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Gonadal sex reversal of the developing marsupial ovary in vivo and in vitro

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SUMMARY

Undifferentiated tammar wallaby ovaries were transplanted under the skin of male pouch young during the period of mitotic division of the XX germ cells. After 25 days, all the germ cells had disappeared and the ovaries contained seminiferous-like cords. Similarly, undifferentiated ovaries cultured for 4 days with recombinant human Müllerian-inhibiting substance (rhMIS) also contained well-differentiated seminiferous-like cords and few or no surviving germ cells. The majority of controls cultured without rhMIS developed as normal ovaries. However, in a few control ovaries seminiferous-like cords developed in those regions of the ovaries that were partially necrotic and contained few germ cells. These results strongly suggest that sex-reversal of the tammar ovary is the direct result of loss of mitotic germ cells, rather than an effect of MIS on female somatic cells. MIS is apparently toxic to these female germ cells in mitosis, but not to male germ cells in mitosis. Thus, in normal development in the tammar, the presence of XX germ cells in the ovary inhibits the formation of seminiferous cords so that the gonad develops as an ovary.

Key words: tammar wallaby, Müllerian-inhibiting substance, anti-Müllerian hormone, testis, ovary, gonad culture, gonad transplantation, germ cell

INTRODUCTION

During early mammalian development, the Sertoli cells of the fetal testis produce Müllerian-inhibiting substance (MIS) (Blanchard and Josso, 1974; Tran and Josso, 1982; Hayashi et al., 1984). MIS induces the regression of the fetal Müllerian ducts, the primordia of the oviducts, uterus and upper vagina. MIS also has a masculinising effect on the developing ovary, prompting suggestions of a role for MIS in testicular differentiation (Vigier et al., 1987, 1989; Behringer et al., 1990). This masculinising effect of MIS was first described in the bovine freemartin, where a female calf is exposed to the testicular hormones of her male co-twin via extensive chorioallantoic vascular anastomoses (Lillie, 1917). The ovaries of the freemartin become depleted of germ cells (Short et al., 1969) and develop structures resembling seminiferous tubules (Jost et al., 1972). In addition to a morphological sex reversal, the freemartin ovary also undergoes an endocrine sex reversal, producing both testosterone (Short et al., 1969; Shore and Shemesh, 1981) and MIS (Vigier et al., 1984). Fetal rat ovaries co-cultured with fetal rat testes (Charpentier and Magre, 1990) or with purified bovine MIS (Vigier et al., 1987) are also induced to form seminiferous-like tubules after the loss of germ cells. In transgenic mice chronically over-expressing high levels of human MIS, both ovarian and testicular development and germ cell survival are perturbed (Behringer et al., 1990), prompting further speculation on a possible role for MIS in testicular differentiation.

In all cases of mammalian ovarian sex reversal, the development of testis-like structures is preceded by a massive loss of germ cells (Holyoke, 1949; Short et al., 1969; Jost et al., 1972; Taketo-Hosotani et al., 1985; Vigier et al., 1987; Prépin and Hida, 1989; Behringer et al., 1990; Charpentier and Magre, 1990; Lyet et al., 1995). Thus, it is unclear whether the masculinising effect of MIS is the direct result of its action on the granulosa cells (Behringer et al., 1990), or an indirect result of its lethal effect on female germ cells (McLaren, 1990). In the ovary, meiotic germ cells are essential for the formation (reviewed in McLaren, 1985, 1991) and maintenance of follicles (reviewed in McLaren, 1990). If the oocytes are lost, the supporting cells may form seminiferous tubules (Burgoyne, 1988; McLaren, 1991). Indeed, the loss of meiotic germ cells alone around the time of folliculogenesis is sufficient to induce the formation of testis-like structures in fetal rat ovaries cultured in the absence of any masculinising influence (Prépin and Hida, 1989).

In marsupials, much of development occurs after birth, permitting easy access to the developing young. In addition, cell-mediated immune responses are undeveloped in the young marsupial (Deane and Cooper, 1988), making them ideal models for tissue transplantation experiments. Testes transplanted under the skin of female recipients continued to grow for at least 150 days and masculinised the female genitalia (Tyndale-Biscoe and Hinds, 1989).

On the day of birth, the gonads of the tammar wallaby are sexually undifferentiated (O et al., 1988; Renfree et al., 1996). By 2 days after birth, the testis contains clearly visible seminiferous tubules (Short et al., 1988) and has begun producing MIS (Hutson et al., 1988), although regression of the Müllerian duct is not apparent for at least another 5 days (Renfree et al., 1992, 1996). The testis begins secreting testosterone 2 days after birth, coinciding with the appearance of Leydig cells (Renfree et al., 1992). Ovarian differentiation is first clearly defined by 8 days after birth, with the formation of the primary ovarian cortex and medulla (Alcorn, 1975; Renfree et al., 1996). However, meiosis

does not commence in the tammar ovary until around 25 days after birth (Alcorn and Robinson, 1983), and it is not until day 50 postpartum that the first primordial follicles appear (Alcorn, 1975). Steroid production by the ovary does not commence until after 200 days of pouch life (Renfree et al., 1992).

The object of this study was to investigate the control of gonadal sex reversal and the relationship between MIS and germ cells in undifferentiated wallaby ovaries transplanted under the skin of male recipients in vivo or cultured with MIS in vitro.

MATERIALS AND METHODS

Animals

Tammar wallabies (*Macropus eugenii*) of Kangaroo Island origin were maintained in our breeding colony at Clayton, Victoria. Pregnant females were checked daily for the presence of newborn young; the day of birth was designated day 0. Neonates were sexed by the presence or absence of scrotal bulges, which are visible only in male young (O et al., 1988).

Gonadal transplants

Gonads for transplantation were dissected from neonatal young of both sexes and transplanted beneath the abdominal skin of 10-day-old recipients of either sex. Initially, 10 gonads were transplanted with attached mesonephros and 22 without mesonephros. Since removal of the mesonephros did not influence gonadal survival or differentiation, the remaining 37 gonads were transplanted without mesonephros.

Gonads from neonates were placed into 1 ml of sterile Dulbecco's Modification of Eagle's Medium (DMEM) / Ham's F12 culture medium (Cytosystems, Castle Hill, NSW, Australia) on ice while the recipient was prepared. Recipient young were anaesthetised by inducing hypothermia, as tammar wallabies are unable to thermoregulate until approximately 6 months of age (Setchell, 1974); this method of anaesthesia for early pouch young of macropodid marsupials is recommended by the National Health and Medical Research Council of Australia (1990). Recipients were removed from the pouch and quickly chilled by placing them onto a moist swab on wet ice. When body movements had ceased, the animal was gently cleaned and supported ventral side up in a beeswax mould attached to an ice-chilled metal tray (Renfree and Tyndale-Biscoe, 1978). Forelimbs and hindlimbs were loosely restrained with saline-moistened strips of swab, held in place with rubber bands. Under a dissecting microscope, a superficial incision was made on both flanks below the last rib, and the gonads were inserted into a small subcutaneous pocket. The incisions were sutured with 6-0 chromic selfdissolving thread (Ethicon, Somerville, N.J. USA), and the recipient immediately warmed by hand to body temperature. Once body movements had recommenced, the animal was reattached to its mother's teat by gently pressing the teat into its mouth with the softened end of a matchstick. Recipients were examined 1 hour later and the next day to confirm that they were still attached to the teat.

After 25 days, the recipients were killed by decapitation and the subcutaneous gonadal transplants excised. Transplants were fixed overnight in Bouin's fluid, washed and stored in 70% ethanol until they were dehydrated, paraffin embedded and serially sectioned at 7 μm. Sections were stained with either Ehrlich's or Harris's haematoxylin and eosin.

Organ cultures

Both gonads were dissected from female tammar neonates either with the mesonephros attached (n=7), or removed (n=5). Each gonad was placed onto a polycarbonate membrane (pore size = 0.8 μm) (Costar, Cambridge, Massachusetts, USA) and floated onto 1 ml of DMEM / Ham's F12 culture medium (Cytosystems) with 10 mM L-glutamine (Cytosystems), 100 U/ml penicillin and 100 μg/ml streptomycin (CSL, Parkville, Victoria, Australia), and 5 μg/ml transferrin (Sigma-Aldrich, Castle Hill, NSW, Australia), 20 μg/ml insulin (Sigma-Aldrich) and 50 ng/ml retinoic acid (Sigma-Aldrich) (Zhou et al., 1993) contained in wells of a 24-well culture plate (Costar). Recombinant human MIS was added at a final concentration of 4-9 μg/ml to one gonad of each pair; the other gonad was cultured in medium alone as a control. In addition, 10 neonatal testes were cultured in control medium, 5 with adjacent mesonephros and 5 without.

Cultures were maintained for 4 days at 37°C, in an humidified atmosphere of 95% air and 5% CO_2. Culture medium was renewed every second day and, after the fourth day, gonads were fixed in Bouin's fluid overnight and processed for histology as above.

RESULTS

Transplant experiments

Only 13 of the 69 of the transplanted gonads underwent differentiation (Table 1). Most transplanted gonads were either resorbed by the recipient, or on histological examination contained only fibrous tissue and were classed as dysgenetic. Removal of the mesonephros did not influence the survival of gonadal transplants or the induction of fibrous growths, and it did not appear to have any impact on testicular or ovarian differentiation. There was no relationship between the presence or absence of mesonephros and the frequency with which ovaries transplanted into males sex reversed. Thus, the results of gonads transplanted with or without mesonephros were pooled.

Development of male gonadal transplants

Of the 14 undifferentiated male gonads transplanted into male recipients, 3 developed as testes and 11 became dysgenetic (Table 1). A further 13 male gonads were transplanted into female recipients. Of these, 4 developed as testes and 9 were dysgenetic. All of the surviving testes contained Sertoli cells, which had formed tightly packed seminiferous cords enclosing clusters of mitotically active germ cells (Fig. 1A,B). Seminiferous cords were bounded by a basement membrane and peritubular myoid cells. The interstitium comprised Leydig cells and the entire testis was encased in a fibrous tunica albuginea (Fig. 1B).

Densely nucleated pseudolymphomas developed alongside all of the surviving gonadal transplants, irrespective of the sex of the transplant or the recipient young (Fig. 1C). In some instances, lymphocytes were present in the gonadal tissue.

Development of female gonadal transplants

3 of the 10 female gonads transplanted into female recipients developed as ovaries (Table 1); the remaining 7 were dysgenetic. Surviving ovaries contained nests of oogonia delineated by bands of mesenchymal tissue (Fig. 2A). There were

Table 1. Development of undifferentiated neonatal gonads transplanted beneath the skin of day 10 pouch young

No. and sex of gonads	Recipient	Gonadal development		
		Ovarian	Testis-like	Dysgenetic
Male				
14	Male	0	3	11
13	Female	0	4	9
Female				
32	Male	0	3	29
10	Female	3	0	7

many more germ cells that had entered meiosis than normally occurs in day 25 ovaries, which may be related to systemic factors in the hosts which were 10 days older than the graft tissue. Some oogonia were still dividing mitotically, others were in various stages of meiosis, while still others were atretic. The nests of mainly meiotic germ cells were in the central core of the graft (Fig. 2A) whereas those germ cells scattered through the fibrous tissue in the periphery of the graft were all mitotic.

Ovaries transplanted into male recipients showed the highest incidence of dysgenesis. 29 of 32 female gonads transplanted into male pouch young became dysgenetic (Table 1). All 3 surviving ovarian transplants contained structures resembling seminiferous cords, with no signs of ovarian development. Each cord was composed of cells that at the light microscope level resembled Sertoli cells, with nuclei perpendicular to the boundary of the tubule and cytoplasm extending into the lumen (Fig. 2B). However, neither Leydig nor peritubular myoid cells could be identified, since the presence of lymphocytes in the transplanted female gonads made it impossible to distinguish the interstitial tissue. All seminiferous-like cords in these sex reversed ovaries lacked germ cells. Some cords were hollow, where the cytoplasm of the Sertoli cells extended only part-way into the lumen. Occa-

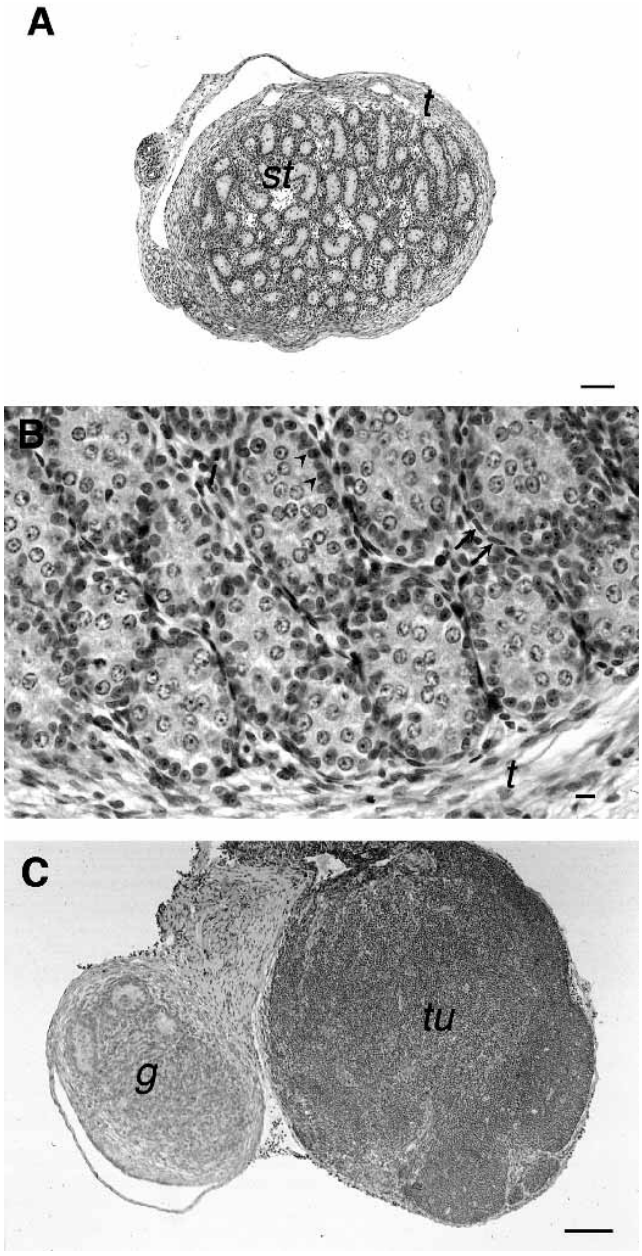

Fig. 1. Structure of testes after transplantation. Similar structures are seen in both male and female recipients. (A) Testis transplants contain numerous closely packed seminiferous tubules (st), and are sheathed in a fibrous tunica albuginea (t). (B) Each seminiferous tubule is composed of Sertoli cells (arrow heads) aligned at right angles to a basement membrane. The cytoplasm of the Sertoli cells extends into the lumina of the tubules and encloses clusters of mitotic germ cells. Peritubular myoid cells (arrows) surround the seminiferous tubules, and Leydig cells are present in the interstitium (i). (C) Pseudolymphomas (tu) developed in association with all transplanted gonads (g). Scale bars 100 μm in A and C; 10 μm in B.

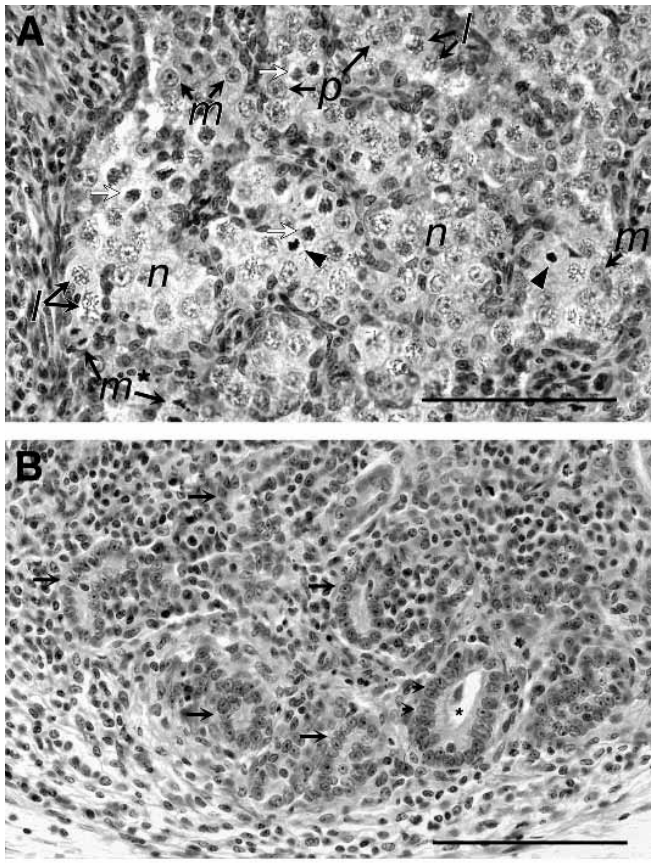

Fig. 2. Structure of ovaries following transplantation into female (A) or male (B) recipients. (A) Section of an ovarian transplant recovered from a female recipient selected to show nests (n) of meiotic germ cells delineated by surrounding mesenchymal cells. The germ cells are in leptotene (l), condensed leptotene-zygotene (white arrows) and pachytene (p). Many germ cells are still mitotic (m; m* metaphase), particularly those at the periphery of the transplant tissue. Some germ cells are atretic (arrow heads). (B) Section of an ovarian transplant recovered from male recipients containing cells that resemble Sertoli cells (arrowheads) and which have become aligned into elongated seminiferous-like tubules (arrows). The voluminous cytoplasm of the Sertoli-like cells extends into the lumina of the tubules, however, the absence of germ cells leaves some tubules hollow (asterisk). The interstitial region of the gonad comprises tumour-like cell (also see Figure 1C). Scale bars 100 μm.

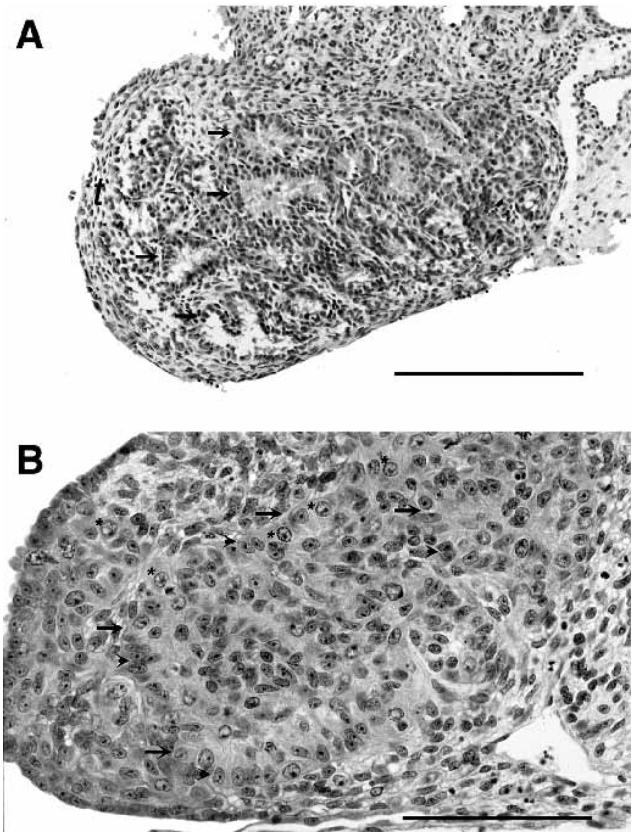

Fig. 3. Effect of culture upon testicular differentiation. (A) Testes from 4-day-old pouch young contain well-organised seminiferous tubules (arrows) and are enclosed in a well-defined tunica albuginea (t). (B) Testes cultured for 4 days in control medium contain Sertoli cells (arrow heads) which are aligned into early seminiferous tubules (arrows). Germ cells (asterisks) are enclosed within the developing seminiferous tubules. Scale bars 50 μm in A; 100 μm in B.

sionally pycnotic cells, which may have been degenerating germ cells, were observed in the lumina of the tubules.

Organ culture experiments

Testicular structure

Gonads from neonatal males maintained in culture for 4 days differentiated as testes, although they were less developed than testes from 4-day-old pouch young (Fig. 3A). No differences in testicular development were observed between testes cultured with mesonephros attached and those cultured without mesonephros. Sertoli cells were clearly distinguishable from other somatic cells by their abundant cytoplasm and were loosely arranged into early seminiferous cords (Fig. 3B). Germ cells were observed in mitosis within the seminiferous cords. Leydig and peritubular myoid cells were not visible, nor was the development of a tunica albuginea evident.

Ovarian structure

Testis-like structures developed in 8 of the 12 female gonads cultured for 4 days with rhMIS (Table 2); 4 of these were cultured with mesonephros attached and 4 without mesonephros. Somatic cells resembling Sertoli cells formed seminiferous-like cords (Fig. 4A), which were better organised than those observed in male gonads cultured for 4 days. The

Table 2. Development of undifferentiated neonatal female tammar gonads with or without attached mesonephros cultured with or without rhMIS for 4 days

		Gonadal development		
Treatment	No.	Ovary	Ovotestis	Testis-like
Cultured with rhMIS				
with mesonephros	7	3	3	1
without mesonephros	5	1	3	1
Cultured without rhMIS				
with mesonephros	7	7	0	0
without mesonephros	5	3	2	0

cords which were elongated, contained very few germ cells and, in many, the cytoplasm of the Sertoli cells did not extend completely into the lumen. The extent to which the female gonads were masculinised varied, with the majority of gonads containing both ovarian and testicular tissue ('ovotestes') (Fig. 4B). Germ cells that persisted were found only in those regions of the gonad that were distinctly ovarian. Two gonads, one cultured with mesonephros and the other without, were devoid of germ cells and consisted entirely of numerous tightly packed seminiferous-like cords (Fig. 4C). A small cluster of pycnotic germ cells was present in the interstitial tissue between the gonad and the mesonephros of one of these explants (Fig. 4D).

The four remaining female gonads exposed to rhMIS but which did not transdifferentiate developed as ovaries with no evidence of seminiferous-like cords (Fig. 4E). The gonadal blastema consisted of a dense cortex in which many mitotically dividing germ cells were found. In contrast, the more fibrous medulla contained relatively few germ cells.

10 of the 12 ovaries cultured as controls in medium without rhMIS developed as ovaries (Table 2). However, 2 of the control ovaries contained seminiferous-like cords histologically similar to those observed in the sex reversed ovaries cultured with rhMIS (Fig. 4F). Both of these gonads contained areas of necrotic tissue and very few germ cells. The few germ cells that persisted were found only in those areas of the gonad devoid of seminiferous-like cords.

DISCUSSION

Undifferentiated female gonads develop seminiferous-like cords in vivo after transplantation into males or after culture with or without MIS in vitro. Loss of germ cells rather than the presence or absence of MIS appears to be the critical factor in ovarian sex reversal. It seems likely that MIS causes ovarian sex reversal because of its toxic effect on female mitotic germ cells.

Ovarian sex reversal can occur in the absence of a masculinising influence such as MIS. Fetal rat ovaries develop seminiferous-like tubules in the absence of MIS when cultured for extended periods (Prépin and Hida, 1989). Furthermore, fetal rat (Buyse, 1935) and mouse (Taketo, 1991; Taketo et al., 1993) ovaries can be induced to form testicular cords equally well when transplanted into female hosts as when placed into male hosts. Thus, although MIS can induce ovarian sex reversal, it is not essential. Indeed, MIS is unnecessary for testicular differentiation since male transgenic mice deficient in MIS can still develop morphologically normal, functional testes (Behringer et al., 1994). It is therefore unlikely that MIS causes differentiation of Sertoli cells. Thus, the greater propensity for ovaries

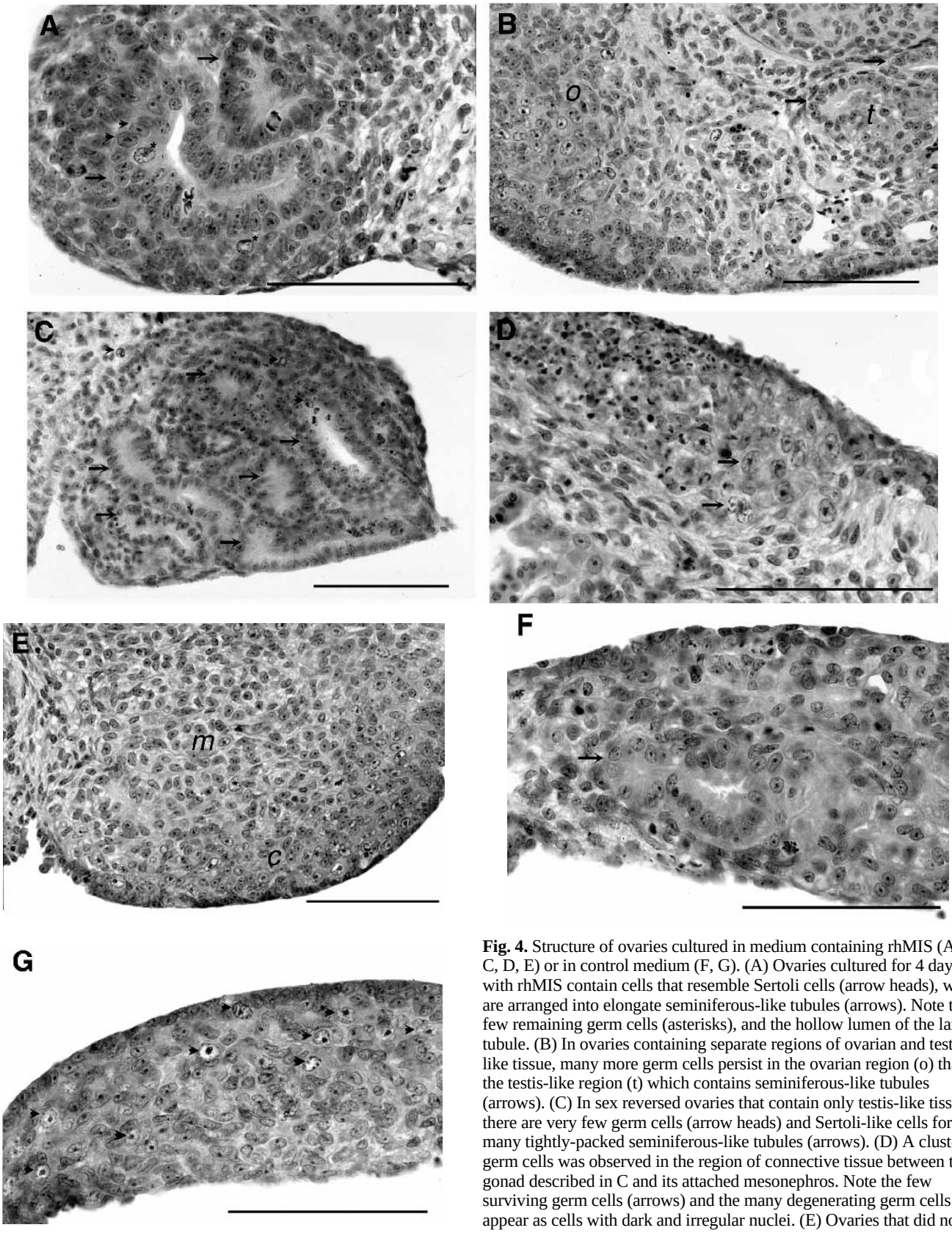

Fig. 4. Structure of ovaries cultured in medium containing rhMIS (A, B, C, D, E) or in control medium (F, G). (A) Ovaries cultured for 4 days with rhMIS contain cells that resemble Sertoli cells (arrow heads), which are arranged into elongate seminiferous-like tubules (arrows). Note the few remaining germ cells (asterisks), and the hollow lumen of the largest tubule. (B) In ovaries containing separate regions of ovarian and testis-like tissue, many more germ cells persist in the ovarian region (o) than in the testis-like region (t) which contains seminiferous-like tubules (arrows). (C) In sex reversed ovaries that contain only testis-like tissue, there are very few germ cells (arrow heads) and Sertoli-like cells form many tightly-packed seminiferous-like tubules (arrows). (D) A cluster of germ cells was observed in the region of connective tissue between the gonad described in C and its attached mesonephros. Note the few surviving germ cells (arrows) and the many degenerating germ cells that appear as cells with dark and irregular nuclei. (E) Ovaries that did not form tubules when cultured with rhMIS continued to develop as ovaries. Note the concentration of germ cells in the cortex (c) of the ovary, with fewer germ cells in the more fibrous medulla (m). (F) Seminiferous-like tubules (arrow) also formed in this control ovary. which contains very few germ cells. (G) The few germ cells (arrowheads) that persisted in the ovary described in F are found in the portion of the ovary that does not contain testis-like structures. Scale bars 100 μm.

to sex reverse in the presence of MIS than in its absence (Vigier et al., 1987, 1989; Prépin and Hida, 1989; Charpentier and Magre, 1990) suggests an indirect action of MIS.

The sex-reversing role of MIS can be attributed to its lethal effect on the female germ cell population. MIS is thought to be toxic to all meiotic germ cells and, in the testis, this may serve to eliminate any germ cells that enter meiosis prematurely (McLaren, 1990). The loss of meiotic germ cells in the ovary would either prevent the somatic cells from differentiating as granulosa cells and forming follicles, or induce transdifferentiation into Sertoli cells of those cells (reviewed in Burgoyne, 1988; McLaren, 1991). Oocytes are also required for the maintenance of follicles and, if the germ cells are lost, the surrounding granulosa cells often transdifferentiate into Sertoli cells. The loss of oocytes alone has been shown to be sufficient to induce ovarian sex reversal. The ovaries of senile rats, which over time have become depleted of germ cells, develop seminiferous-like tubules from the existing follicles (Crummeyrolle-Arias et al., 1976). A similar sex reversal of follicle cells into Sertoli cells has been observed in the mole, *Talpa occidentalis*, in which the ovaries of fertile females contain portions of testicular tissue (Jiménez et al., 1993). The authors suggest that the spherical rather than elongate shape of the seminiferous tubules is indicative of follicles transforming into seminiferous tubules, probably after the loss of oocytes. Thus, in rats, mice and moles, the formation of testis-like structures in the ovary is due to the elimination of the meiotic germ cells responsible for the formation and maintenance of follicles (Burgoyne, 1988; Merchant-Larios and Taketo, 1991; Taketo-Hosotani et al., 1984, 1985; McLaren, 1991).

Sex reversal of the tammar ovary does not entirely fit this model. Testicular cords formed in neonatal ovaries after 4 days in culture (whether MIS was present or not). These testis-like structures cannot be due to the loss of meiotic germ cells since, in neonatal tammars, the oocytes are still mitotic and enter meiosis only after day 24 postpartum. It is unlikely that they are the result of transdifferentiation, because the first primordial follicles do not appear in the tammar ovary until around 50 days after birth and prefollicular basement membrane bound ovarian cords are normally not seen until day 8-10 postpartum (Alcorn, 1975; Alcorn & Robinson, 1983). Seminiferous-like cords in the tammar ovary thus appear to have developed spontaneously in response to the loss of mitotic germ cells from apparently undifferentiated supporting cells. Although the majority of previous studies have described the effects of MIS on meiotic germ cells, Vigier et al. (1987) comment upon the development of testis-like structures before the onset of folliculogenesis in fetal rat ovaries exposed to MIS. They show that MIS inhibited proliferation of mitotic germ cells without killing them, but meiotic germ cells were lost. Charpentier and Magre (1990) describe the formation of seminiferous-like tubules in fetal rat ovaries in culture 3 to 5 days before granulosa cells are recognisable in ovaries in vivo, so the development of seminiferous-like tubules in these ovaries cannot be the result of abnormal folliculogenesis or of transdifferentiation. Similarly, reaggregated fetal mouse ovaries depleted of germ cells before the onset of meiosis and follicle development also develop testis-like structures (Hashimoto et al., 1990). Thus, the tammar data confirms that the loss of germ cells alone, whether mitotic or meiotic, is sufficient to induce ovarian sex reversal. Consequently, as Charpentier and Magre (1990) found in the rat, prefollicle cells are able to differentiate into Sertoli-like cells and form seminiferous-like tubules before the onset of folliculogenesis.

During male development, MIS is produced by the testis at the time that the germ cells are dividing mitotically and it may be involved in the development of gonocytes to type A spermatogonia (Zhou et al., 1993). In contrast, MIS production by the ovary does not commence until long after the germ cells are arrested in meiotic prophase (reviewed in Catlin et al., 1993).

Sex differences between XX and XY germ cells in mammals have been reported extensively previously (reviewed in Short, 1971, 1994; McLaren, 1995), but the toxic effects of MIS on female germ cells has been attributed to their earlier entry into meiosis and not to any intrinsic differences between XX and XY germ cells. Similarly, in the tammar, MIS production by the testis coincides with male germ cell mitosis, and exposure to endogenous MIS of the host had no influence on the male germ cells of grafted testes. In contrast, the XX mitotic germ cells did not survive after grafting into males or after exposure to MIS in culture.

This sex difference in the effect of MIS on germ cells may be the result of intrinsic differences between XX and XY germ cells, or an indirect result of a sex difference in the surrounding somatic cells. In eutherians, sex differences in the somatic cells can lead to differences in germ cell differentiation. Thus, XY germ cells can develop as oocytes in an ovarian situation (Eicher and Washburn, 1986), and XX germ cells can develop as prospermatogonia in a testis (Palmer and Burgoyne, 1991; Burgoyne et al., 1995). In both fetal rat and fetal rabbit, the MIS receptor gene is strongly expressed in pregranulosa but not germ cells, suggesting that MIS acts indirectly on the germ cells via the somatic support cells (Baarends et al., 1994; di Clemente et al., 1994).

The critical factor in the sex reversal of the tammar ovary, as in eutherian mammals, appears to be the loss of germ cells and not any direct effect of MIS on the female somatic cells. However, the tammar data confirm that there is no difference between the loss of meiotic versus mitotic germ cells. The data also support the argument that premeiotic XX germ cells in the tammar neonate act to inhibit the differentiation of Sertoli cells from the supporting cell lineage.

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