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Fertile females of the mole *Talpa occidentalis* are phenotypic intersexes with ovotestes

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SUMMARY

We investigated the origin of XX sex reversal in the insectivorous mole *Talpa occidentalis*. Cytogenetic, histological and hormonal studies indicate that all XX individuals analyzed from two different populations are true hermaphrodites, with ovotestes. This suggests that XX sex reversal may be the norm in this species. The intersexes are functional fertile females and the trait is transmitted and maintained in the population. Intersexes lack the Y chromosome gene *SRY* (sex determining region Y gene), shown to be the testis determining gene. These results suggest that XX intersex moles may have arisen from a mutation of a gene located downstream from *SRY/TDY* in the testis determining pathway.

Key words: mammalian sex determination, sex reversal, true hermaphroditism, ovotestis, fertility, *ZFY*, *SRY*, *Talpa occidentalis*

INTRODUCTION

In mammals, the male phenotype depends on the presence of a Y chromosome. A gene on the Y chromosome known as *TDF* (testis determining factor) induces the undifferentiated gonads to develop into testes. In the absence of the Y chromosome, ovaries are formed and the female phenotype develops. Deviations from this pattern result in total or partial sex reversal (XX males and XY females or intersexes, respectively), which have been described in a variety of mammals, including mice and humans (see review by Lyon et al., 1981).

In a previous article (Jiménez et al., 1988), we described a new case of XX sex reversal in an insectivorous mole *(Talpa occidentalis)* and reported a very high frequency of individuals with abnormal sexual development. Most of the individuals analyzed showed intersex features, including a female external phenotype and evident bilateral ovotestes. Two other individuals examined in this study were XX males.

In some mammalian species, XY females can be fertile, e.g., mares (Sharp et al., 1980) and the rodents *Myopus schisticolor* (Fredga et al., 1976) and *Microtus cabrerae* (Burgos et al., 1988). However, all XX males and most intersexes described (Selden et al., 1984) are sterile because of the degeneration of germinal cells. Hence, the situation in *T. occidentalis* is unlikely to persist in a natural population if all the affected individuals are sterile. It is also unlikely that such a high frequency of abnormal individuals should result from independent mutational events in normal males or females.

We report here that most if not all XX individuals of this species possess ovotestes and behave as functional fertile females. Thus, incomplete XX sex reversal may be the normal condition in *T. occidentalis*, and appears to be transmitted by each female to its XX offspring. Histological and ultrastructural studies of testicular tissue developed in the absence of Y chromosome sequences are of special interest with regard to the steps in testis determination which lie downstream from *TDY* action, as they will tell us which testicular structures can be formed under these circumstances.

MATERIALS AND METHODS

Individuals analyzed and anatomical features

A total of 218 moles were trapped live in Vega de Granada and 5 others in Alcudia de Guadix (Granada province, Spain), 131 of which showed the male phenotype (anus and meatus clearly separated, presence of penis, testes, epididymis and seminal vesicles), whereas the other 87 individuals showed the female external phenotype (anus and meatus together with or without an evident vaginal opening between them), and internally had a uterus with two branches ending in more or less evident ovotestes.

Estimation of fertility

For males, fertility and sexual activity were estimated, as described elsewhere (Jiménez et al., 1988), on the basis of testis weight (average of both testes), diameter and development of seminiferous tubules, sperm content of the epididymis and relative age. In females, sexual activity and fertility were judged by the condition of the vulva, the development of the uterus and mammary glands, the existence of oogenesis in gonads and the presence or absence of embryos.

Estimation of age

In each particular animal, age was estimated by calculating a dental wear index, according to our method (Jiménez et al., 1988).

Karyotyping

Chromosome preparations were made to investigate sex chromosome constitution. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, concanavalin A (Sigma) at the final concentration recommended by the supplier for each particular batch, and 0.1 μg/ml colchicine, for 1 hour at 37°C. This was followed by hypotonic treatment with 0.56% KCl, then cells were fixed in methanol: acetic acid, (3:1) and spread onto cold wet slides. Chromosomes were G-banded according to our usual method (Burgos et al., 1986).

Histological and electron microscope preparations

In males, both testes and epididymes were fixed in Bouin's fluid. In females, the uterus, vagina and gonads were fixed in the same manner. Gonads were dehydrated, embedded in paraffin wax (Paraplast), cut in serial sections 5-10 μm thick and conventionally stained with haematoxylin and eosin. In two animals, the entire reproductive tract was serially sectioned from the lower vagina to the gonads, in order to search for ontogenic Wolffian duct structures. In addition, semithin sections were cut from thick slices fixed in Bouin's fluid and stained with toluidine blue. These thick slices were obtained from the centralmost plane through the longitudinal axis of the gonad.

In three XX and two XY individuals, gonads were separated from the oviducts, cut into small pieces (1 mm^3, approximately), which were classified according to their location within the gonad, prefixed in 1:1 2% paraformaldehyde/2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) and postfixed in 1% OsO_4 in 0.2 M S-collidin for 2 hours.

Fixed pieces were embedded in Epon 812 and thin sections were stained with uranyl acetate and lead citrate. Observations were made at 60 kV with a Zeiss EM 10C transmission electron microscope at the University of Granada Technical Services. Some semithin sections were also obtained from these pieces and stained with toluidine blue.

Serum testosterone concentrations

42 individuals (25 males and 17 females) were killed by cervical dislocation and blood was immediately extracted from the heart. After incubation at 37°C during 30 minutes for coagulation, blood was centrifuged at 1500 ***g*** during 10 minutes and the serum was stored at −80°C until use. Serum testosterone concentrations were measured by radioimmunoassay (RIA) using commercially available kits Sorin. Duplicate measurements were made for all animals in the same RIA. The intraassay coefficient of variation was 7%.

Southern blots

Southern blot analyses were done on genomic DNA extracted from lymphocytes. DNA was digested with restriction enzymes and run on an 0.8% agarose gel, then transferred to Hybond N^+ (Amersham). Two probes were used in these experiments: *ZFY*, a gene of unknown function on the Y chromosome (Page et al., 1987), and *SRY* (Sinclair et al., 1990), which is known to be the testis determining gene (*TDF*) on the Y chromosome. Probes were labelled with [^{32}P]dCTP, then hybridized to the filters; the filters were washed at high stringency and exposed to X-ray film overnight.

RESULTS

Sex features and fertility

Males were invariably XY with no sign of abnormal sexual development. However, as described elsewhere (Jiménez et al., 1990), marked variability was observed in all parameters relating to fertility. This was due in some cases to seasonal fluctuations in sexual activity and to youth in some individuals.

All XX individuals had a female external phenotype, although internally most of them clearly showed bilateral ovotestes. Furthermore, histological examination demonstrated that all XX individuals had some testicular tissue in their gonads, and could therefore be considered true hermaphrodites (see below). Six of these XX phenotypic intersexes were found to be functional females, as two of them (T-141 and T-176) were pregnant (Fig. 1) and the other four (T-78, T-106, T-185, T-195) were suckling. In these individuals, the uterus was dilated and was receiving an abundant blood supply. The rest of the XX individuals (81) showed variable features in their reproductive tracts. 33 of them were juvenile animals (one year old or younger) with a small, poorly vascularized uterus, whereas the rest (48) were adult individuals (1-5 years old) in which the uterus was enlarged and well vascularized only during the period of sexual activity (from October to June; see Jiménez et al., 1990). This age distribution was consistent with that proposed by other authors for the related species *Talpa europaea* (see Lodal and Grue, 1985).

Regardless of whether the individual was fertile, wide variations were observed in the size of the ovotestes. As a result of variability, the weight of the ovotestes varied considerably between individuals, (74.9 mg in T-336 and 3.4

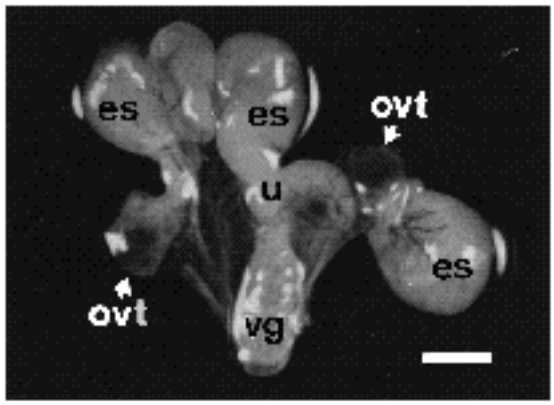

Fig. 1. Anatomy of the reproductive organs in a pregnant, phenotypically intersex female mole (T-176). Two embryonic sacks (es) on the left and one on the right branch of the uterus (u), and two large ovotestes (ovt) are visible; (vg) vagina. Scale bar, 5 mm.

mg in T-108 were the extreme values). Regardless fertility, most of the reproductive tracts of these phenotypic true hermaphrodites contained normally developed Müllerian duct derivatives such as the vagina, uterus and oviducts (Fig. 2A-C), although these individuals also showed Wolffian duct derivatives, such as bilateral rudimentary epididymes (Fig. 2D,E). These were variable in size and appeared generally as a relatively short, slightly folded epididymal tube, closely associated to the gonadal envelope by connective tissue, including adipose tissue. The degree of development varied between individuals, from relatively complex (Fig. 2D) to very thin and short (Fig. 2E). The epididymal nature of these tubes was shown by their location near the testicular pole of the ovotestis, as well as by the features of the cells in the tubular wall (Fig. 2F). These were cuboid epithelial cells projecting ciliae in the lumina, similar to those forming the wall of the epididymal tube in normal XY males (not shown). These epididymes lacked an outlet at the non-gonadal end, due to the absence of a ductus deferens. No sign of this structure was found in the two individuals whose entire urogenital tract was serially sectioned.

In all XX animals, gonads were bilateral ovotestes in the position of the normal mammalian ovary. These ovotestes were composed of a small portion of morphologically normal ovarian tissue and a variably sized portion of abnormal testicular tissue (Fig. 3A). In general, testicular tissue predominated and was not separated from the ovarian region by a continuous border of connective tissue.

The gonadal envelope varied depending on the subjacent gonadal tissue. On the testicular portion, a typical tunica albuginea was present (Fig. 3B). This was composed of a multilayer of flattened epithelial cells with abundant collagen fibres, as observed in electron microscope preparations (not shown). Ovarian tissue was covered by a monolayer of prismatic cells giving rise to a typical ovarian epithelium (Fig. 3C).

The ovarian tissue of these ovotestes developed normally, as expected in individuals behaving as functional fertile females (Fig. 3A). Numerous primary and secondary follicles and one or more Graffian follicles were observed in the ovotestes of all mature females during the period of sexual activity. In contrast, immature juvenile females contained only primordial follicles.

The testicular portion of the ovotestes was composed of a mass of testicular tissue organized into lobules separated by connective tissue trabeculae (Fig. 3A) and a variably developed rete testis, generally located near the nonovarian pole of the gonad, in the vicinity of the epididymis (Fig. 3D). The rete testis was composed of many confluent tubules of normal appearance with evident lumina, which gradually increased in diameter as they neared the tunica albuginea. These tubules, as well as those of the epididymes, were always empty, though not obliterated. Occasionally, these conducts were partially filled with cell debris, as seen in normal epididymes from XY males during the period of testicular inactivity (from July to September, see Jiménez et al., 1990).

Testicular tissue contained numerous, generally small seminiferous cord-like structures immersed in an abundant, dense matrix of interstitial tissue (Fig. 3A). Serially sectioned ovotestes showed these structures to be very short solid cords or spheres. The diameter of the sexual cords was

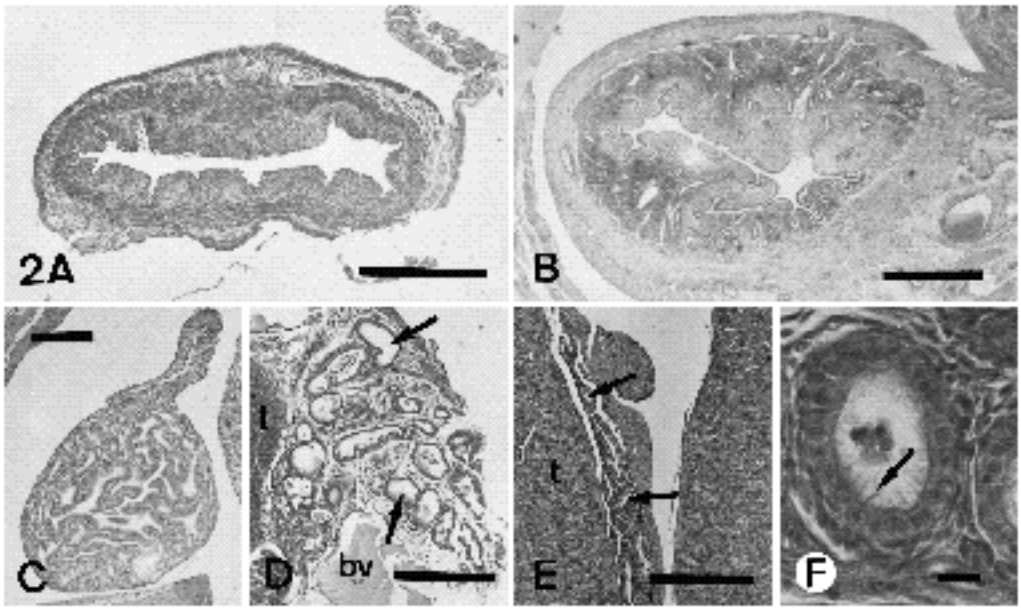

Fig. 2. Haematoxylin and eosin-stained transversal histological sections of Müllerian (A-C) and Wolffian (D-F) duct derivatives in adult females of *T. occidentalis*. Vagina (A), uterus (B) and oviduct (C) in pregnant female T-176, showing normal morphology.
(D,E) Abnormal epididymes showing different degrees of development on the basis of the number of tubular sections (arrows), which are abundant in D and scarce in E; (t) testicular tissue, (bv) blood vessel. (F) Higher magnification of an epididymal tube showing the internal epithelium formed by a monolayer of cuboid cells projecting cilia toward the lumina (arrow). Scale bars, 1 mm in A, 500 μm in B and C, 250 μm in D and E, 10 μm in F.

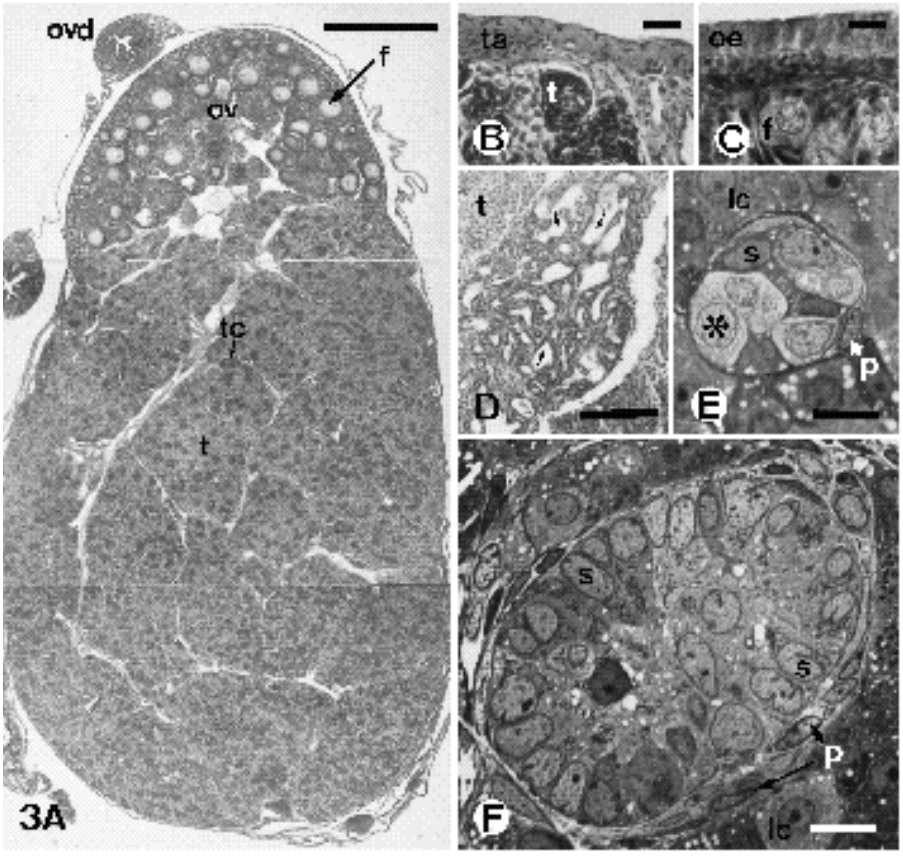

Fig. 3. Histology of the ovotestes in *T. occidentalis* females. (A) Haematoxylin and eosin-stained longitudinal section of an ovotestis in a non-pregnant, non-suckling female. A relatively small portion of normal-appearing ovarian tissue (ov) with numerous primordial and growing follicles, and a large portion of abnormally developed testicular tissue (t) are visible; (ovd) oviduct, (f) ovarian follicle, (tc) testicular cords. (B) Higher magnification of the tunica albuginea (ta), located on the testicular tissue (t) and composed of a multilayer of flattened cells. (C) Detail of the typical ovarian epithelium (oe) formed by a monolayer of prismatic cells; (f) ovarian follicle. (D) Rete testis showing variable sized ducts (arrows), located at the nonovarian pole of the testicular portion of the ovotestis (t). (E,F) Toluidine blue-stained semithin sections of small (E) and large (F) testicular cords, located at the nonovarian and ovarian poles of the testicular portion of the ovotestis, respectively. Note that normal Sertoli cells (s) are present in both cases, whereas degenerating Sertoli cells (*) are seen only in E; (p) peritubular myofibroblasts, (lc) Leydig cell-based interstitial tissue. Scale bars, 250 μm in A, 10 μm in B, E and F, 5 μm in C, 100 μm in D.

variable; they were generally larger near the ovarian pole of the gonad. Diameter gradually decreased toward the nonovarian pole, where they were almost completely disorganized in many animals (Fig. 3E,F). In both poles, sexual cords were completely covered by loosely arranged, flattened cells similar to the myofibroblast forming the envelope of the seminiferous tubules in normal XY male testes (Fig. 3E,F). A more detailed quantitative study of the variation in the sexual cords and the size of the testicular portion of the ovotestes will be published elsewhere.

Histological examination of the three embryos in the pregnant female T-176 showed that one of them (T-178) had readily identifiable developing testes (Fig. 4A), whereas the other two embryos (T-177 and T-179) showed no sign of testicular differentiation (Fig. 4B), although they were as voluminous as the testes in T-178. Consequently, T-178 would probably have developed as an XY male, whereas T-177 and T-179 may have become XX phenotypic intersexes/functional females. The presumed chromosome constitution of these embryos was confirmed by the Southern blot data (see below). Individual T-141, which was the other pregnant female analyzed in this study, had three underdeveloped embryos with no sign of sex differentiation, so that they did not provide any relevant information.

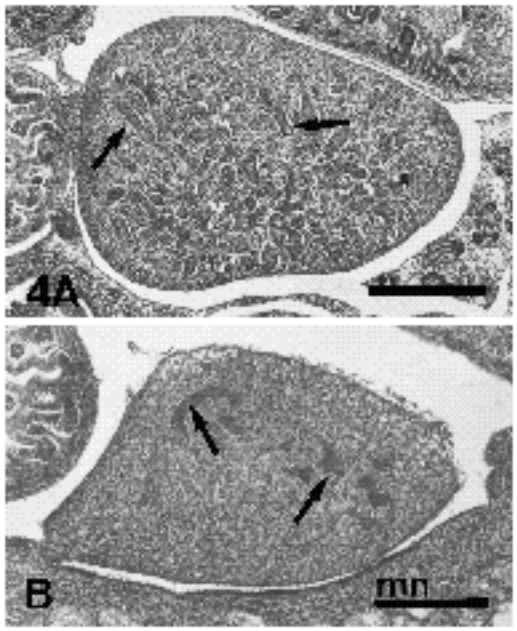

Fig. 4. Haematoxylin and eosin-stained transversal histological sections of developing gonads in *T. occidentalis* embryos. (A) Normal appearing testis in XY male embryo T-178 showing advanced development. Elongated, solid testicular cords (arrows) are clearly visible. (B) Gonad from an XX sister (T-177) of T-178, at the same embryonic stage. Note that no differentiated structure is yet apparent, with the exception of large blood vessels (arrows); (mn) mesonephros. Scale bars, 250 μm.

Electron microscopic observations

Among the cells that participated in the organization of the sexual cords, sustentacular cells, whose features generally coincided with those of the immature Sertoli cells, predom-

inated. These cells defined the outer surface of the sexual cords, which was very regular and continuous from one cell to the next (Fig. 5A). The basal lamina was well formed and externally covered by peritubular cells, which emitted long, thin cytoplasmic processes that completely covered the sexual cord, isolating the latter from the interstitial tissue (Fig. 5A).

As in normal testes, in the sexual cords of the ovotestes of *T. occidentalis*, Sertoli cells also established abundant tight junctions. Zonulae adherens and gap junctions were frequent in the regions of contact between Sertoli cells (Fig. 5A). However, Sertolian specialized junctions were absent between these cells, so that they resembled fetal Sertoli cells.

Sertoli cells contained relatively abundant smooth and rough endoplasmic reticulum regularly dispersed throughout the cytoplasm, markedly elongated mitochondria containing a very dense matrix, and lipid droplets (Fig. 5A). The nuclear outline was typically irregular, showing deep infoldings. The nucleus contained little heterochromatin, which was mostly accumulated along the nuclear membrane.

Another type of cell in the sex cords was clearly identifiable by its large size, large, round nucleus and clear cytoplasm (Fig. 5B). Several features in these cells suggested that they were Sertoli cells undergoing autolysis. The cytoplasm was swollen and almost devoid of organelles, and contained abundant cell debris as well as large vacuoles. The plasma membrane was frequently digested over extensive lengths. The nucleus was also swollen and contained several heterochromatic clusters regularly distributed throughout the nucleoplasm. Due to the increased size

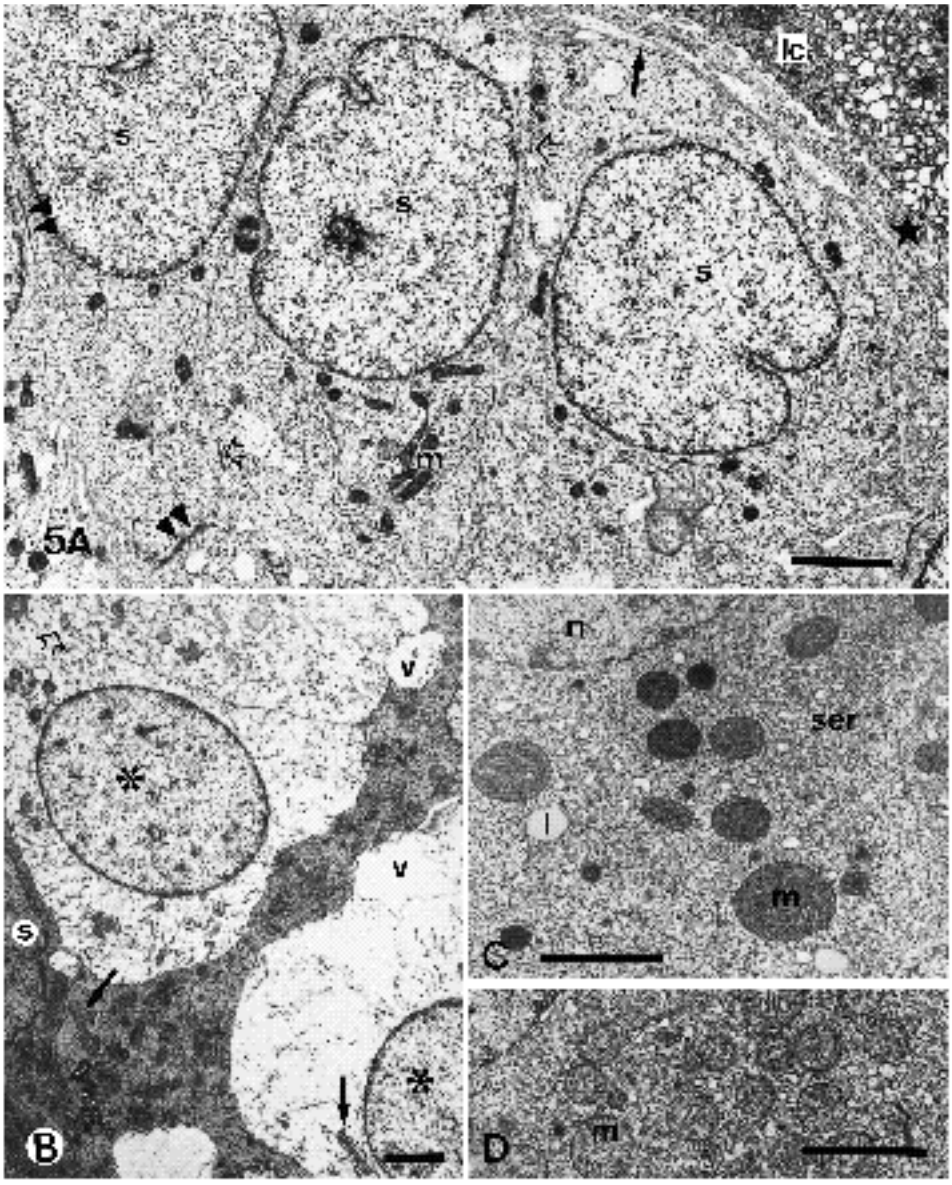

Fig. 5. Ultrastructure of testicular tissue in the ovotestes of *T. occidentalis* XX fertile females. (A) Electron micrograph of a portion of testicular cord at the ovarian pole of the gonad. The cord is composed of Sertoli cells (s) and completely surrounded by a well-formed basal lamina (arrow) and by cytoplasmic processes from myofibroblasts (star). The testicular cord is thus isolated from the Leydig cell-based (lc) interstitial tissue. Sertoli cells have a typically irregular nuclear outline, elongated mitochondria with a dense matrix (m), and abundant smooth and rough endoplasmic reticulum; (open arrows) zonulae adherens, (double arrowheads) gap junctions. (B) Electron micrograph of a portion of testicular cord located at the nonovarian pole of the ovotestis. Two types of Sertoli cells are clearly distinguishable. Degenerating, autolytic Sertoli cells (asterisks) show a swollen, round nucleus and clear cytoplasm almost devoid of organelles, with large vacuoles (v) and a plasma membrane frequently digested over extensive lengths (open arrow). Sertoli cells of the other type (s) are compressed, with a darker than normal cytoplasm. Associations between elongated mitochondria and cisternae of rough endoplasmic reticulum (arrows) are a common cytological feature in both Sertoli cell types in these testicular cords. (C) Electron micrograph of a Leydig cell from the ovarian pole of the testicular portion in the ovotestis. The nucleus (n) is round and the cytoplasm contains many large, round mitochondria (m) with tubular cristae and a dense matrix, lipid droplets (l), and abundant smooth endoplasmic reticulum (ser). (D) Part of a Leydig cell from the nonovarian pole. Mitochondria (m) are generally smaller and clearer than those shown in C, and lipid droplets are less abundant. Scale bars, 5 μm.

of these cells, they compressed the adjacent Sertoli cells, whose cytoplasm became darker than normal. The arrangement of the stretched and flattened Sertoli cells about the degenerating cells recalled the way Sertoli cells surround germinal cells in the seminiferous tubules of normal testes. The identification of these cells as degenerating Sertoli cells is strongly supported by the existence of the same cytological marker in both Sertoli cell types: a longitudinal association between mitochondria and one or two cisternae of rough endoplasmic reticulum (Fig. 5B).

Germinal cells were absent in the sexual cords of the testicular tissue in the ovotestes of *T. occidentalis.*

Finally, interstitial tissue was composed mainly of Leydig cells that were practically indistinguishable from those of normal testes (Fig. 5C). The abundant cytoplasm in these cells was densely filled with numerous organelles. The smooth endoplasmic reticulum was well developed and composed of numerous small tubules and vesicles evenly distributed throughout the cytoplasm. Lipid droplets were abundant and the nucleus was generally round, with a single and large nucleolus and several peripheral heterochromatic clusters. The large, round mitochondria bore tubular cristae and a dense matrix. In the nonovarian pole (Fig. 5D), the number of lipid droplets was markedly lower and mitochondria were smaller and showed a clear matrix with fewer tubular cristae, features suggesting reduced functionality.

Scope of the trait in the species *Talpa occidentalis*

To investigate whether sex reversal in *T. occidentalis* was exclusive to the population in Vega de Granada, we analyzed five individuals from a population in Alcudia de Guadix, on the assumption that these two populations, located about forty kilometres apart, have been physically and genetically separated since the formation of the Sierra Nevada mountains. Two of the individuals from the later population were XX intersexes similar to those described above. The other three individuals were normal XY males.

Serum testosterone

The presence of abundant, morphologically normal Leydig cells in the testicular portion of the ovotestes in XX individuals of this species suggested the possibility of testosterone production, which was confirmed by RIA for serum testosterone detection. Table 1 summarizes the results of this study. Interestingly, some females, especially juveniles captured during the period of sexual inactivity and with the largest ovotestes, showed serum testosterone levels that were even higher than those of adult males captured during the same period. In addition, wide variations were detected in hormone concentrations in males and females, depending on both age and capture period. However, because variation was also high within each particular group, little can be concluded about the evolution of testosterone production throughout the year or the animal's life. In any case, it seems clear that whereas in males the hormone levels paralleled the sexual activity cycle as expected, in females, the opposite process seemed to occur. Furthermore, correlation analyses between gonadal weights and serum testosterone concentrations yielded a very interesting finding: surprisingly, hormone levels were better correlated with ovotestis weight in females (R=0.866; P<0.001) than with testicular weight in males (R=0.417; P=0.038). This good correlation clearly suggests that the testosterone detected in females is mainly produced by the testicular portion of the ovotestes, as this is the only gonadal component responsible for the variability in ovotestis size (our unpublished data). Hence, testosterone is probably produced by the Leydig cells present in this testicular tissue.

Southern hybridizations

Southern blot analyses showed that XY males of *T. occidentalis* had a single copy of both the *ZFY* and *SRY* genes, which were lacking in XX individuals (Fig. 6). This demonstrates that both *ZFY* and *SRY* genes are Y linked in this species. As in other mammalian species (Page et al., 1987), the X chromosome of *T. occidentalis* carries a *ZFY*-related sequence, which has been referred to as *ZFX*. These results clearly show that the XX individuals of *T. occidentalis* lack the *SRY* gene, and hence the testis determining region of the Y chromosome.

DISCUSSION

Nature of the sex reversal in *Talpa occidentalis*

The most striking finding in the present study is the absence

Table 1. Testosterone concentrations (ng/ml) measured by RIA in serum from individuals of *T. occidentalis*

Sex	Capture period	Age group[a]	n	Gonad weight[b]	Testosterone (ng/ml)
				Ovotestes	
Females	Sexual[c] activity	juvenile	5	7.09±2.03	0.61±0.49
		adult	7	20.51±4.87	1.16±0.79
	Sexual[d] inactivity	juvenile	2	55.20±35.28	5.50±4.38
		adult	3	22.27±3.99	2.62±2.76
				Testes	
Males	Sexual activity	juvenile	5	37±35.46	1.15±0.74
		adult	15	307±65.73	12.60±11.40
	Sexual inactivity	juvenile	1	60	3.9
		adult	4	117.5±76.65	4.9±5.33

[a]Animals were considered juvenile (one year old or younger) or adult (1-5 years old) on the basis of their dental wear index and anatomical sex features.
[b]Values given are the combined weights of both gonads (ovotestes in females and testis in males). Means and standard deviations are shown.
[c]From October to June.
[d]From July to September.

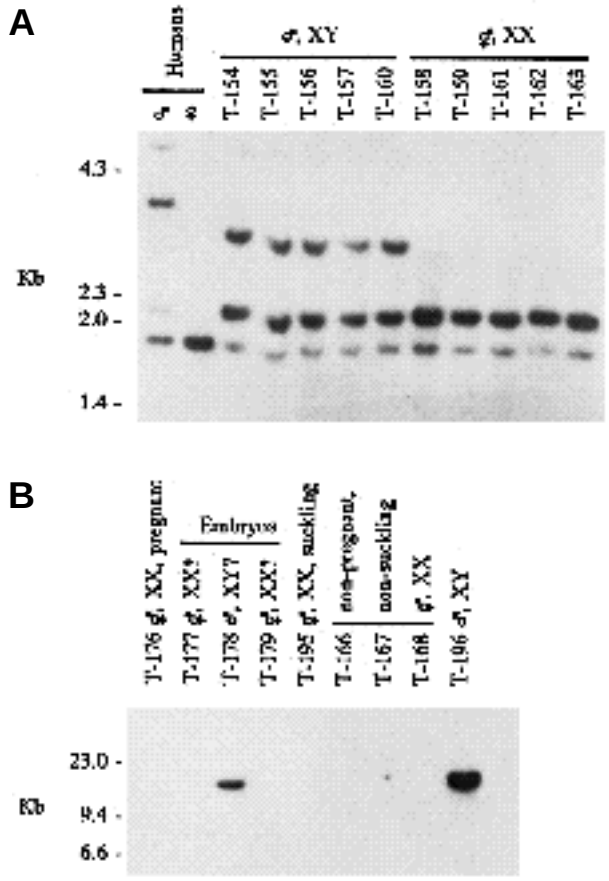

Fig. 6. Southern blot analysis of genomic DNA from XX and XY individuals of *Talpa occidentalis* using Y-chromosome-specific probes. (A) *Eco*RI-digested DNA samples hybridized with the *ZFY* probe. A single strong band is detected in XX intersexes, whereas two lighter ones are found in XY males, one of them of the same molecular weight as the band from intersexes. This demonstrates the existence of one copy on the Y chromosome (*ZFY*) and another related sequence (*ZFX*) on the X. Lanes 1 and 2 show the human *ZFY* pattern for comparison. (B) *Hin*dIII-digested DNA samples hybridized with the *SRY* probe. Only XY males (embryo T-178 on lane 3 and an adult male on lane 9) show a single band. *SRY* sequences are therefore exclusive for the Y chromosome in *T. occidentalis.*

of phenotypically normal females in a mammalian species, a situation that has not been described before. From a structural point of view, testicular tissue in the ovotestes of *T. occidentalis* intersexes showed normal features, with two exceptions: (1) Sertoli cells showed a fetal-like morphology and failed to form true sexual cords, which were generally very short or spherical, and (2) germinal cells were absent. In fact, the rest of structures and cell types forming the abnormal testicular tissue (Leydig cells, peritubular myofibroblasts, rete testis and the complex vascularized testicular connective tissues, including the tunica albuginea) appeared to be normally differentiated, showing features very similar to those of XY male moles (not shown) or those described for human and other mammalian species (Nistal and Paniagua, 1983; Nistal et al., 1986).

Our observations also raise questions about the functionality of these cell types. Although serum testosterone in females could be attributed to low aromatase activities due to impaired ovarian differentiation affecting theca cells, several features rule out this possibility in *T. occidentalis*. (1) Aromatase activity would be expected to be normal in individuals behaving as fertile females. (2) Serum testosterone levels are not correlated with the size of the ovarian portion, but with the testicular portion of the ovotestes. (3) The morphology and location of cells forming the interstitial tissue in the testicular portion of these ovotestes are inconsistent with the characteristics of theca steroidogenic cells. Hence, Leydig cells in the ovotestes of this species probably produce testosterone and are therefore well differentiated, not only in morphology but also in function. In addition, this hormone is needed for the formation of structures such as the epididymes, which, although incompletely developed, are present in all the XX individuals analyzed. Moreover, it is currently assumed that Sertoli cells trigger the differentiation of the rest of testicular structures, including the Leydig cell-based interstitial tissue, the peritubular basal membrane formed by myofibroblasts and the tunica albuginea (see Burgoyne et al., 1988). The existence of such structures in the testicular portion of the ovotestes in *T. occidentalis* females would suggests that the Sertoli cells in these individuals may also be functional. Otherwise, we would have to accept that Leydig cell differentiation, for example, is not Sertoli cell dependent.

In our opinion, all these features demonstrate that a portion of testicular tissue is formed in the gonads of all XX individuals of this species, which nevertheless behave as normal fertile females.

However, this raises an apparent conflict with our earlier data (Jiménez et al., 1988), based on the analyses of individuals from the same population, in which 11 of them were considered normal females. In fact, all 87 XX moles analysed in the present study had ovotestes, but showed different amounts of testicular tissue. This variability makes it very difficult to determine the condition of ovotestis in those gonads where the amount of testicular tissue is very small. Thus, such ovotestes looked like normal ovaries upon macroscopic examination. This is the probable cause of the discrepancy in the data, as not all gonads in the earlier study were histologically analyzed.

Because *SRY* is thought to be expressed only in the supporting cell lineage of the gonadal primordium, leading to the differentiation of Sertoli cells and the subsequent formation of testicular tissue in XY individuals (see Burgoyne et al., 1988; Palmer and Burgoyne, 1991a,b; Burgoyne and Palmer, 1993; Patek et al., 1991), XX sex reversal is considered to occur in those cases in which XX Sertoli cells are present. This is the case of *T. occidentalis*, in which all XX individuals have a portion of testicular tissue in their gonads, where the Sertoli cells are likely to be differentiated.

The cytogenetic and histological observations presented here clearly demonstrate that the Y chromosome of *T. occidentalis* contains genetic information that is essential for normal and complete development of testicular structure and function. However, testicular tissue was also present in the gonads of all XX individuals, although it was incompletely developed. Southern blot analyses showed that these XX

intersexes lack Y-derived sequences associated with the testis determining gene *SRY*. This situation differs from that described in *Sxr* mice (Cattanach et al., 1982; McLaren and Monk, 1982) and in some human XX males and hermaphrodites (Guaellaen et al., 1984; Page, 1986; Goodfellow and Darling, 1988). In these XX individuals, the male phenotype is a result of Y-derived sequences that have been transferred to the X chromosome by crossing-over during male meiosis. As has been described for most affected humans (Berkovitz et al., 1992), the situation in *T. occidentalis* corresponds to a case of XX true hermaphroditism, in which maleness occurs in the absence of Y-derived genes.

Possible origin of sex reversal in *T. occidentalis*

Histological examination of embryos showed that testicular tissue in the ovotestes is formed later than that in normal male testes. This would suggest that XX Sertoli cells are the result of a process of transdifferentiation of the follicular cells in the absence of *TDF*. XX Sertoli cells have been found in freemartin gonads (Jost et al., 1975), mouse ovaries grafted to male kidneys (Taketo-Hosotani et al., 1985), ageing rat ovaries (Crumeyrolle-Arias et al., 1976, 1986), fetal rat ovaries cultured in the presence of Anti-Müllerian Hormone (AMH) (Vigier et al., 1987) and juvenile ovaries from mice transgenic for human AMH (Behringer et al., 1990). In all these cases, it has been demonstrated that, when oocytes disappear from the ovaries, follicles can transdifferentiate into testis cord-like structures. This change may be caused by AMH, which, during normal embryonic development in the male, inhibits the Müllerian ducts, thus precluding the formation of female sexual organs such as the oviducts, uterus and upper vagina (Tran and Josso, 1982). During the transdifferentiation process, AMH might act either directly on follicular cells (Behringer et al., 1990) or indirectly by depleting oocytes (McLaren, 1990).

However, because all intersexes of *T. occidentalis* retained their Müllerian duct derivatives, it is clear that AMH did not act during fetal stages when Müllerian ducts are sensitive to AMH. Nevertheless, the fact that very young, immature intersexes showed wide variations in the number of primordial follicles, such that some of them had very few oocytes before sexual maturity (unpublished data), strongly suggests that oocyte depletion indeed occurs to variable degrees in different individuals. This in turn suggests that Sertoli cells probably arise late in gestation, after the formation of ovarian tissue.

This hypothesis is consistent with other features in these ovotestes, such as the abnormal development of epididymes, as Leydig cells are probably formed late, or the abnormal testicular cord morphology, as an original arrangement of supporting cells around an oocyte in a follicle may explain the spherical instead of elongated shape of these cords. Studies of the embryonic stages of gonadal development in this species are needed to determine whether oocyte depletion in fact takes place and, if so, when germ cells are lost and when transformation to testicular tissue occurs. It will then be easier to look for explanations for other striking features in these ovotestes, such as the variation in the amount of testicular tissue, the polarity in the organization of testicular cords and the cell degeneration processes that appears to affect Sertoli and Leydig cells.

The *SRY* gene on the Y chromosome induces the indifferent embryonic gonad to develop as a testis. Hormonal output from the testis results in a male phenotype. However, many other non-Y-linked genes are probably involved in the testis-determining pathway. A mutation in one of these genes resulting in constitutive expression could produce the XX sex reversal observed in *T. occidentalis*. Because the trait was present in all XX individuals analyzed in two different populations, such a mutation is probably fixed in this species.

Elucidating the genetic basis for ovotestis development in the XX fertile female moles (sp. *T. occidentalis*) may increase our understanding of mammalian testis development and sex determination.

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REFERENCES

Behringer, R. R., Cate, R. L., Froelick, G. J., Palmiter, R. D. and Brinster, R. L. (1990). Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. *Nature* **345**, 167-170.

Berkovitz, G. D., Fechner, P. Y., Marcantonio, S. M., Bland, G., Stetten, G., Goodfellow, P. N., Smith, K. D. and Migeon, C. J. (1992). The role of the sex-determining region of the Y chromosome (SRY) in the etiology of 46,XX true hermaphroditism. *Hum. Genet.* **88**, 411-416.

Burgos, M., Jiménez, R. and Díaz de la Guardia, R. (1986). A rapid, simple and reliable combined method for G-banding mammalian and human chromosomes. *Stain Technol.* **61**, 257-260.

Burgos, M., Jiménez, R. and Díaz de la Guardia, R. (1988). XY females in *Microtus cabrerae* (Rodentia, Microtidae). *Cytogenet. Cell Genet.* **49**, 275-277.

Burgoyne, P. S., Buehr, M., Koopman, P., Rossant, J. and McLaren, A. (1988). Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX-XY chimeric mouse testes. *Development* **102**, 443-450.

Burgoyne, P. S. and Palmer, S. J. (1993). Cellular basis of cell determination and sex reversal in mammals. In *Gonadal Development and Function* (ed. S. G. Hiller), pp. 17-30. New York: Raven Press.

Cattanach, B. M., Evans, E. P., Burtenshow, M. D. and Barlow, J. (1982). Male, female and intersex development in mice of identical chromosome constitution. *Nature* **300**, 445-446.

Crumeyrolle-Arias, M., Scheib, D. and Ascheim, P. (1976). Light and electron microscopy of the ovarian interstitial tissue in the senile rat: Normal aspect and response to HCG of 'deficiency cells' and 'epithelial cords'. *Gerontology* **22**, 185-204.

Crumeyrolle-Arias, M., Zaborski, P., Scheib, D., Latouche, J. and Ascheim, P. (1986). Differentiation of Sertoli-like cells in senescent ovaries of both intact and hypophysectomized rats and its relation to ovarian H-Y antigen expression. In *Modern Trends in Aging Research*, vol. **147**, (eds. Y. Courtois, B. Faubeux, B. Forette, D. L. Knook and J. A. Tréton), pp. 117-120. John Libbey Eurotext Ltd.

Fredga, K., Gropp, A., Winking H. and Frank, F. (1976). Fertile XX and XY-type females in the wood lemming (*Myopus schisticolor*). *Nature* **261**, 255-257.

Goodfellow, P. N. and Darling, S. M. (1988). Genetics of sex determination in man and mouse. *Development* **102**, 251-258.

Guaellaen, G., Casanova, M., Bishop, C., Geldwerth, D., Andre, G., Fellous, M. and Weissenbach, J. (1984). Human XX males with single copy DNA fragments. *Nature* **307**, 172-173.

Jiménez, R., Burgos, M., Caballero, L. and Díaz de la Guardia, R. (1988). Sex reversal in a wild population of *Talpa occidentalis* (Insectivora, Mammalia). *Genet. Res.* **52**, 135-140.

Jiménez, R., Burgos, M., Sánchez, A. and Díaz de la Guardia, R. (1990). The reproductive cycle of *Talpa occidentalis* in the southeastern Iberian Peninsula. *Acta Theriol.* **35**(1-2), 165-169.

Jost, A., Perchellet, J. P., Prépin, J. and Vigier, B. (1975). The prenatal development of bovine freemartins. In *Symposium on Intersexuality* (ed. R. Reinborn) pp. 392-406. Berlin: Springer-Verlag.

Lodal, J. and Grue, H. (1985). Age determination and age distribution in populations of mole (*Talpa europaea*) in Denmark. *Acta Zool. Fenn.* **173**, 279-281.

Lyon, M. F., Cattanach, B. M. and Charlton, H. M. (1981). *Mechanisms of Sex Differentiation in Mammals* (ed. C. R. Austin and R. G. Edwards), pp 329-386. New York: Academic Press.

McLaren, A. (1990). Of MIS and the mouse. *Nature* **345**, 111.

McLaren, A. and Monk, M. (1982). Fertile females produced by inactivation of an X chromosome of 'sex reversed' mice. *Nature* **300**, 446-448.

Nistal, M. and Paniagua, R. (1983). The postnatal development of the human Sertoli cells. *Z. mikrosk-anat. Forsch.* **97**, 739-752.

Nistal, M., Paniagua, R., Regadera, J., Santamaría, L. and Amat, P. (1986). A quantitative morphological study of human Leydig cells from birth to adulthood. *Cell. Tissue Res.* **246**, 229-236.

Page, D.C. (1986). Sex reversal: Deletion mapping the male determining function of the human Y chromosome. *Cold Spring Harbor Symp. Quant. Biol.* **51**, 229-235.

Page, D. C., Mosher, R., Simpson, E. M., Fisher, E. M. C., Mardon, G., Pollack, F., McGillivray, B., Chapelle de la A., and Brown, L. G. (1987). The sex-determining region of the human Y chromosome encodes a finger protein. *Cell* **51**, 1091-1104.

Palmer, S. J. and Burgoyne, P. S. (1991a). XY follicle cells in the ovaries of X0/XY and X0/XY/XYY mosaic mice. *Development* **111**, 1017-1019.

Palmer, S. J. and Burgoyne, P. S. (1991b). In situ analysis of fetal, prepuberal and adult XX-XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. *Development* **112**, 265-268.

Patek, C. E., Kerr, J. B., Gosden, R. G., Jones, K. W., Hardy, K., Muggleton-Harris, A. L., Handyside, A. H., Whittingham, D. G. and Hooper, M. L. (1991). Sex chimaerism, fertility and sex determination in the mouse. *Development* **113**, 311-325.

Selden, J. R., Moorhead, P. S., Koo, G. C., Wachtell, S. S., Haskings, M. E. and Patterson, D. F. (1984). Inherited XX sex reversal in the cocker spaniel dog. *Hum. Genet.* **67**, 62-69.

Sharp, A. J., Wachtell, S. S. and Benirschke, K. (1980). H-Y antigen in a fertile female horse. *J. Reprod. Fertil.* **58**, 157-160.

Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A. M., Lovell-Badge, R. and Goodfellow, P. N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346**, 240-244.

Taketo-Hosotani, T., Merchant-Larios, H., Thau, R. and Koide, S. S. (1985). Testicular cell differentiation in fetal mouse ovaries following transplantation into adult male mice. *J. Exp. Zool.* **236**, 229-237.

Tran, D. and Josso, N. (1982). Localisation of anti-Müllerian hormone in the rough endoplasmic reticulum of the developing bovine Sertoli cells using immunocytochemistry with a monoclonal antibody. *Endocrinology* **111**, 1562-1567.

Vigier, B., Watrin, F., Magre, S., Tran, D. and Josso, N. (1987). Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it in vitro. *Development* **100**, 43-55.

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