Title: 'Spontaneous' sex reversal in organ cultures of the embryonic male gonad of the bird

By GREGORY F. ERICKSON

From the University of California, San Diego, La Jolla

SUMMARY

The left embryonic testis of the bird (4-8 days of incubation) was organ cultured in medium that contained 10% foetal calf serum. Under these conditions, the germinal epithelium (GE) of the 4-day gonad differentiates into an ovarian cortex and the male primordial germ cells (PGCs) complete a developmental sequence similar to normal oocytes, i.e. they divide mitotically, develop a Balbiani body, divide synchronously in groups of two, four, and eight germ cells, and some enter pre-leptotene. No medullary tissue develops in the 4-day explants. The pieces of 6- and 8-day gonad differentiate into true ovotestes in which the GE develops into a cortex and the medulla develops into seminiferous cords. The PGCs in the cortex differentiate as oocytes and those in the seminiferous cords differentiate as spermatogonia. The possibility that biologically active oestrogens are present in the growth medium is discussed.

INTRODUCTION

During normal development of the bird, Gallus domesticus, the primordial germ cells (PGCs) in the testis and ovary complete a similar developmental sequence up to meiotic prophase. This sequence includes mitosis, the aggregation of mitochondria around the Golgi forming the Balbiani body, a series of synchronous mitoses, and a synchronous meiosis (Swift, 1916; Erickson, 1974). This sequence is completed in female PGCs during embryonic development, but it is not completed in the male until the bird reaches sexual maturity.

The factors that regulate this developmental sequence in the female have been shown to reside in the germinal epithelium (GE) of the 4-day gonad (Erickson, 1974). Although the nature of these factors has not been defined, they appear to reside within the prospective follicle cells and their expression is independent of the pituitary and medullary estrogens.

The factors that control the differentiation of the male PGCs also reside in the gonad. Wolff & Haffen (1952a,b) have shown that PGCs differentiate into spermatogonia in organ cultures of the indifferent gonad, and Steinberger & Steinberger (1966) observed the differentiation of spermatogonia to pachytene in cultures of newborn mice testes. Precisely what cells in the testis control the differentiation of the PGCs is not clear, but Ellingson & Yao (1970) suggest the control resides within the seminiferous tubule.

1 Author's address: Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115, U.S.A.
It is well established that the male PGCs in the bird have the ability to differentiate as oocytes. When the indifferent male gonad is incubated with biologically active oestrogens, the GE develops into an ovarian cortex and the PGCs complete the developmental sequence of oocytes (for review see Burns, 1961; Narbaitz, 1971). This present paper describes an experiment in which the indifferent male gonad of the bird develops 'spontaneously' in vitro into a true ovotestis. The GE develops into ovarian secondary cords and the primary cords of proliferation develop into seminiferous cords. Even though these cords are continuous, the male PGCs in the cortex differentiate as oocytes and those in the seminiferous cords differentiate as spermatogonia.

**MATERIALS AND METHODS**

**Animals**

White Leghorn chicken embryos at 3–17 days of incubation were used in this study. The sex of embryos older than 8 days of incubation was determined by the difference in size and shape of the male and female left gonad (Weniger, 1961). The sex of embryos less than 8 days of incubation was determined by karyotype (Owen, 1965).

**Culture of gonads**

The male left gonad from 6- and 8-day embryos was dissected from the mesonephros and cultured as pieces of intact gonad. The 6-day gonads were cut into thirds and the 8-day gonads were cut into fourths. The 4-day gonad was left attached to the mesonephros, cut into thirds, and cultured as units of gonad, mesonephros, and Müllerian–Wolffian ducts. Because the sex of 4- and 6-day embryos could not be determined immediately, the pieces of gonad from each embryo were cultured separately until the sex was established. At that time the pieces of male gonad from embryos at the same stage of development were pooled and cultured together.

The methods for culturing the tissue have been described elsewhere (Erickson, 1974). Briefly, the tissue was cultured submerged in 5 ml of growth medium on a layer of agar. The growth medium consisted of Ham's F-12 nutrient mixture supplemented with 10% foetal calf serum, 100 units/ml of penicillin, 5 μg/ml of streptomycin sulphate, and 1% L-glutamine (stock was 200 mM).

The pieces were cultured for a time equal to 17 days in ovo. That is, pieces of 4-day gonad were cultured for 13 days, pieces of 6-day gonads for 11 days, and 8-day gonads for 9 days. (Additional pieces of 8-day gonad were collected after 2 and 5 days in vitro.) In the text this is designated by $T_{(r)}$, where $T$ stands for the age of the gonad and $(x)$ designates the number of days in culture, i.e. 4-day ($T_8$) denotes a piece of 4-day gonad at the time it was put in culture and 4-day ($T_{13}$) denotes the piece after 13 days in culture.
Table 1. The number of germ cells in normal male left gonads of the chick embryo*

<table>
<thead>
<tr>
<th>Age gonads (days)</th>
<th>Total number of germ cells</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
</tr>
<tr>
<td>4</td>
<td>460</td>
</tr>
<tr>
<td>5</td>
<td>1082</td>
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<tr>
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<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>2808</td>
</tr>
<tr>
<td>10</td>
<td>7401</td>
</tr>
<tr>
<td>17</td>
<td>8740</td>
</tr>
</tbody>
</table>

* Each mean value represents measurements on at least three individual gonads.

At the end of the culture period, the pieces were serially sectioned and stained with hematoxylin and eosin. The quantitative procedures have been described previously (Erickson, 1974).

RESULTS

In ovo study

The normal differentiation of the left testis of the bird has been described in detail by Swift (1916), and the observations in this study confirm his findings.

To interpret the quantitative changes occurring in the germ cells in vitro, a study was made on the number of germ cells in the left testis between 3 and 17 days of incubation. The results are presented in Table 1. They show that the PGCs increase regularly during the indifferent period (3–6 days), but there is only a slight increase after sexual differentiation (8 days). A quantitative study of the PGCs in the rat embryonic testis show that they continue to increase regularly after sexual differentiation (Beaumont & Mandl, 1963). This may reflect a difference in the mitotic activity of the PGCs in the testis of the rat and the chick.

Comparing the data presented here with a similar quantitative study done on the PGCs in the female embryonic ovary (Erickson, 1974), shows that the left testis begins with half the number of PGCs as the left ovary. This confirms the findings of Limborgh (1968) and supports his thesis that the first indication of sexual differentiation in the bird is a difference in the number of PGCs in the male and female left gonads.

In vitro study

4-day gonad. When the 4-day gonad is put in vitro, it consists of a layer of GE and a small amount of condensing mesenchyme (Fig. 1). After 13 days in culture, the GE differentiates into an ovarian-like cortex that contains typical secondary cords packed with somatic and germ cells (Fig. 2). Except for a few scattered
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mesenchymal cells, no medullary tissue is obvious in these explants. It is not clear why the medulla does not differentiate in these explants, but it could be correlated with the failure of the primary cords to proliferate from the GE. A similar observation has been reported in cultures of the 4-day female left gonad (Erickson, 1974).

The PGCs in the 4-day ($T_{13}$) explants differentiate similarly to those in a normal ovary, i.e. they divide mitotically (Table 2), develop a Balbiani body, and divide synchronously in groups of two, four and eight germ cells (Fig. 2). No meiotic prophase figures are seen, but some nuclei (Fig. 7) are similar to the pre-leptotene cells described by Hughes (1963). This indicates that these cells complete pre-meiotic DNA synthesis and are entering meiotic prophase (Callebaut, 1967).

These results show that under identical culture conditions, the 4-day male gonad differentiates qualitatively and quantitatively like the 4-day female left gonad (Erickson, 1974).

6-day gonad. At the time the 6-day gonad is put in culture, the GE is in the process of proliferating the primary cords into the underlying medullary mesenchyme (Fig. 3). These cords are the prospective seminiferous cords and consist of a basement membrane enclosing the prospective Sertoli cells and numerous PGCs. At this stage the 6-day gonad is still an indifferent gonad (Wolff & Haffen, 1952a, b).

After 11 days in vitro, each piece differentiates into a true ovotestis (Fig. 4). The GE differentiates into female secondary cords and the primary cords develop into continuous seminiferous cords. As indicated in Fig. 5, the seminiferous cords appear to remain attached to the secondary cords of the cortex. A similar finding has been reported by Wolff & Haffen (1952b). This suggests the primary cords did not separate from the GE and supports the observation of Firket (1914) that the seminiferous cords (and Sertoli cells) originate from the GE.

**Figures 1-4**

Fig. 1. Cross-section of a 4-day ($T_{0}$) male left gonad showing the germinal epithelium (GE) and prospective medulla (M). The GE contains several layers of somatic cells and numerous PGCs (arrows). × 320.

Fig. 2. The 4-day gonad in vitro appears as a translucent finger-like body that protrudes from the mesonephros. This is a cross-section of the 4-day ($T_{13}$) gonad. The GE (shown in Fig. 1) differentiates into female secondary cords that are packed with somatic and germ cells. These PGCs differentiate as oocytes and some divide synchronously (arrows). × 800.

Fig. 3. Cross-section of the indifferent 6-day ($T_{0}$) gonad. The germinal epithelium (GE) is proliferating the primary cords or prospective seminiferous cords (arrows) into the underlying medulla (M). At this stage the male GE is developing as a female cortex that contains prospective follicle cells (Rahil & Narbaitz, 1972). × 320.

Fig. 4. A typical 6-day ($T_{11}$) male gonad that differentiates into an ovotestis. The seminiferous cords (SC) are surrounded by a female-like cortex (C). × 200.
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Although the seminiferous cords are clearly delineated, the seminiferous epithelium is not well developed. The prospective Sertoli cells do not appear to increase significantly in number and they do not align themselves along the basement membrane (compare Figs. 5, 6). However, the PGCs in these cords appear to differentiate normally into spermatogonia, i.e. they divide mitotically and develop a Balbiani body. This observation is different from that reported in the 6-day (T\(_{11}\)) female ovotestis in which the female PGCs in the male-like cords differentiate as oocytes, i.e. they divide mitotically, develop a Balbiani body, divide synchronously, and some enter meiotic prophase (Erickson, 1974).

As shown in Table 2 and Figs. 7 and 8, the male PGCs in the ovarian-like cortex differentiate into oocytes similar to those described in the 4-day (T\(_{13}\)) gonad.

8-day gonad. Even though the 8-day (T\(_{8}\)) gonad is sexually differentiated, it still has patches of female-like ovarian cortex. These patches are similar ultrastructurally to the GE in a 6-day (T\(_{6}\)) ovary and can be stimulated with estrogens to differentiate into a normal cortex (Burns, 1961; Rahil & Narbaitz, 1972).

In culture, the pieces of 8-day testis (Fig. 9) differentiate similarly to the pieces of 6-day (T\(_{11}\)) gonad except each 8-day (T\(_{8}\)) piece has patches of ovarian cortex interspersed with tunica albuginea (Fig. 10). As shown by the (T\(_{2}\)) and (T\(_{5}\)) pieces, both the cortex and seminiferous cords are clearly delineated after 2 days.

Figures 5-10

Fig. 5. A 6-day (T\(_{11}\)) explant showing the continuation of the basement membrane (arrows) of the cortex (C) and the seminiferous cords (SC). The prospective Sertoli cells in the SC do not increase significantly in number and do not align themselves along the basement membrane (compare with Fig. 6). Even though the cords are continuous, the germ cells in the SC differentiate as spermatogonia and those in the C differentiate as oocytes. \(\times 320\).

Fig. 6. A portion of a normal 17-day testis. The GE shown in Fig. 3 has differentiated into a tunica albuginea (TA) that contains no germ cells. There is a dramatic increase in the number of Sertoli cells between 7 and 17 days and they are now aligned in a regular way along the basement membrane. The spermatogonia have a prominent Balbiani body (arrow) and some begin to attach to the basement membrane. \(\times 560\).

Fig. 7. A male germ cell in the cortex of a 6-day (T\(_{11}\)) gonad that differentiates as an oocyte. The nucleus appears in pre-leptotene (open arrow) suggesting that premeiotic DNA synthesis has occurred. The prominent, eosinophilic Balbiani body (thin arrow) is clearly seen. Similar cells appear in the ovarian-like cortex in the 4-day (T\(_{13}\)) and 8-day (T\(_{8}\)) explants. \(\times 1400\).

Fig. 8. Four (1, 2, 3, 4) of a group of eight synchronously dividing germ cells in the cortex of a 6-day (T\(_{11}\)) explant. \(\times 800\).

Fig. 9. Pieces of living 8-day (T\(_{8}\)) gonads. Each piece is surrounded by a translucent germinal epithelium that increases in thickness \textit{in vitro}. The 6-day (T\(_{11}\)) pieces appear similar. \(\times 20\).

Fig. 10. Cross-section of an 8-day (T\(_{8}\)) explant. Patches of ovarian cortex (arrows) are seen interspersed with tunica albuginea. The small amount of cortex relative to the 6-day (T\(_{11}\)) pieces is a result of the decreased amount of incipient cortex in the 8-day (T\(_{8}\)) gonad. \(\times 200\).
Table 2. Quantitative data on germ cells in pieces of male gonad cultured in vitro*

<table>
<thead>
<tr>
<th>Gonad piece</th>
<th>Corresponding age in ovo (days)</th>
<th>Number of germ cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>4-day $T_0$</td>
<td>4</td>
<td>153</td>
</tr>
<tr>
<td>$T_{13}$</td>
<td>17</td>
<td>521</td>
</tr>
<tr>
<td>6-day $T_0$</td>
<td>6</td>
<td>662</td>
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<tr>
<td>$T_{11}$</td>
<td>17</td>
<td>7795</td>
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<td>8-day $T_0$</td>
<td>8</td>
<td>702</td>
</tr>
<tr>
<td>$T_2$</td>
<td>10</td>
<td>1804</td>
</tr>
<tr>
<td>$T_5$</td>
<td>13</td>
<td>1838</td>
</tr>
<tr>
<td>$T_9$</td>
<td>17</td>
<td>3945</td>
</tr>
</tbody>
</table>

* $T_0$ represents the number of germ cells in each piece at the time it was put in vitro and was calculated from data presented in Table 1. For example, the whole 4-day ($T_0$) gonad has a mean number of 460 germ cells. Since the gonad was cut into thirds, each piece at the time it was put in vitro is assumed to contain an average of 153 germ cells.

$\dagger$ Each in vitro mean value represents measurements on at least ten pieces of gonad.

The results reported here show that under these culture conditions the GE in the male embryonic gonad differentiates into a true ovarian cortex, and that the PGCs in the cortex complete a normal developmental sequence of oocytes and the PGCs in the seminiferous cords complete the developmental sequence of spermatogonia.

The quantitative data presented in Table 2 show a progressive increase in the PGCs in each 8-day piece, but the number of PGCs at the end of the culture period is only half that in the 6-day ($T_{11}$) pieces. This is clearly correlated with the difference in the amount of cortex between the 6-day ($T_{11}$) and 8-day ($T_0$) explants.

DISCUSSION

The experimental studies on sexual differentiation of the bird indicate that the male indifferent GE (unlike the female) is not determined to differentiate into an ovarian cortex in isolation (for reviews see Wolff & Haffen, 1965; Haffen, 1960). Culturing the indifferent male gonad on the chorioallantoic membrane (Willier, 1933, 1939) or in an oestrogen-free medium (Wolff & Haffen, 1952a, b; Weniger, 1961) results, in most cases, in a typical testis. In a
convincing set of experiments, Wolff & Haffen (1959, 1965) have shown that pieces of male duck GE cultured alone do not differentiate into a cortex, but they do when cultured with female medullary tissue. These data, together with the fact that oestrogens stimulate the male GE to differentiate into an ovarian cortex (for review see Burns, 1961), suggest that significant amounts of biologically active oestrogens were present in the FCS used in the present study.

Unfortunately, the particular lot of FCS used in this study is no longer available for analysis. However, it has been shown that the concentration of biologically active oestrone ($E_1$) and oestradiol ($E_2$) is very high in the serum of both male and female foetal calves throughout the course of gestation (Challis et al. 1974). Since commercial FCS is pooled from foetuses of different age and sex (Dr R. McKinney, Microbiological Associates), it seems reasonable to assume that each lot of FCS contains relatively high levels of $E_1$ and $E_2$. Although one must be cautious, the data of Challis et al. (1974) suggest that the growth medium used in this present study (10% FCS) contained on the order of 20–200 pg/ml of biologically active oestrogens ($E_1 + E_2$).

Although this appears to be an extremely small amount of oestrogen, it must be emphasized that it is not known how target cells in the male GE respond when exposed directly to these levels of oestrogens. Willier, Gallagher & Koch (1935) have shown that the degree of intersexuality induced in the male gonad in ovo is dose-dependent; a large dose results in complete sex reversal while a small dose produces an ovotestis similar to that described in this paper. It seems reasonable to assume that Willier’s findings were a function of the final concentration of circulating oestrogens to which the male GE was exposed. His results provide support for the hypothesis presented here that the FCS used in this study contained a small amount (pg levels) of biologically active oestrogen that stimulated the male GE to develop into an ovarian cortex.

These results indicate the caution an author must exercise in interpreting the results of an experiment in which embryonic tissues and cells are cultured in medium that contains animal sera. Even though a particular growth medium might contain what is thought to be only negligible amounts of a specific hormone(s), the concentration at the in vitro cell level may be sufficient to trigger a developmental response.

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REFERENCES


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