compared the contents of both proteins in the entire ovary by immunoblotting (Fig. 9). We detected five

Figure 7 Detection of *Ubely1* and *Rbmy1a1* transcripts by RT-PCR in fetal gonads. S, 100 bp ladders. (A) Fetal gonads at 12.5 dpc. Both *Ubely1* and *Rbmy1a1* transcripts were detectable in all B6.Y^{TIR} gonads examined, which were yet sexually undifferentiated, and B6.XY testes but not B6.XX ovaries. Neither of the transcripts were detectable in the RT-negative control or in the absence of cDNA input. β -actin transcripts were detectable in all samples but the negative controls. (B) Fetal gonads at 17.5 dpc. *Ubely1* transcripts were detectable in all B6.Y^{TIR} gonads examined, either ovaries (Ov) or ovotestes (OT), as well as B6.XY testes, but not B6.XX ovaries. *Rbmy1a1* transcripts were detectable in B6.Y^{TIR} ovotestes and B6.XY testes but not in B6.Y^{TIR} or B6.XX ovaries. Neither of the transcripts were detectable in the absence of cDNA input.

Figure 8 Relative *Ubely1* and *Rbmy1a1* transcript levels in B6.Y^{TIR} and B6.XY gonads during fetal development. B6.Y^{TIR} gonads at 13.5 and 17.5 dpc were categorized into ovotestes (OT) or ovaries (Ov) based on the morphology under the dissecting microscope with transmitted light. The number in parentheses indicates the number of gonads examined. ^{a,b}Statistical differences by ANOVA ($P < 0.05$ and 0.001 for *Ubely1* and *Rbmy1a1* respectively).

bands of 65, 67, 79, 81, and 95 kDa with the antibody against GCNA1 in both XX and XY ovaries at 14.5, 17.5, and 20.5 dpc. The band intensities were comparable at 14.5 and 17.5 dpc, whereas the intensity of 95 kDa band in the XY ovary was consistently lower (70%) than the intensity of the same band in the XX ovary at 20.5 dpc ($n = 2$). Other bands were comparable. We also detected a single band of 88 kDa with the antibody against MVH. The band intensity in the XY ovary was higher than that in the XX ovary at 14.5 and 17.5 dpc although significant difference was found only at 17.5 dpc ($P < 0.05$). The band in the XY ovary was considerably fainter than that in the XX ovary at 20.5 dpc in two out of three experiments, but no significant difference was found ($n = 3$).

Discussion

Pairing between the X- and Y-chromosomes in the oocyte

Our current studies confirmed that the X- and Y-chromosomes are not paired in the oocyte. We have previously reported similar findings (Amleh *et al.* 2000). However, in the present study, we examined a larger number of oocytes over a wider range of developmental stages (17.5–20.5 dpc). In some XY oocytes, the sex

Figure 9 Immunoblotting of GCNA1 (top) and MVH (bottom) in XX and XY ovaries at 14.5, 17.5, and 20.5 dpc. A pair of ovaries were lysed and applied to each lane. Approximate molecular weight of each band is given on the left.

chromosomes were close together; however, no pairing was seen between them. It has been well established that pairing between homologous chromosomes is essential for successful meiosis in many species. In particular, the X- and Y-chromosomes pair in the male germ line and their failure to do so leads to a block of spermatogenesis in mice (Miklos 1974, Burgoyne *et al.* 1992, Everett *et al.* 1996, Eaker *et al.* 2001). Similar checkpoint mechanism has been predicted in the female germ line (Speed 1982, Burgoyne & Baker 1985, Setterfield *et al.* 1988). One objective of our studies was to clarify the association of X–Y pairing failure with the progression of meiotic prophase and the elimination of oocytes.

Oocyte loss during meiotic prophase in XY ovaries

Consistent with our previous studies, we observed that oocytes began to disappear in the central region of the XY ovary at 17.5 dpc (Taketo-Hosotani *et al.* 1989, Villalpando *et al.* 1993). At this developmental stage, a considerable proportion of oocytes were seen at the pachytene stage, most likely in the central region. Therefore, it was conceivable that a pachytene checkpoint operated in response to the defects in XY oocytes and eliminated them in the XY ovary. However, to our surprise, the total number of GCNA1-positive cells in the XY ovary did not differ from that in the control XX ovary up to 19.5 dpc. One simple explanation is that while the lack of oocytes in the central region of the XY ovary is visually impressive, it does not affect the total number of germ cells. Alternatively, the excessive loss of oocytes in the XY ovary may have been compensated by recruitment of more oogonia into meiosis. Because oogonia enter meiosis in a central to peripheral wave, newly recruited oocytes might be concentrated in the peripheral region, leaving the central region empty. It is also conceivable that an excessive loss of oocytes at advanced stages may suppress the loss of oocytes which follow them in meiotic progression.

We observed significantly fewer oocytes in the XY ovary than the XX ovary at 20.5 dpc. The temporal accumulation of zygotene oocytes and deficit in diplotene oocytes at

21.5 dpc suggest that oocytes at the pachytene stage were continuously eliminated in the XY ovary up to this developmental stage. Since we did not observe similar oocyte loss in the XO female, we speculate that the presence or expression of the Y-chromosome, but not the absence of the second X-chromosome, is responsible for the oocyte loss in the XY ovary. The *Sry* gene, the primary testis-determining gene on the Y-chromosome, is both transcribed and translated in the B6.Y^{TIR} gonad (Lee & Taketo 2001, Taketo *et al.* 2005). Therefore, one may speculate that SRY expression caused the oocyte loss in the XY ovary. However, SRY is expressed only in somatic cells and downregulated in the B6.Y^{TIR} ovary by 14.5 dpc. Furthermore, the genes involved in testicular differentiation downstream of *Sry*, such as *Sox9* and *Mis*, are expressed only in the testicular region of the XY ovotestis and not in the ovarian region (Moreno-Mendoza *et al.* 2004, Taketo *et al.* 2005). Therefore, it is unlikely that *Sry* expression is responsible for the oocyte loss in the XY ovary.

An excessive loss of oocytes in the XO neonatal mouse ovary has previously been reported (Burgoyne & Baker 1985). It must be noted that the XO female used in these studies carried the paternal X-chromosome, whereas the XO female mouse in our study carried the maternal X-chromosome. Although GCNA1-labeling may have underestimated the number of germ cells in neonatal ovaries, MVH-labeling also did not show any difference in the density or distribution of germ cells between XO and XX ovaries at 24.5 dpc in our studies. We believe that the above discrepancy can be attributed to the parental origin of the single X-chromosome in the XO female. We must acknowledge that the comparison between XO females with their XX littermates is not as simple as it appears. For example, the XO female carrying the paternal X-chromosome is growth retarded when compared with its XX littermate (Burgoyne *et al.* 1983, Thornhill & Burgoyne 1993). On the other hand, the XO female carrying the maternal X-chromosome grows normally. However, its XX sisters inherit the mutant X^{Paf}-chromosome from their father, which may influence ovarian development (Lane & Davisson 1990, Burgoyne & Evans 2000).

Transcriptional activity of sex chromosomes in XY ovaries

It has been established that the X- and Y-chromosomes occupy the sex body, separate from autosomes, and transcriptionally repressed in the spermatocyte. By contrast, the second X-chromosome is reactivated prior to the onset of meiosis and remains active in the oocyte. To determine the behavior of the unpaired X- and Y-chromosomes in the oocyte, we used γ H2AX labeling, which has been shown to be associated with asynapsis and transcriptional repression of chromosomes in both spermatocytes and oocytes (Turner *et al.* 2005). Our results suggest that while the single X-chromosome was

labeled for γ H2AX in the majority of XY oocytes, the Y-chromosome was not. Since we could not definitely identify the X-chromosome, we examined the frequency of normal XX oocytes in which a single chromosome core was labeled for γ H2AX. Such oocytes were limited to 10%. Therefore, we speculate that the X-chromosome is transcriptionally repressed, while the Y-chromosome is active in the majority of XY oocytes.

We confirmed that a Y-encoded gene, *Ubely1*, was transcribed in the XY ovary. However, another Y-encoded gene, *Rbmy1a1*, was downregulated in the XY ovary. Thus, the Y-chromosome appeared to be subjected to gene-specific transcriptional regulation during meiotic prophase in the oocyte. As we examined the entire gonad for technical limitations, we need to confirm that both genes are transcribed in germ cells. It has been reported that the expression of *Rbmy1a1* is germ cell dependent in the fetal testis (Mahadavaiah *et al.* 1998). The expression of *Ubely1* is also germ cell dependent in the adult testis (Odorisio *et al.* 1996), but its specificity in the fetal testis remains to be examined.

The single X-chromosome in the XO oocyte was also frequently labeled for γ H2AX. However, the proportion of XO oocytes without γ H2AX labeling was 28% in our studies, consistent with the previous report (Turner *et al.* 2005), and significantly higher than 12% of XY oocytes ($P < 0.01$). It has been proposed that oocytes in the XO ovary escape the checkpoint mechanism by the ability of the univalent X-chromosome to form a non-homologous association with itself or with an autosome (Speed 1986). This hypothesis was further supported by the finding that while the asynapsed X-chromosome is transcriptionally repressed, the self-synapsed X-chromosome is transcriptionally active in the XO oocyte (Turner *et al.* 2005). We do not yet know whether the lower proportion of oocytes with γ H2AX labeling can explain the fertility or longer retention of oocytes in the XO female mouse when compared with the XY female mouse.

Expression of GCNA1 and MVH proteins in XY ovaries

We found that both GCNA1- and MVH labeling in the XY ovary was considerably weaker than the control XX ovary at 14.5–15.5 dpc as well as after birth. The absence of both GCNA1- and MVH labeling was not due to the absence of germ cells in the XY ovary. Since GCNA1 localizes in the nucleus whereas MVH in the cytoplasm, we considered the possibility that the germ cells in the XY ovary were defective in overall protein synthesis or metabolism. Immunoblotting did not show reduced protein contents of either GCNA1 or MVH in the XY ovary at 14.5 dpc. The weaker GCNA1 staining may be caused by a delay in the nuclear translocation of GCNA1 (Alton & Taketo 2007). By contrast, we found reduced protein contents of both GCNA1 and MVH in the XY ovary at 20.5 dpc. This reduction cannot be solely attributed to the smaller number of

germ cells because not all bands were reduced in parallel. We do not anticipate a specific function of MVH since a *Mvh* null-mutant female mouse is healthy and fertile (Tanaka *et al.* 2000). The GCNA1 gene has not been cloned and its physiological function remains unknown. Since both GCNA1 and MVH staining were normal in the XO ovary, the defect in the germ cells of XY female cannot be attributed to the single X-chromosome. We speculate that the expression of Y-encoded genes may be responsible for the modified protein levels in the XY oocyte.

In conclusion, we would like to predict that the transcriptional activity of sex chromosomes during meiotic prophase may have a long effect on oocyte growth and maturation, and lead to the incompetence for embryonic development. However, we do not yet know whether the X-chromosome with γ H2AX labeling is actually repressed, and if so, how long the repression remains in the XY oocyte after the pachytene stage. More work is needed to understand the dynamics of oogenesis in the XY female mouse.

Materials and Methods

Animals

All animal experiments were conducted in accordance with the Guide to the Care and Use of Experimental Animal issued by the Canadian Council on Animal Care and with the approval from the Animal Research Committee of McGill University. The B6.Y^{TIR} mouse was generated by placing the Y-chromosome of a variant of *Mus musculus domesticus* (caught in Tirano, Italy) onto the C57BL/6J (B6) genetic background and further backcrossing to B6 females (Nagamine *et al.* 1987). B6.Y^{TIR} male mice (N40–44 backcross generations) were crossed with B6 females (Jackson Laboratory, Bar Harbor, ME, USA) to produce XY females and their XX littermates. A *Paf* carrier mutant male on the C3Heb/Fej (C3H) background was purchased from the Jackson Laboratory and backcrossed to B6 females in our mouse colony. *Paf* carrier males (six to eight backcross generations) were crossed with B6 females to produce XO (with maternal X-chromosome) and XX^{*Paf*} sisters. B6 males were also crossed with B6 females to produce B6 progeny in some experiments. The gestational age was defined as dpc, assuming that the copulation occurred at midnight of the day when the copulation plug was found.

Isolation of ovaries

Pregnant females were killed between 12.5 and 18.5 dpc and their fetuses were removed. After delivery, usually on 19.5 dpc, newborn mice were taken for experiments. Ovaries, with or without the adjacent mesonephroi, were collected and kept in Eagle's minimum essential medium with Hank's salts (GIBCO BRL life Technologies), supplemented with 0.25 mM HEPES buffer (MEM-H), while a piece of liver was taken from each mouse and stored at -20°C for genotyping.

Genotyping

For genotyping of the fetuses/offspring from the cross between B6 females and B6.Y^{TIR} males or B6 females and B6 males, liver sample was digested overnight in a lysis buffer and the lysate was used for PCR amplification of the Y-encoded *Zfy* gene as described previously (Amleh *et al.* 2000). For genotyping of the fetuses/offspring from the cross between B6 females and *Paf* carrier males, liver sample was used to extract total RNA using TRIzol (Invitrogen), and the precipitate was dissolved in DEPC-treated water. The RNA sample was then subjected to cDNA synthesis and subsequent PCR amplification of the *Xist* transcript, using the conditions and primers described by Kay *et al.* (1994). Examples of genotyping are shown in Supplementary Fig. 4, which can be viewed online at www.reproduction-online.org/supplemental/. *Xist* is transcribed in XX females but not in XO females or XY males.

Immunohistochemistry

Immediately after isolation, ovaries with attached mesonephroi at 14.5–16.5 dpc or ovaries alone at later developmental stages were fixed in a fresh mixture of absolute ethanol and acetic acid at 3:1 ratio for 1 h at room temperature, and then rinsed and stored in 70% (v/v) ethanol at 4 °C. The fixed ovaries were embedded in paraffin and sectioned at 5 µm using a standard protocol. The slides were kept in glass jars with silica gel at 4 °C. Selected sections from the middle part of each ovary were deparaffinized and labeled with either a rat-MAB against GCNA1 (Enders & May 1994) at 1:50 dilutions or a rabbit polyclonal antibody against MVH (Tanaka *et al.* 2000) at 1:1000 dilutions at room temperature overnight. Next day, the slides were washed and incubated with either a goat anti-rat IgM or goat anti-rabbit IgG antibody, both conjugated with biotin (Pierce Endogen, Rockford, IL, USA), at 1:1000 dilutions for 30 min. After washings, the slides were incubated with the avidin–biotin horseradish peroxidase complex from ABC Vectastain Kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). The slides were washed and incubated with 0.2 mg/ml 3′3-diaminobenzidine and 0.005% (v/v) H₂O₂ (provided in a kit from Sigma–Aldrich) for 6 min. The slides were rinsed with ddH₂O and dehydrated for mounting in Histoclad (Clay Adams, Franklin Lakes, NJ, USA). Some sections were stained with hematoxylin and eosin using a standard protocol. The staining was examined under a transmission microscope (Zeiss Axiophot, Carl Zeiss Canada, Don Mills, ON, Canada). All images were captured with a digital camera (Retiga 1300, QImaging, Burnaby, BC, Canada) and processed with Northern Eclipse digital imaging software, version 6.0 (Empix Imaging, Mississauga, ON, Canada).

Spread chromatin preparations and immunocytochemical labeling

Immediately after isolation, ovaries without mesonephroi were subjected to spread chromatin preparations as previously described (McClellan *et al.* 2003, Alton & Taketo 2007). The slides were washed in holding buffer and incubated with both the anti-GCNA1 antibody at 1:50 dilutions and a rabbit

polyclonal antibody against a mixture of SC components (Dobson *et al.* 1994) at 1:1000 dilutions at room temperature overnight. In some preparations, a human CREST autoimmune serum at 1:1000 dilutions was added in place of the anti-GCNA1 antibody. Next day, the slides were washed and incubated with a goat anti-rat antibody conjugated with FITC (Pierce Endogen) and the goat anti-rabbit antibody conjugated with biotin. For detection of CREST, a goat anti-human antibody-conjugated DTAF (Pierce Endogen) was used. After washings, the slides were incubated with avidin conjugated with Cy3 (Pierce Endogen) at 1:1000 dilutions. Finally, the slides were washed in PBS, ddH₂O, and mounted in Prolong Antifade Mounting Medium (Molecular Probes, Eugene, OR, USA) containing 0.4 µg/ml 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI; Behringer, Mannheim, Germany).

The slides were first viewed under u.v. light to identify the area occupied by DAPI-positive cells. Then, GCNA1-positive cells in the entire area were counted. The area was then divided into four quadrants. In one quadrant, over 100 GCNA1-positive cells, if available, were identified and their meiotic prophase substages were assessed by the pattern of SC labeling as previously described (McClellan *et al.* 2003). Fluorescence signals were captured by digital camera as described above.

For the detection of γH2AX, spread chromatin preparations were incubated with a rabbit polyclonal antibody against γH2AX (AbCam, Cambridge, MA, USA) at 1:2000 dilutions, a mouse polyclonal antibody against a mixture of SC proteins (a gift from Dr. P Moens) at 1:1000 dilutions, and the CREST autoimmune serum at 1:1000 dilutions, overnight. After washings, the slides were incubated with a goat anti-rabbit antibody conjugated with FITC (Pierce Endogen), the goat anti-mouse antibody conjugated with biotin, and a goat anti-human antibody conjugated with rhodamine (Pierce Endogen), all at 1:1000 dilutions. Finally, the slides were incubated with the avidin conjugated with Cy3 at 1:1000 dilutions and processed for mounting with DAPI as described above. Each slide was scanned to identify about 100 SC-positive cells. In the slides from XX and XO ovaries, the proportion of pachytene oocytes with γH2AX labeling over one restricted region and that of pachytene oocytes without γH2AX labeling were estimated. In the slides from XY ovaries, pachytene oocytes in which the Y-chromosome was recognizable were selected and divided into three groups: (1) γH2AX labeling was seen over the Y-chromosome as well as one restricted region (presumably the X-chromosome), (2) γH2AX labeling was seen over one restricted region but not the Y-chromosome, and (3) γH2AX labeling was absent in the entire area.

Semi-quantitative RT-PCR

Gonads were isolated from fetuses at 11.5–17.5 dpc from the cross between B6 females and B6.Y^{TIR} males or between B6 females and B6 males, and separated from the adjacent mesonephroi. A piece of liver was collected from each fetus and used for genotyping as previously described. Total RNA was isolated from each gonad using TRIzol and dissolved in 16 µl DEPC-treated H₂O. After denaturation at 90 °C for 5 min, 2 µl aliquot of RNA solution were subjected to cDNA synthesis (RT) in a volume of 10 µl as described previously (Lee & Taketo

2001). To provide negative controls, aliquots were processed for RT without the reverse transcriptase (RT⁻). Each cDNA sample was processed for PCR amplification in a total volume of 50 μ l as described previously with 2.0 mM MgCl₂ using a Perkin-Elmer Cetus thermocycler (Model 9600). Each cycle included denaturation at 94 °C for 15 or 20 s, annealing at 52 °C for 15 s, and extension at 72 °C for 30 s, except that the first denaturation was extended to 5 min and the last extension was extended to 7 min. The primers for the amplification of *Ubely1*, *Rbmy1a1*, and β -actin have been published in Tokunaga *et al.* (1986), Hendriksen *et al.* (1995) and Elliott *et al.* (1996) respectively. After PCR amplification, 10–20 μ l of each reaction mixture was applied to 2% agarose gel electrophoresis in TAE buffer and visualized with ethidium bromide fluorescence. The intensity of each band was quantified using an Alphamager 2000 system (Alpha Innotech Co., San Leandro, CA, USA). The RT-PCR amplified products were verified by restriction enzyme digestion and DNA-sequencing (data not shown).

Immunoblotting of GCNA1 and MVH in fetal gonads

All chemicals and equipment for electrophoresis and transfer were purchased from Bio-Rad Laboratories unless specified. Each pair of fetal ovaries was snap-frozen in a microfuge tube immediately after collection and stored at –80 °C. Each sample was lysed in 20 μ l sample buffer containing a reducing agent by boiling for 10 min, vortex-mixed, and centrifuged. The supernatant, as well as a broad-range biotinylated molecular weight standard mix, was loaded on 4–12%(w/v) gradient Bis-Tris SDS-PAGE using a Criterion Pre-Cast XT system. Proteins from the SDS-PAGE were transferred electrophoretically to 0.45 μ m nitrocellulose membranes in 10 mM 3- (cyclohexylamino)-1-propanesulfonic acid (CAPS) (USB, Cleveland, OH, USA) buffer (pH 10.0), containing 10% (v/v) methanol using a Trans-Blot Cell apparatus. The membranes were blocked with Start Block blocking buffer (Pierce Endogen) containing 0.05% (v/v) Tween-20, and incubated with either the MAB against GCNA-1 at 1:1000 dilutions or the rabbit polyclonal antibody against MVH at 1:10000 dilutions, both in PBS containing 0.1% (v/v) Tween-20. After washings, the membranes were incubated with either the goat anti-rat IgM or the goat anti-rabbit IgG, both conjugated with biotin, at 1:10000 dilutions. Again after washings, the membranes were incubated with the avidin conjugated with HRP (Cell Signaling, Danvers, MA, USA) at 1:1000 dilutions. The enzymatic activity was detected with a Lumi-Light ECL kit (Roche Diagnostics, Laval, QC, Canada).

Statistical analysis

For estimating the total number of germ cells, at least three females from two litters were examined and the mean and S.E.M. was calculated in each group. The percentages of meiotic substages or those of various γ H2AX-labeling patterns were similarly estimated. Student's *t*-test was used to compare the results between XY and XX sisters or XO and XX sisters. The relative transcript levels were compared by ANOVA.

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