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Original Article

The role of the mesonephros in cell differentiation and morphogenesis of the mouse fetal testis

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ABSTRACT In mouse fetal gonads, the adjacent mesonephros is required for seminiferous cord formation *in vitro* (Buehr *et al., Development 117:* 273-281, 1993). Here, we have investigated the role of mesonephric cells in seminiferous cord formation and in differentiation of Sertoli and Leydig cells. Undifferentiated male gonads with and without mesonephros at 11.5 dpc were cultured and immunocytochemical staining of Müllerian-inhibiting substance (MIS) was used as the criterion for Sertoli cell differentiation. For Leydig cells, testosterone (T) radioimmunoassay and cytochemical detection of Δ5-3ß-hydroxysteroid-dehidrogenase (HSD) were undertaken. An ultrastructural study was also performed. In our culture conditions, the timing of differentiation of both Sertoli and Leydig cells was similar to that in the fetus. Although mesonephros is required for seminiferous cord formation, differentiation of Sertoli and Leydig cells proceeds in its absence at 11.5 dpc. Moreover, when 3H-thymidine labeled mesonephroi were grafted to unlabeled gonads, endothelial and peritubular myoid-like cells migrated into the gonad. We propose that these cells might be the mesonephric cells required for seminiferous cord formation.

KEY WORDS: *mouse fetal testis, mesonephros, Sertoli cells, Leydig cells, endothelial cells*

Introduction

The recent demonstration of *Sry* as the testis-determining factor *(Tdy)* (Koopman *et al.*, 1991), and that its encoded product is likely a DNA-binding protein (Harley *et al.*, 1992) which cannot function as a diffusible morphogen, emphasizes the importance of short range cell interactions at the onset of male gonad differentiation. *Sry* is expressed at 10.5 and 11.5 days post coitum (dpc), when gonads of both XX and XY embryos are morphologically alike (Koopman *et al.*, 1990). Two main processes characterize the onset of testis differentiation: seminiferous cord formation, and differentiation of Sertoli and Leydig cells. Since these processes immediately follow *Sry* expression, knowing the relationship between the two processes is required for an understanding of the cascade of events switched on by *Sry*.

At the sexually undifferentiated stage, the mammalian gonad is already a complex structure formed by diverse cell lineages (see Wartenberg *et al.*, 1991 for a recent review). Briefly, most primordial germ cells (PGCs) appear closely associated with epithelial-like cells which begin to deposit basal lamina leading to the segregation of two tissue compartments: epithelial and stromal, (Merchant-Larios and Taketo, 1991).

At the onset of testis differentiation, the epithelial compartment becomes organized as seminiferous cords, due mainly to an increased amount of stromal tissue. Mesenchymal, endothelial and myoid cells form the latter tissue at this stage of development. Since *Sry* is expressed at the undifferentiated stage, it might be responsible for these changes. Burgoyne (1988) has proposed that *Tdy* (now *Sry*) acts cell-autonomously to bring about Sertoli cell differentiation and that all other aspects of fetal testicular development are directed by Sertoli cells. Experimental evidence obtained from rat fetal gonad in organ culture, however, has led other authors to the concept that cell differentiation and morphogenesis in the mammalian gonad might follow separate control mechanisms (Magre and Jost, 1984; Jost and Magre, 1988).

Although the mesonephric contribution to preSertoli cells in the gonad still remains controversial (Wartenberg *et al.*, 1991), a recent experimental study in the mouse has demonstrated that at least some stromal cells (myoid, fibroblast-like cells) have a mesonephric origin (Buehr *et al.*, 1993). Moreover, in this work, it was found that seminiferous cord formation *in vitro* apparently requires the attached mesonephros, since isolated male gonads at 11.5 dpc fail to differentiate cords.

Abbreviations used in this paper: dpc, days post coitum; HSD, Δ5-3ß-hydroxysteroid dehydrogenase; MIS, Müllerian-inhibiting substance; PGCs, primordial germ cells; RIA, radioimmunoassay; T, testosterone; *Tdy*, Testis determining factor.

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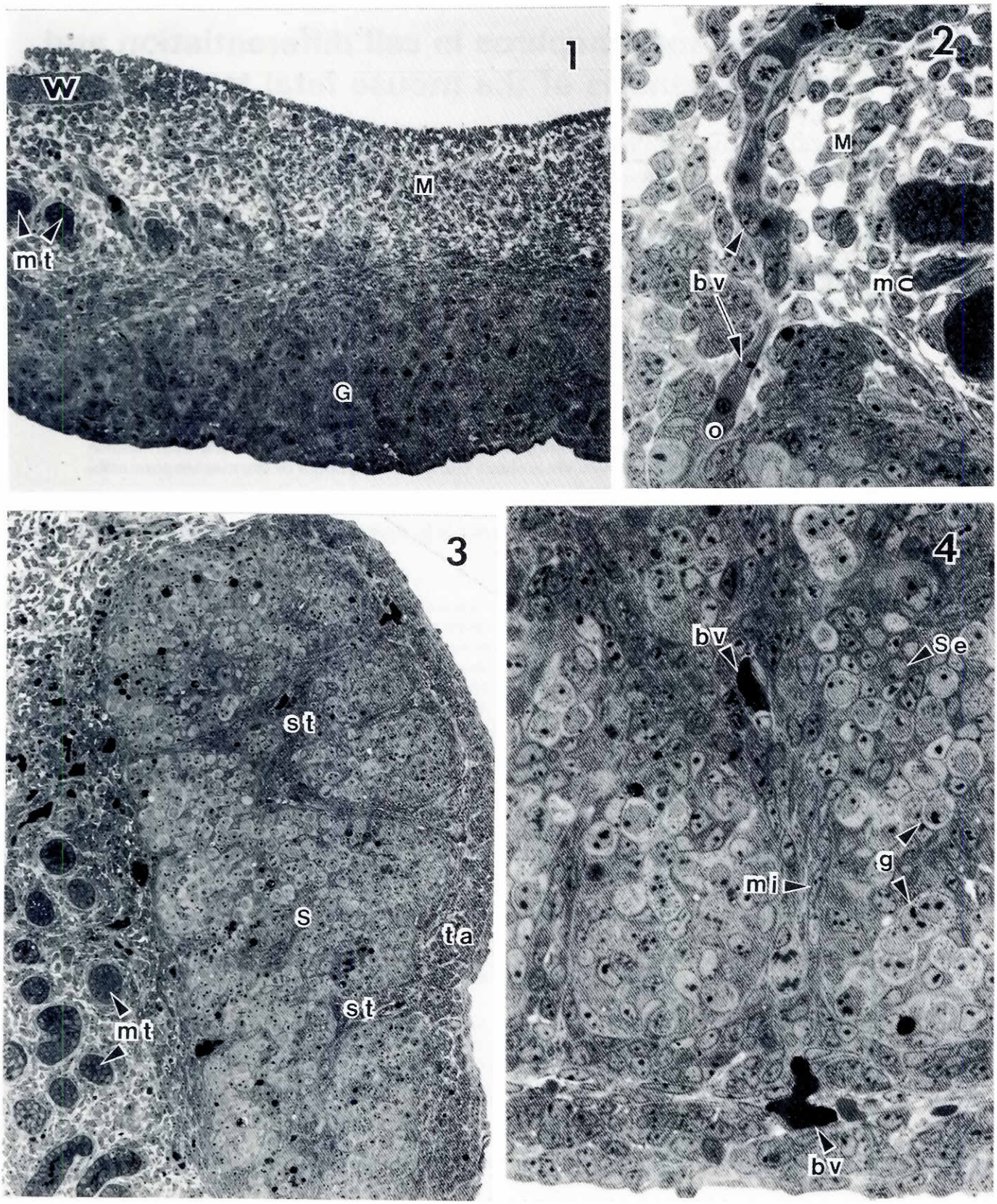
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In the work presented here, we have extended the analysis of the role played by the mesonephros in testis differentiation. Three criteria were considered: formation of seminiferous cords and differentiation of Sertoli and Leydig cells. After undifferentiated gonads with or without mesonephroi were placed in organ culture, immunohistochemical staining for Müllerian-inhibiting substance (MIS) and laminin were used to assess Sertoli cell differentiation and cord formation, respectively. For Leydig cell differentiation, both cytochemical detection of Δ5-3ß-hydroxysteroid dehydrogenase (HSD) activity and radioimmunoassay (RIA) of testosterone (T) were undertaken. Moreover, cells in the isolated mesonephros were labeled with H3-thymidine, and their penetration into grafted testis was studied by autoradiography.

We found that although the mesonephros is required for seminiferous cord formation, Sertoli and Leydig cells differentiate in the gonads without mesonephros. Moreover, since labeled endothelial and myoid cells were detected around the seminiferous cords in grafted gonads, we postulate an active role of these mesonephric cells in normal seminiferous cord formation. Furthermore, since no labeled Leydig cells were found in isolated gonads, we suggest that their precursors are present in the gonadal anlage at 11.5 dpc when the *Sry* is still being expressed.

Results

Morphology of urogenital complexes at 11.5 dpc

When male urogenital complexes were isolated from embryos at 11.5 dpc, when the hind-limb was at stages 4 and 5 (McLaren and Buehr, 1990), the mesonephric and gonadal regions were already clearly distinguishable (Fig. 1). In the former, the Wolffian duct, mesonephric tubules, loose mesenchymal cells and blood vessels were the most conspicuous tissues. On the other hand, the gonadal anlage was formed by a heterogeneous population of densely packaged cells through which some blood vessels from the mesonephric region penetrated (Fig. 2). For cultures of isolated testes, they were separated from the mesonephros and only the densely packaged gonadal tissue was cultured. Observation of this region under the electron microscope revealed that epithelial-like cells closely attached to primordial germ cells began to deposit thin basal lamina which separated them from a densely arranged stromal tissue (data not shown). In the latter, blood vessels and spindle shaped cells were beginning to delineate the epithelial cords.

Time course of gonadal development in culture

Table 1 summarizes the time course of development from mouse testes at 11.5 dpc with or without mesonephros. Seminiferous cord formation detected in semithin sections was clearly defined in the testis with mesonephros after 24 h *in vitro* (Fig. 3). Thereafter, seminiferous cords became completely surrounded by basal lamina to which early myoid-like cells were attached. Blood vessels, fibroblasts and mesenchymal cells in the stromal compartment made the seminiferous cords more conspicuous (Fig. 4). Laminin was strongly detected in basement membranes around the seminiferous cords and irregularly distributed in the stromal compartment (Fig. 5).

TABLE 1

DEVELOPMENT OF FETAL MOUSE TESTIS AT 11.5 dpc IN ORGAN CULTURE

Time in culture (hours)	Condition	MIS	Δ53ß-HSD	Seminiferous cords
12	MG	(+) n= 4	(–) n= 2	(+) n= 4
	G	–	–	–
24	MG	(+) n= 5	(–) n= 2	(+) n= 5
	G	(+) n= 5	(–) n= 2	(+) n= 5
48	MG	(+) n= 3	(–) n= 2	(+) n= 3
	G	(+) n= 2	(–) n= 2	(–) n= 2
72	MG	(+) n= 4	(+) n= 4	(+) n= 3
	G	(+) n= 4	(+) n= 4	(–) n= 3
96	MG	(+) n= 8	(+) n= 8	(+) n= 6
	G	(+) n= 5	(+) n= 5	(–) n= 4

MG, urogenital complexes; G, isolated gonad; n, number of testes.

On the other hand, in the testis without the mesonephros, cord-like formation was rarely found. When it occurred, the presence of stromal tissue was evident (probably due to «mesonephric contamination"). Observations under the light microscope revealed that although some prospermatogonia remained among somatic cells of different shape and density, neither endothelial nor myoid-like cells could be identified (Fig. 6). Laminin was detected in random distribution and no signs of epithelial formation were present (Fig. 7).

Although immunocytochemical staining for MIS was negative in testes at 11.5 dpc before culture and after the first 4 h *in vitro*, low intensity of MIS staining was first detected in Sertoli cells after 12 h of culture (Fig. 8). Thereafter, from 24 h onwards, MIS staining intensity increased and remained intense until 96 h, the time limit in which fetal testes were maintained in culture (Fig. 9).

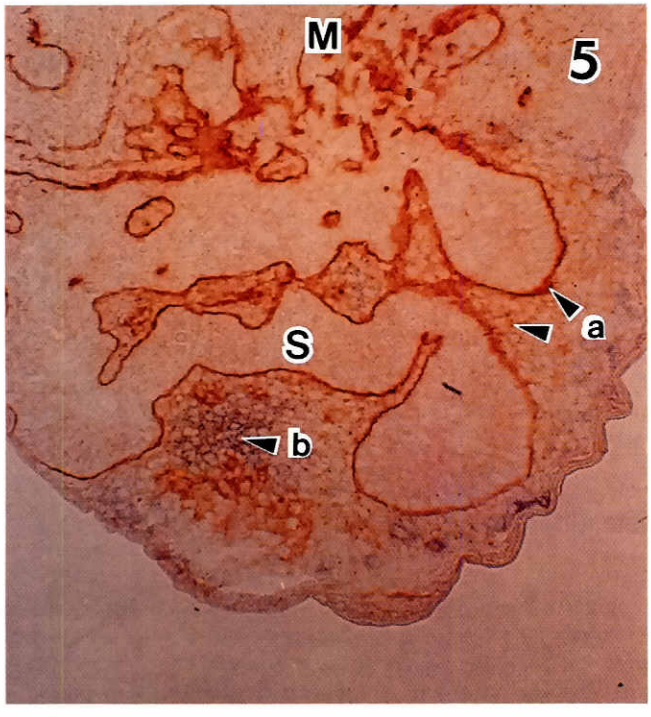
As indicated in Table 1, the ontogeny of MIS stained cells was similar between the testes cultured with or without the adjacent mesonephros. Distribution of MIS positive cells, however, was different depending upon the presence of mesonephros. In urogenital complexes, MIS positive cells were organized regularly into cord-like formations in the gonadal region (Fig. 9). In isolated testes, by contrast, MIS positive cells appeared as irregular clusters in random distribution (Fig. 10).

Fig. 1. Longitudinal section of the urogenital ridge at 11.5 dpc before culture. *Part of the Wolffian duct (W) and some mesonephric tubules (mt) among loose mesenchymal cells are seen in the mesonephric region (M). The gonadal anlage (G) appears as a compact arrangement of somatic and germ cells. (x200).*

Fig. 2. A blood vessel (bv) penetrating into the gonad (G) from the mesonephric region (M). *Part of two mesonephric tubules (mt) can be seen. (x600).*

Fig. 3. Urogenital ridge after 24 h of culture. *Seminiferous cords (S) start to be evident by the presence of stromal tissue (st) around them. The tunica albuginea (ta) and several mesonephric tubules (mt) are also shown. (x200).*

Fig. 4. Well-formed seminiferous cords (SC) in a testis cultured 96 h with the adjacent mesonephros. *Germ cells (g), Sertoli cells (Se), myoid-like cells (mi) and Leydig cells (L) are shown. (x750).*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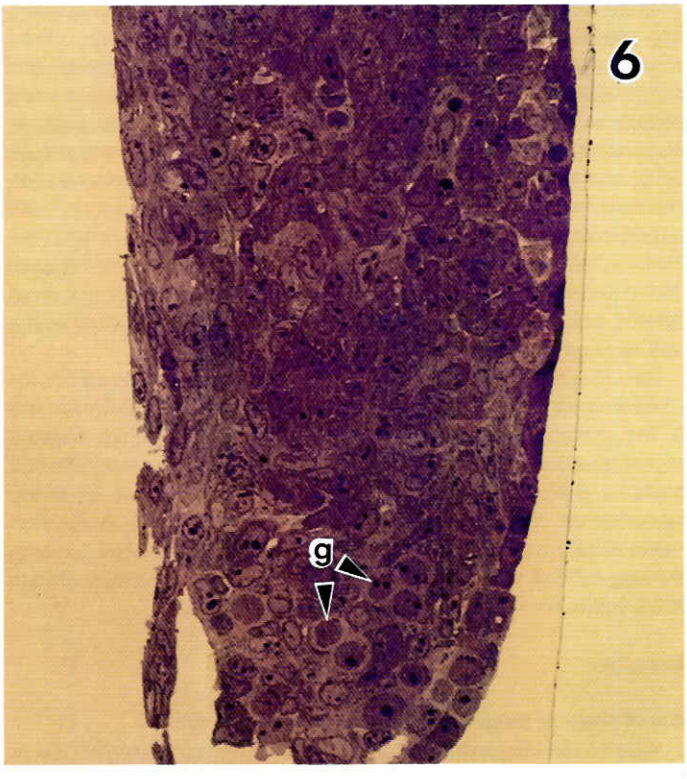
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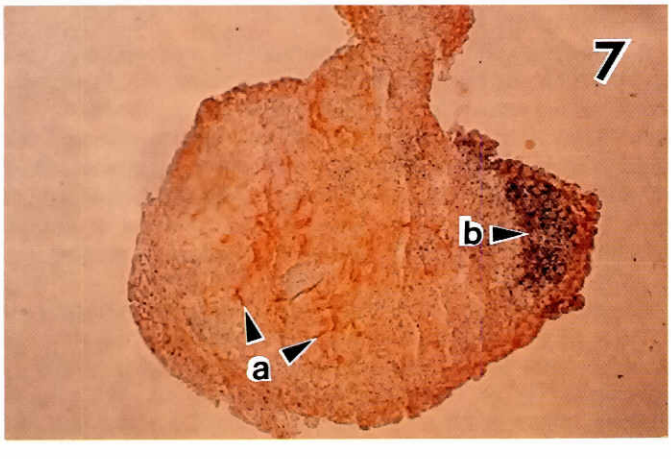
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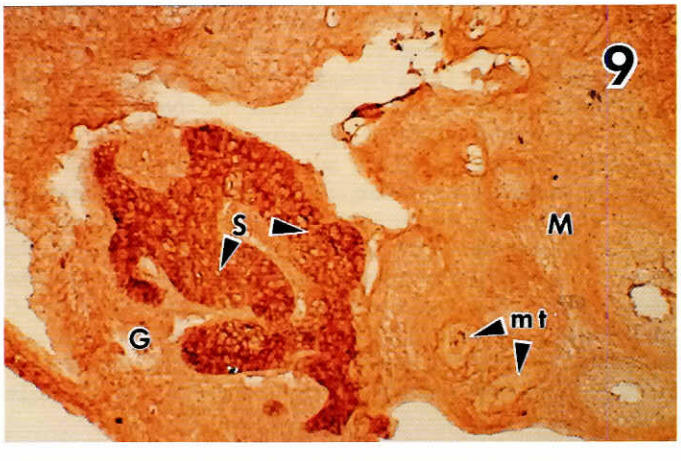
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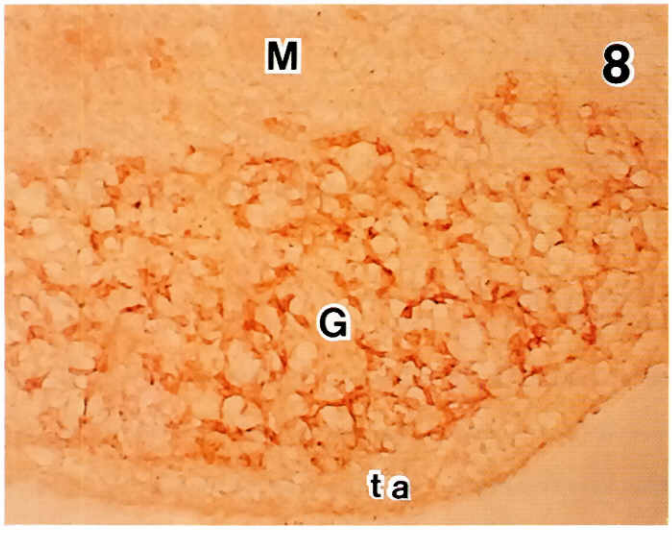
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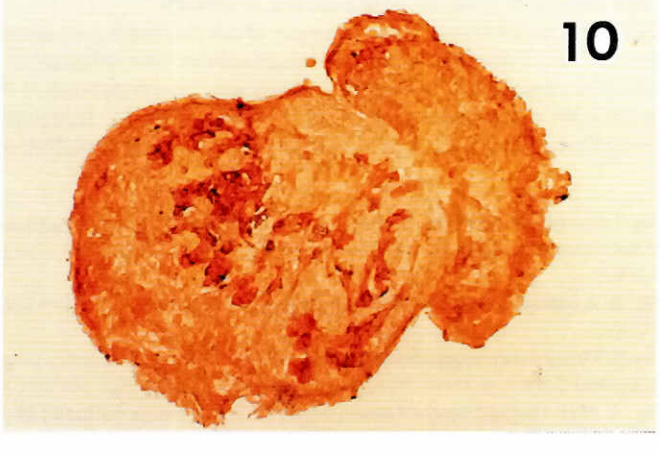
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Δ5-3ß-HSD activity was first histochemically detected after 48 h of culture in both urogenital complexes and isolated testes. This enzymatic activity remained positive in all organs cultured for 72 and 96 h (Table 1). As in the case of MIS, distribution of Δ5-3ß-HSD activity differed between testes cultured with or without mesonephros. In the former, Δ5-3ß-HSD positive cells were localized in stromal cells among the seminiferous cords (Fig. 5). In isolated testes, Δ5-3ß-HSD positive cells appeared to be randomly localized and often formed a cluster on one side of the explant (Fig. 7).

Examinations of the explants at the electron microscope level reaffirmed and extended the observation at the light microscope. Although deposition of basement membrane components around the seminiferous cords began by 11.5 dpc (stages 4-5), its completion occurred *in vitro* only when the adjacent mesonephros was left in the explant (Fig. 11). Otherwise, only uncompleted basement membrane or irregular deposits of its components appeared between somatic cells (Fig. 12). In the explants of urogenital complexes, Sertoli and myoid-like cells were well differentiated after 96 h in culture (Fig. 11). In the isolated testes maintained for the same period *in vitro*, only Sertoli-like and Leydig cells distributed among undifferentiated stromal cells were seen, endothelial and myoid-like cells were not found (data not shown).

Testis-mesonephros grafts

After 4 h exposure to 3H-thymidine in the culture medium, around 30% of cells in the isolated mesonephros were labeled. The least number of labeled cells was found among the epithelial cells of mesonephric tubules (around 10%). Nuclei of mesenchymal and endothelial cells appeared densely labeled (Fig. 13). After 4-6 days of culture, less than 5% cells remained labeled in the grafted explants. Some stayed in the mesonephros and others penetrated into the grafted gonad (Figs. 14 and 15).

The labeled cells were distributed in the stromal compartment and identified as mesenchymal, peritubular myoid-like cells and endothelial cells. The peritubular myoid cells were identified by their attachment to the basement membrane of the seminiferous cords (Fig. 14). Endothelial cells were identified as elongated cells forming blood vessels with a cavity sometimes filled with erythroblasts (Fig. 15).

Radioimmunoassay of testosterone

The levels of T accumulated in culture media steadily increased during incubation from 48 to 96 h (Fig. 16). The effect of mesonephros on testosterone production statistically was not significant (F ratio= 2.86; d.f.= 5.64; $P<0.05$).

Discussion

Studying the development of rat fetal testis *in vitro*, some authors have proposed that differentiation of Sertoli cells, Leydig cells and seminiferous cords in mammalian fetal testis might be controlled by independent mechanisms (Magre and Jost, 1984; Patsavoudi *et al.*, 1985). When rat fetal gonads were cultured in the presence of fetal calf serum, Sertoli and Leydig cell differentiated but seminiferous cords failed to form (Magre *et al.*, 1981). In the mouse fetal gonads cultured in presence of the alpha globin fraction of human serum, Sertoli cells differentiated, but Leydig cells and seminiferous cords were not formed (Jost and Magre, 1988). Furthermore, Taketo *et al.* (1984), studying mouse fetal gonad development in organ culture, found that addition of dibutyryl-cAMP prevents seminiferous cord formation without affecting the physiological differentiation of Leydig cells. Although these observations suggest that differentiation of Sertoli cells, Leydig cells and seminiferous cords can be inhibited independently *in vitro*, the role of the adjacent mesonephros was not considered.

On the other hand, it has been recently found that mouse fetal testes develop better when they are cultured with the adjacent mesonephros than without. It was suggested that this organ may play an important role in differentiation of the male gonad, since isolated male gonads fail to form seminiferous cords in the absence of the attached mesonephros (Buehr *et al.*, 1993).

In the present study it was confirmed that the adjacent mesonephric region is required for the morphogenetic process leading to formation of seminiferous cords in the mouse gonad *in vitro*. Moreover, our autoradiographic analysis demonstrates that at least two kinds of stromal cells penetrate the testis from the mesonephric region: peritubular myoid-like and endothelial cells. Therefore it is reasonable to postulate that these cells and/or their extracellular products are the mesonephric contribution to seminiferous cord formation and indicate why at the onset of testis differentiation this region is required for normal segregation of epithelial and stromal compartments *in vitro*. It would be interesting to see if factors such as alpha globin (Jost and Magre, 1988) and dibutyryl-cAMP (Taketo *et al.*, 1984), which prevent seminiferous cord organization in mouse fetal testis, also disturb the migration of mesonephric stromal cells onto the gonad. Moreover, it has been observed that seminiferous cords form *in vitro* even after dissociation-aggregation of isolated testes at 12.5 dpc (Escalante-Alcalde and Merchant-Larios, 1992). Therefore, the presence of mesonephric cells in the male gonad necessary for cord formation appears to be critical at 11.5 dpc.

Fig. 5. Testis cultured 96 h with the adjacent mesonephros (M). *Detection of laminin (a) and Δ53ß-HSD (b) were performed in the same section. Laminin appears around the seminiferous cords (S) and in some regions of the stromal compartment. Formazan deposits reveal the presence of Leydig cells in the stroma. (x200).*

Fig. 6. Semithin section of a gonad cultured 96 h without the adjacent mesonephros. *Some germ cells (g) randomly distributed among a heterogeneous mass of somatic cells are shown. (x600).*

Fig. 7. Detection of laminin (a) and Δ53ß-HSD activity (b) in an isolated gonad after 96 h of culture. *Irregular deposits of both laminin and steroidogenic cells can be seen. (x200).*

Fig. 8. MIS detection in a male gonad cultured 12 h. *Positive Sertoli cells are in the gonadal region (G) between the mesonephros (M) and the forming tunica albuginea (ta). (x200).*

Fig. 9. Urogenital complex cultured 96 h. Immunohistochemical staining for MIS reveals well-organized seminiferous cords (S) in the gonadal region (G). *In the mesonephric region (M) some mesonephric tubules (mt) can be seen. (x100).*

Fig. 10. Immunohistochemical staining for MIS in an isolated testis cultured 96 h. *Positive cells appear irregularly distributed. (x100).*

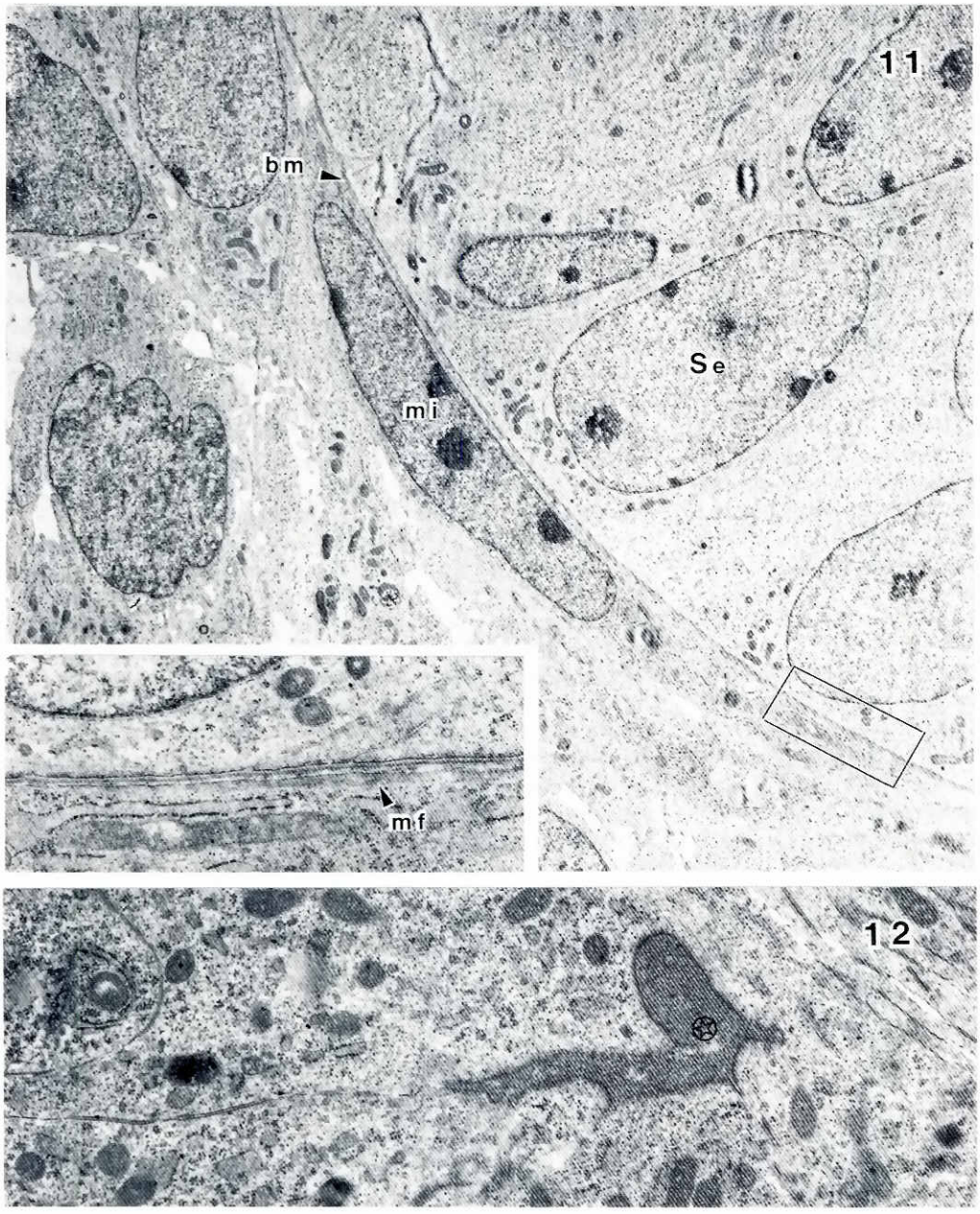


Fig. 11. Electron micrograph taken from a testis cultured 96 h with the adjacent mesonephros. *The deposit of basement membrane (bm) between well-differentiated Sertoli cells (Se) and a peritubular myoid-like cell (mi) is shown. (x7500). The inset is a higher magnification of the area labeled by a rectangle in the lower left corner. It shows the deposit of microfilaments (mf) in the cytoplasm of the myoid-like cell. (x25,000).*

Fig. 12. Electron micrograph showing an irregular deposit of basement membrane material (star) between the cytoplasm of somatic cells. *Isolated gonad cultured 96 h. (x24,000).*

In the present study it was found that around 35% of cells in the isolated mesonephros were labeled with 3H-thymidine in culture. Only a few cells remained labeled (less than 5%) in the grafts after 4-6 days of culture, probably because either most labeled cells died or the label was diluted by cell proliferation. In any case, the fact that the majority of cells that remained labeled in the graft were endothelial indicates that their proliferation occurred in the mesonephric region and that after they penetrated into the gonad they divided less frequently than other labeled mesonephric cells. This interpretation is in line with the concept that the angiogenic process involves at least two major independent components: locomotion and proliferation of endothelial cells (Folkman and Klagsbrun, 1987). Thus, at the onset of testis differentiation, a signal from the as yet morphologically undifferentiated male gonad might induce penetration of blood vessels, which in turn might guide other cells such as myoid precursors from the mesonephros into the gonad in order to shape the seminiferous cords. Two other lines of circumstantial evidence support our interpretation: i) At the onset of sex differentiation (12.5 dpc), a male gonad is first distinguished from a female by its higher vascularisation (Merchant-Larios and Taketo, 1991). ii) Endothelial and myoid cells were absent in cultures of isolated gonads which failed to form seminiferous cords (Buehr *et al.*, 1993; and present observations).

In the rat testis, it has been found that the basement membrane surrounding the seminiferous tubules is probably produced in concert by the Sertoli and peritubular myoid cells (Skinner *et al.*,

1985). Under culture conditions peritubular myoid cells produce both laminin and fibronectin, whereas Sertoli cells produce only laminin (Davis *et al.*, 1990). In the present study we found that although laminin was produced in isolated testis, formation of a basement membrane was impaired. Therefore, this failure might be due to the absence of peritubular myoid cells from the mesonephric region.

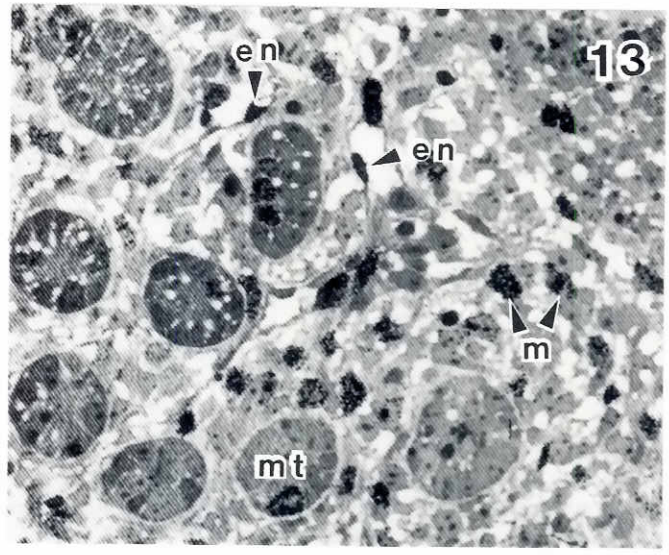
Our time course study has shown that Sertoli cells and Leydig cells differentiated *in vitro* almost at the same age as in the embryo. Moreover, the presence of the adjacent mesonephros did not have any influence on either the timing or differentiation of these two important cell lineages. In our study, in the urogenital complexes or isolated gonads at 11.5 dpc (stages 4-5; McLaren and Buehr, 1990), the first Sertoli cells detected by immunohistochemical staining for MIS appeared very soon, after 12 h in culture. This time course appears to be comparable to that of the male gonads *in vivo*. Intensity of the staining increased after 24 h both *in vivo* and *in vitro*, the only remarkable difference being that in isolated gonads, Sertoli cells were distributed as irregular clusters of different sizes and cord-like formations were rarely found.

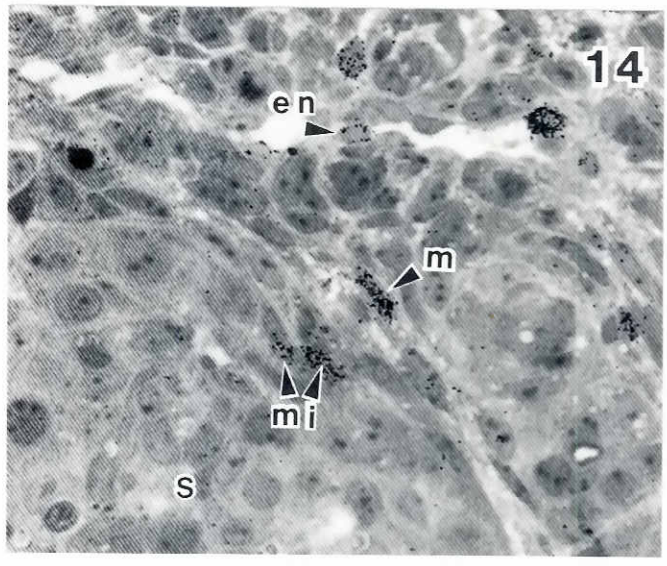
Using *in situ* hybridization, Münsterberg and Lovell-Badge (1991) have shown that MIS transcripts are present in Sertoli cells of mouse fetal testis at 12.5 dpc. Taketo (1991) using immunocytochemical staining also has demonstrated that MIS is first detected at 12.5 dpc. Hence, it appears that translation of MIS closely follows transcription of its gene. Taking MIS expression as evidence of Sertoli cell differentiation, our present study demonstrates that seminiferous cord organization is not required for Sertoli cell differentiation.

Leydig cell differentiation in mouse fetal testis has been studied by several criteria: cytochemical (Hitzemann, 1962), ultrastructural (Russo and de Rosas, 1971) and morphological (Vergouwen *et al.*, 1991). In all these reports, Leydig cells appear in the stromal compartment after seminiferous cord formation. Moreover, although Taketo *et al.* (1991a) have reported that mouse fetal testis secrete significant amounts of testosterone at 12.5 dpc, a correlation between morphological and physiological differentiation of Leydig cells during ontogeny of mouse testes has not been made.

Although Leydig cells appear in the stromal compartment, our present observations suggest that their precursors did not enter the gonad from the mesonephric region after 11.5 dpc as other stromal cell precursors do. Thus, Leydig cell precursors are among the densely packaged cells of the gonadal anlage at 11.5 dpc, when *Sry* is still being expressed (Koopman *et al.*, 1990).

It has been established that «*Sry* acts specifically within cells of the developing indifferent gonads to cause their differentiation along the testicular pathway» (Lovell-Badge, 1992). PreSertoli cells





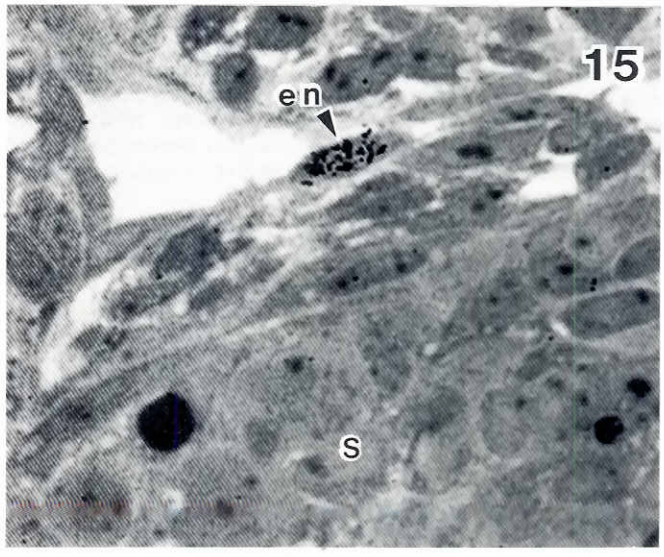


Fig. 13. Autoradiography of an isolated mesonephros cultured 6 h with 3H-thynidine. *A high percentage of cells are labeled. Some epithelial cells in the mesonephric tubules (mt), mesenchymal cells (m) and endothelial cells (en) can be identified. (x200).*

Fig. 14. This graft shows several labeled cells in the stromal tissue. *Endothelial (en), mesenchymal (m) and peritubular myoid-like cells (mi) can be identified. Seminiferous cord (S). (x750).*

Fig. 15. Autoradiography of a male gonad grafted to a 3H-thymidine labeled mesonephros and cultured 96 h. *A labeled endothelial-like cell (en) appears as an elongated cell facing a cavity. (x900).*

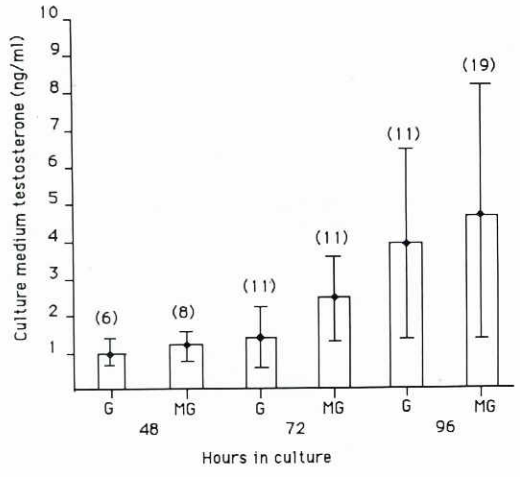


Fig. 16. T production from testicular explants at 11.5 dpc. *G= isolated gonad. MG= gonad+mesonephros. Each column indicates the mean value+SD. The number in parenthesis indicates the number of explants examined. Statistical parameters: F. ratio= 2.86; d.f.= 5.64; P<0.05.*

have been proposed as the gonadal cells in which *Sry* is expressed and regulates the production of factors (ligand or receptor) required for differentiation of Sertoli cells. According to the present results, expression of angiogenic factors in male undifferentiated gonads may be an important event in the cascade initiated by the *Sry* gene.

Materials and Methods

Animals

Two mouse strains, CD-1 and MF1 were used in this study. The latter strain was used only for the grafting experiments. Females were sacrificed at mid-day of the 11th day after the vaginal plug was found (the day of the vaginal plug was defined as day postconception, dpc) and so the embryos were considered to be at 11.5 dpc. The hind-limb bud was used as a criterion of developmental stage according to McLaren and Buehr (1990), and only embryos at stages 4 and 5 were used (stage 19 of Theiler, 1989). Since at 11.5 dpc mouse gonads were as yet morphologically undifferentiated, sex was determined by the presence or absence of the sex chromatin in amniotic cells, following the method of Burgoyne *et al.* (1983).

Organ culture

Each gonadal explant from male embryos was cultured with or without the adjacent mesonephros on a Nucleopore filter floating on 0.5 ml culture medium as described previously (Taketo and Koide, 1981). The basic culture medium was McCoy 5a modified (Microlab, México) containing 10% fetal calf serum (Sigma, USA), 200 UI/ml penicillin G sodium, and 200 µg/ml streptomycin sulfate.

Cultures were maintained for intervals of 12, 24, 48, 72 and 96 h at 37°C in 0.5% CO_2/95% air. The number of explants used for the different determinations is shown in Table 1.

Light and electron microscopy

At the end of the culture the explants were fixed in Karnovsky's aldehyde solution (1965) without Ca^{++} (pH 7.4), postfixed with 1% OsO_4 in Zetterqvist's buffer (1956), and embedded in Epon 812. Semithin (1 µm) and thin (60-90 nm) sections were stained with toluidine blue and uranyl acetate and lead citrate, respectively.

Histochemistry and immunocytochemistry

Cultured explants were embedded in O.C.T. medium (Tissue-tek), frozen at -70°C (dry-ice/acetone), and serial sections of 7 µm thickness were cut with a cryostat. After air-drying on microscope slides (covered with Vectabond, Vector, USA) overnight, sections were incubated at 37°C with dehydroepiandrosterone as a substrate (Levy *et al.*, 1959). After washing in PBS, the slides were mounted in glycerol and examined by light microscope. To verify the formation of seminiferous cords in the same sections, the coverslide was carefully removed and then processed for immunohistochemical detection of laminin, as follows: the sections were incubated for 30 min in 0.3% H_2O_2 in methanol and transferred to the reagents for immunocytochemistry according to the instructions of Vectastain ABC Kit (Vector, USA). The primary antibody against laminin was diluted 1:500 with bovine serum albumin in PBS.

For MIS detection, the explants were fixed in Zamboni's solution (Zamboni and DeMartino, 1967) and processed according to the method of Taketo *et al.* (1991b). Briefly, after fixation, tissues were washed and stored in 7% sucrose in PBS (0.1 M, pH 7.4) at 4°C overnight. The tissues were then incubated in increasing concentrations of polyethyleneglycol (PEG, mol wt, 400) at room temperature. After four changes in PEG (mol wt, 1000) at 47°C, the explants were embedded in PEG (mol wt, 1450). Five to eight sections (7 µm thick) from each sample were floated on distilled water and treated with 0.1% Triton X-100 in water for 5 min, and then transferred to the reagents for immunocytochemistry according to the instructions of Vectastain ABC Kit (Vectastain Lab, USA). As negative controls, sections were incubated in non-immune rabbit serum instead of primary antiserum.

Antibodies

Polyclonal anti-laminin antibody was purchased from Sigma (USA). Rabbit polyclonal antibody against MIS was prepared from human recombinant MIS, according to the method previously described (Ueno *et al.*, 1989).

Grafting experiments and autoradiography

The mesonephric region was cut away from the gonadal region with a fine needle, following the method described by Buehr *et al.* (1993).

Care was taken to cut sufficiently proximal to ensure that no gonadal tissue remained attached to the mesonephros. For labeling the mesonephric cells, isolated mesonephroi were placed in 2.5 µCi/ml of 5'-3H-thymidine (Amersham, UK) in culture medium for 4-6 h. After washing twice with culture medium containing 0.1 mg/ml of cold thymidine, the mesonephroi were grafted to unlabeled isolated gonads. The two organs were laid side by side and pressed gently into a narrow groove cut in the agar block on which the tissue rested. Any graft pair that had not fused after 24 h in culture was discarded. Grafts were cultured at 37°C in an atmosphere of 5% CO_2 in air for 4-6 days, and then fixed and embedded for high resolution microscopy as the cultured explants described above.

For autoradiography, semithin sections (1.5 µm) were placed on slides, and then covered with Ilford K5D liquid emulsion. After exposure for three weeks, they were developed with Kodak D-19 and stained after fixation (Kodak rapid fixative). Geimsa stain diluted 10% in PBS at 50°C for 20 sec was used to stain the sections through the photographic emulsion.

Radioimmunoassay for T

Testosterone secreted for each explant into culture medium was determined by radioimmunoassay using a kit from Diagnostic Systems Laboratories, Inc. (USA). The limit of sensitivity of testosterone assay was 0.01 ng/ml. Statistical analysis was performed using one-way analysis of variance. Tukey test was used to compare individual mean differences.

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