In situ analysis of fetal, prepuberal and adult XX<-XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY

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Summary

The testes of fetal, prepuberal and adult XX<-XY chimaeras were examined using in situ hybridisation to identify the β-globin transgenic marker contained in one component of each chimaera. This enabled the proportion of XX and XY cells contributing to the major cell lineages of the testis to be estimated from sectioned and air-dried material. A few XX Sertoli cells were found in all three age groups, but the XX contribution was always much lower than in other somatic cell types. Significantly, in fetal XX<-XY testes, Sertoli cells were the only cell type to show a bias in favour of the XY component. This strengthens the view that Tdy acts solely in the lineage that gives rise to Sertoli cells. However, the finding of some fetal XX Sertoli cells means that one of the steps in the Tdy-initiated process of Sertoli cell determination is capable of locally recruiting XX cells.

Key words: testis determination, XX<-XY chimaeras, XX Sertoli cells, mouse testes.

Introduction

In order to divert the indifferent fetal gonad away from ovarian development into the testicular pathway, the Y chromosomal testis determinant Tdy must alter the fate of at least three gonad-specific cell lineages – the germ cell lineage, the steroid cell lineage and the supporting cell lineage (see Burgoyne, 1988, for a review). It is assumed that these three lineages are bipotential prior to Tdy action.

Burgoyne et al. (1988a) examined the contribution of XX and XY cells to these lineages in prepuberal and adult XX<-XY male mouse chimaeras. The germ cells, not surprisingly, were exclusively XY because two X chromosomes are incompatible with spermatogonial development after birth (see West, 1982). However, the Leydig cells (steroid cell lineage) had XX and XY contributions in similar proportions to non-gonadal tissues. Importantly, the Sertoli cells (supporting cell lineage) had XX and XY contributions in similar proportions to non-gonadal tissues. Specifically, the analysis of Sertoli cells from prepuberal XX<-XY chimaeras using isozymes of GPI-1 as lineage markers revealed an intense XY band and a barely detectable XX band, while an examination of adult testicular material from XX<-XY chimaeras by in situ hybridisation detected no XX Sertoli cells. It was concluded that the minor XX band from prepuberal testes was probably due to contamination of the Sertoli cell fraction with peritubular myoid cells. These results led Burgoyne (1988) to propose that Tdy acts to bring about testis determination by triggering, cell-autonomously, the differentiation of the supporting cell lineage into Sertoli cells, and that the commitment of the other components of the testis to the male pathway is directed by the Sertoli cells without further Tdy involvement.

If Sertoli cells are exclusively XY in adult XX<-XY testes, this need not necessarily reflect the situation in fetal XX<-XY gonads when Sertoli cells first form. The objective of the present study was to extend the in situ analysis of XX<-XY testes to cover a range of stages from fetus to adult.

Materials and methods

Mice

A transgenic mouse stock, (line 83) produced by Lo (1986), was used to mark cells of one component of each chimaera. This line is homozygous for a single insertion of 1000 copies of the β-major globin gene close to the telomere of chromosome 3. The stock is derived from a mixed CBA, C57BL/6, SJL background and is maintained as a small closed colony but is not formally inbred (These mice will be referred to as GT). Chimaeras were produced from two different sets of crosses:

1) CBA-T6,XXxGT,XY<->BALB/c,XXxMF1,XY\textsuperscript{del}
2) GT,XXxMF1,XYY\textsuperscript{del}=(CBAxC57BL/6)F\textsubscript{1},XXxCBA-T6,XY\textsuperscript{meta}

The chromosome markers include: the T6 translocation (Ford et al. 1956), the metacentric Y chromosome variant (Y\textsuperscript{meta}, Winking, 1978; Burgoyne et al. 1988b) and the Y\textsuperscript{del} chromosome, which is an RIII Y chromosome with a large long arm deletion, maintained on an MF1 (random bred albino) background. These markers allow the positive
identification of all possible combinations in cytogenetic preparations. The Y chromosome is the Tdy-negative mutant Y of Lovell-Badge and Robertson (1990). This cross was set up for a different project but four of the resulting chimaeras that lacked the mutant Y chromosome are included here.

Pairs of embryos were aggregated at the 8- to 16-cell stage, then transferred on the following day to pseudopregnant F_{1} (CBA×C57BL/6) mothers. The age of the fetal chimaeras is taken from the plug date of the female recipients.

**Cytogenetic analysis**

Air-dried metaphase spreads were prepared from bone marrow of adult and prepuberal chimaeras by standard methods. Fetal chimaeras were karyotyped from samples of liver disaggregated with a glass Pasteur pipette and then processed in the same way as the bone marrow.

**In situ hybridisation**

All tissues for *in situ* histology were fixed in 3:1 ethanol/glacial acetic acid, rinsed in ethanol, cleared in cedarwood oil and blocked out in 58°C m.p. paraffin wax. Sections were cut at 5μm and mounted on glycerine/albumin coated slides. Air-dried adult testis preparations were made by a modification of Meredith's method (Levy and Burgoyne, 1986).

The transgenic insert of the GT mice was identified using the probe pM/3G2 (Tilghman et al. 1977), which contains a 7 kb insert spanning the β-major globin sequence. The entire plasmid was biotin labeled using the BRL (Bethesda Research Laboratories) nick translation kit and BRL biotin-11-dUTP. *In situ* hybridisation was carried out according to the method of Rossant et al. (1986) and bound biotin was detected using horseradish peroxidase conjugated with streptavidin, followed by incubation in a solution of diaminobenzidine. Positive hybridisation is recognised by a discrete area of brown precipitate in the nucleus.

The proportion of labelled cells in the different lineages of the fetal testis was estimated from the sections either by scoring all the cells in a series of randomly selected tubules, or in the interstitial regions by using a gridded eyepiece graticule. To rectify the bias due to false negative cells, correction factors were produced by applying the same procedure to testis sections from a fetus hemizygous for the transgenic insert (i.e. all cells carry the transgene).

### Table 1. Estimated proportions of XX cells in cell lineages from fetal XX<->XY chimaeras

<table>
<thead>
<tr>
<th>Chimaera*</th>
<th>Non-testicular cells</th>
<th>Testicular cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age dpc</td>
<td>Liver</td>
</tr>
<tr>
<td>1 XX&lt;-&gt;XY</td>
<td>16.5</td>
<td>23(13)</td>
</tr>
<tr>
<td>2 XY&lt;XY</td>
<td>13.5</td>
<td>32(50)</td>
</tr>
<tr>
<td>3 XX&lt;XY*</td>
<td>15.5</td>
<td>61(51)</td>
</tr>
<tr>
<td>4 XX&lt;XY*</td>
<td>15</td>
<td>74(50)</td>
</tr>
<tr>
<td>MEAN</td>
<td>47.5</td>
<td>48.0</td>
</tr>
</tbody>
</table>

* The chimaeras were all from cross 1. The component carrying the transgenic marker is indicated by an asterisk.
† For the liver, the counts are based on cytogenetic markers in air-dried mitotic cells. For the other cell types, the estimates are based on the *in situ* analysis of the transgenic marker, corrected for the incidence of false negatives. The percentage of false negatives by cell type, as estimated in control sections were: mesonephric tubules 29% (344), myoid cells 30% (244), Leydig cells 21% (146), T1-prospermagonia 43% (249) and Sertoli cells 27% (313).
‡ At 13.5 dpc, many of the cells in the testis cords were in prophase or metaphase of mitosis. Because of the possibility of confusion between dividing Sertoli cells and germ cells, the Sertoli cell counts are based on the unambiguously identifiable, peripherally located interphase cells, and the T1-prospermagonia counts on centrally located cells whether or not they were dividing.

**Results**

The results of the *in situ* analysis of 12 chimaeric males are presented here: 10 with an XX<XY genotype and 2 XY<XY controls. These 12 fall into three age categories: fetal (13.5–16.5 days post coitum, dpc), prepuberal (13–15 days pp), and adult (46–52 days pp). A close examination of the testis sections after *in situ* hybridisation revealed that XX Sertoli cells do in fact exist at all three ages (see Figs 1 and 2). Sertoli cells were identified on the basis of their irregular nuclear morphology, the distribution and density of heterochromatin and their position in the tubule. The presence of XX Sertoli cells was confirmed for the adult chimaeras by the *in situ* analysis of air-dried testis preparations, in which Sertoli cells are easily recognised by the characteristic ‘triple spots’ of a light blue nucleolus flanked by two heterochromatic blocks. In these preparations, a small population of such cells were found with clear hybridisation (chimaeras 2–4, e.g. Fig. 2D) or lacking hybridisation (chimaera 1) indicating their XX nature.

Hybridisation was always found, both in the control XY<XY testis preparation and in the experimental chimaeras, closely associated with one of the heterochromatic blocks. The fact that these regions are known to contain centromeric sequences (Hsu et al. 1971), plus the fact that the target transgenic insert is close to the telomere of chromosome 3, may indicate something about the organisation of chromatin in the Sertoli cell nucleus.

The estimates of the contribution of XX cells to specific cell types in the fetal testes are presented in Table 1. The chimaeras are arranged in order of increasing XX contributions to liver. With the exception of the Sertoli cell lineage, there is no prejudice against XX cells contributing to testicular cells. Overall, the proportion of XX peritubular myoid cells (43%) and Leydig cells (48%) is very similar to the proportion of XX cells in non-testicular tissues (47–48%), while the overall proportion of XX T1-prospermagonia...
Fig. 1. (A) Section of 16.5 dpc XX→XY testis showing some labelled XX T1 prospematogonia inside cords lined with unlabelled XY Sertoli cells. (B) Section of the same testis with two labelled XX cells which, on the basis of position and morphology, are presumed to be Sertoli cells (arrowed). Bar, 10 μm.
Fig. 2. (A) Prepuberal XY<->XY control testis section showing the patchy distribution of labelled germ cells and Sertoli cells. (B) Prepuberal XX<->XY testis section from a mouse with 29% XX contribution to the bone marrow. A patch of labelled XX Sertoli cells (arrowed) can be seen in an otherwise XY dominated testis. (C) Section of testis from adult XX<->XY chimaera 4. Four XX Sertoli cells (small arrows) can be seen inside a tubule producing condensed spermatids. The other labelled cells are peritubular myoid cells (large arrow). (D) Air-dried testis preparation from chimaera 4 showing Sertoli cells with the characteristic heterochromatic blocks. One of the Sertoli cells has a hybridisation signal over one of the heterochromatic blocks (arrow) indicating the XX nature of the cell. Bar, 10 μm.
The majority of Sertoli cells from this age lack the typical nuclear morphology seen in the adult. In the four adult XX<->XY testes, they presumably differentiated contemporaneously with the XY Sertoli cells, and as a consequence of Tdy action. What does this tell us about the mode of Tdy action? It should be emphasised that, although the Sertoli cells were not exclusively XY, there was a strong XY bias that was already established by 13.5 dpc. There was no XY bias in any of the other cell types in the fetal testes. This strengthens the conclusion by Burgoyne et al. (1988a) that Tdy must act by directing cells of the supporting cell lineage to form Sertoli cells. However, since a small proportion of fetal XX Sertoli cells were found, at some point between the expression of Tdy and the formation of Sertoli cell cords, there must be a step that can locally recruit XX cells. In a normal XY male, this capacity for local recruitment is irrelevant since all cells contain Tdy. Recently, a new gene (Sry) has been identified on the mouse Y chromosome (Gubbay et al. 1990), which is almost certainly Tdy. The expression of Sry is first seen at 10.5 dpc in the genital ridge and is germ cell independent (Koopman et al. 1990), which is compatible with Sry being expressed in the Sertoli cell precursors. The sequence of Sry suggests that it encodes a DNA-binding protein which would be expected to act cell autonomously. This would imply that the capacity for recruiting some XX cells to form Sertoli cells in XX<->XY testes is a characteristic of a gene product in the Sry-initiated 'cascade', rather than of Sry itself.

The XX Sertoli cells that form in XX<->XY chimaeric gonads should not be equated with those that form in ovarian tissue in a number of situations where there has been germinal failure (reviewed by Burgoyne, 1988 and 1991). Taketo-Hosotani et al. (1985) and Taketo-Hosotani and Sinclair-Thompson (1987); for example, have described the 'transdifferentiation' of follicle cells into Sertoli cells, following germ cell loss, when fetal mouse ovaries were grafted under the kidney capsule of

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### Table 2. Estimated proportions of XX cells in the cell lineages of adult XX<->XY chimaeras

<table>
<thead>
<tr>
<th>Chimaera Cross</th>
<th>Age dpp</th>
<th>Bone marrow</th>
<th>Pachytene spermatocytes</th>
<th>MI spermatocytes</th>
<th>Sertoli cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 XX&lt;-&gt;XY*</td>
<td>1</td>
<td>52</td>
<td>10(50)</td>
<td>0(514)</td>
<td>8(124)*</td>
</tr>
<tr>
<td>2 XX&lt;-&gt;XY</td>
<td>2</td>
<td>52</td>
<td>22(50)</td>
<td>0(6)</td>
<td>2(1078)</td>
</tr>
<tr>
<td>3 XX&lt;-&gt;XY</td>
<td>2</td>
<td>46</td>
<td>61(533)</td>
<td>0(500)</td>
<td>1(6039)</td>
</tr>
<tr>
<td>4 XX&lt;-&gt;XY</td>
<td>2</td>
<td>52</td>
<td>84(51)</td>
<td>0(537)</td>
<td>1(62052)</td>
</tr>
</tbody>
</table>

*The component carrying the transgenic marker
†The bone marrow counts are based on cytogenic markers in air-dried mitotic cells. For the other cell types, the counts are based on the in situ analysis of the transgenic marker in air-dried testicular cells
‡This is based on a single unlabelled cell, which probably lost the marker as a result of metaphase breakage

(65 %) is somewhat higher. By contrast, there is a clear prejudice against XX Sertoli cells in all four chimaeras, with a mean XX contribution of 10 %. Comparing the proportion of XX Sertoli cells in each chimaera with the pooled estimates for all other cell types indicates a reduction of between 4- and 7-fold. The extent of the reduction in the contribution of XX cells to the Sertoli cell lineage is not related either to fetal age or the strain of the XX component.

The two prepuberal XX<->XY chimaeras had XX contributions to the bone marrow of 29 % and 39 %. The labelled Sertoli cells occurred as clonal patches but the patches were rare, confirming the strong XY bias (Fig. 2B). The proportion of XX XX<->XY testis Sertoli cells has not been estimated for these two chimaeras. The quantitation procedure used for the sections is inappropriate when the cells occur in rare patches and, in air-dried preparations of dissociated testicular tissue, the majority of Sertoli cells from this age lack the typical nuclear morphology seen in the adult. In the four adult chimaeras (Table 2), the proportion of XX Sertoli cells was very low (1.6–2.1 %) and surprisingly consistent despite a wide range of XX contribution to the bone marrow (10–84 %).

Extensive earlier work has demonstrated that XX germ cells do not survive in XX<->XY testes. Of the 2381 pachytene and MI cells scored here, one MI cell lacking the transgene marker was identified in the chimaera that had the transgene in the XY component. This probably represents artefactual loss of the bivalent carrying the marker. It was noted that the adult XY<->XY control, in which the XY component lacking the transgene was heterozygous for the T6 translocation, has no unlabelled MI spermatocytes, although this component was well represented in pachytene spermatocytes (13 %), Sertoli cells (30 %) and bone marrow cells (43 %). It has previously been reported that heterozygosity for this translocation causes extensive meiotic failure (Baranov and Dyban, 1968).

**Discussion**

This study demonstrates that some XX Sertoli cells are present in fetal, prepuberal and adult XX<->XY chimaeras. The presence of XX Sertoli cells in adult XX<->XY chimaeras is contrary to the results of Burgess et al. (1988a). However, the incidence of XX Sertoli cells in the adults was less than 3 % and it is possible that such a low contribution, occurring as it does in the form of rare patches, may have been missed in the earlier study. The presence of XX Sertoli cells in an adult XX/XY male has previously been inferred by Singh et al. (1987) and is also reported for a series of adult XX<->XY chimaeras by Patek et al. (1991). Since XX Sertoli cells are already present in the 13.5 dpc XX<->XY testes, they presumably differentiated contemporaneously with the XY Sertoli cells, and as a consequence of Tdy action. What does this tell us about the mode of Tdy action? It should be emphasised that, although the Sertoli cells were not exclusively XY, there was a strong XY bias that was already established by 13.5 dpc. There was no XY bias in any of the other cell types in the fetal testes. This strengthens the conclusion by Burgoyne et al. (1988a) that Tdy must act by directing cells of the supporting cell lineage to form Sertoli cells. However, since a small proportion of fetal XX Sertoli cells were found, at some point between the expression of Tdy and the formation of Sertoli cell cords, there must be a step that can locally recruit XX cells. In a normal XY male, this capacity for local recruitment is irrelevant since all cells contain Tdy. Recently, a new gene (Sry) has been identified on the mouse Y chromosome (Gubbay et al. 1990), which is almost certainly Tdy. The expression of Sry is first seen at 10.5 dpc in the genital ridge and is germ cell independent (Koopman et al. 1990), which is compatible with Sry being expressed in the Sertoli cell precursors. The sequence of Sry suggests that it encodes a DNA-binding protein which would be expected to act cell autonomously. This would imply that the capacity for recruiting some XX cells to form Sertoli cells in XX<->XY testes is a characteristic of a gene product in the Sry-initiated 'cascade', rather than of Sry itself.

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adult male, or even adult female, hosts. In the latter instance, there was no Y chromosome present in the graft or host, so it can hardly be considered of direct relevance to the mode of Tdy action.

The fact that Sertoli cells are the only cell type to show an XY bias in the fetal testis supports the view of Burgoyne (1988) that the commitment of other testicular cell types to the male pathway is directed by Sertoli cells without further Y involvement. This does not preclude a role for the Y in the subsequent differentiation or function of these other cell types, or indeed, of Sertoli cells. For example, the mouse Y has at least three distinct functions during spermatogenesis (reviewed by Burgoyne, 1991). Patek et al. (1991) suggest that the Y may also be required for normal Leydig cell functions. This suggestion is based on their finding of an unusually high incidence of tubules with germinal failure together with a particularly high proportion of XX Leydig cells in their series of XX<->XY chimaeras, leading them to propose that the germinal failure is brought about by XX Leydig cell dysfunction. An alternative possibility is that this particular chimaeric combination (in which the XY component was always derived from embryonic stem cells of the 129 inbred strain and the XY component from C57BL/6xCBA F2) not only favours XX Leydig cells but also favours XX germ cells. The tubules with germinal failure would then be attributable to the loss of the XX germ cells around the time of birth. We therefore prefer to reserve judgement on a possible role for the Y in Leydig cells until information on the XX contribution to the germ line is available for fetal XX<->XY testes of this particular chimaeric combination.

A puzzling feature of the present data was the consistency in the proportion of XX Sertoli cells (1.6–2.1%) in the adult chimaera despite a wide range of XX contributions to the mice as a whole (10–84%). While the consistently lower frequency of XX Sertoli cells in the adult chimaeras, as compared to the fetal chimaeras, may indicate selection against XX Sertoli cells subsequent to their formation, it is hard to imagine a selective mechanism that acts to produce such a low but invariant proportion of XX cells.

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References


Hsu, T. C., Cooper, J. E. K., Mace, M. L. and Brinkley, B. R. (1971). Arrangement of centromeres in mouse cells. Chromosoma 34, 73–87


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