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Original Article

Abnormal sex - duct development in female moles: the role of anti - Müllerian hormone and testosterone

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ABSTRACT We have performed a morphological, hormonal and molecular study of the development of the sex ducts in the mole *Talpa occidentalis*. Females develop bilateral ovotestes with a functional ovarian portion and disgenic testicular tissue. The Müllerian ducts develop normally in females and their regression is very fast in males, suggesting a powerful action of the anti - Müllerian hormone in the mole. RT - PCR demonstrated that the gene governing this hormone begins to be expressed in males coinciding with testis differentiation, and expression continues until shortly after birth. Immunohistochemical studies showed that expression occurs in the Sertoli cells of testes. No expression was detected in females. Wolffian duct development was normal in males and degenerate in prenatal females, but developmental recovery after birth gave rise to the formation of rudimentary epididymides. This event coincides in time with increasing serum testosterone levels and Leydig cell differentiation in the female gonad, thus suggesting that testosterone produced by the ovotestes is responsible for masculinisation of female moles. During postnatal development, serum testosterone concentrations decreased in males but increased in females, thus approaching the levels that adult males and females have during the non - breeding season.

KEY WORDS: *Müllerian duct, Wolffian duct, anti - Müllerian hormone, testosterone, Talpa occidentalis*

Introduction

Most components of the mammalian reproductive tract derive from the sex ducts formed during prenatal development. These are the Müllerian and the Wolffian ducts, precursors of the female and male accessory sexual organs, respectively, and located in the mesonephros. The development of these ducts depends on the type of gonads present in the embryo. In a female embryo, the lack of testis testosterone leads to the involution of the Wolffian ducts and the subsequent development of female structures (oviducts, uterus and upper vagina) from the Müllerian ducts. As this may occur also in castrated individuals, the process is clearly not ovary - dependent (see Jost, 1947; Austin and Edwards, 1981). Rather, it occurs as the result of a default programme of development. By contrast, male development requires the presence of two testicular hormones. The anti - Müllerian hormone (AMH), also known as the Müllerian inhibiting substance (MIS), is produced by Sertoli cells as soon as they begin to differentiate and induces the Müllerian duct regression (Tran and Josso, 1982). The testosterone produced by Leydig cells triggers the Wolffian duct development, which results in the differentiation of the male tract and accessory organs such as the vas deferens, seminal vesicles and epididymides (see review by Haqq and Donahoe, 1998). AMH is a member of the transforming growth factor – β family, for which two different receptors have been described. These are called type I and type II AMH receptors and express in AMH target organs (reviewed in Josso *et al.*, 2001).

Several natural and experimental observations have lead to the suggestion that AMH may be involved in testis differentiation. Freemartins are XX cattle embryos masculinised probably by the passage of AMH from male twins through placental anastomosis (Jost el a., 1975). Similar effects result from the administration of exogenous AMH to female gonads *in vitro* (Vigier *et al.*, 1987; Charpentier and Magre, 1990), or the chronic expression of the AMH gene in XX transgenic mice (Behringer *et al.*, 1990). In all these cases, ovaries undergo a process by which follicle cells «transdifferentiate» into Sertoli cells and form testis cord - like structures. However, rather than being a direct consequence of AMH, transdifferentiation is more probably the result of the oocyte

Abbreviations used in this paper: AMH, anti - Müllerian hormone.

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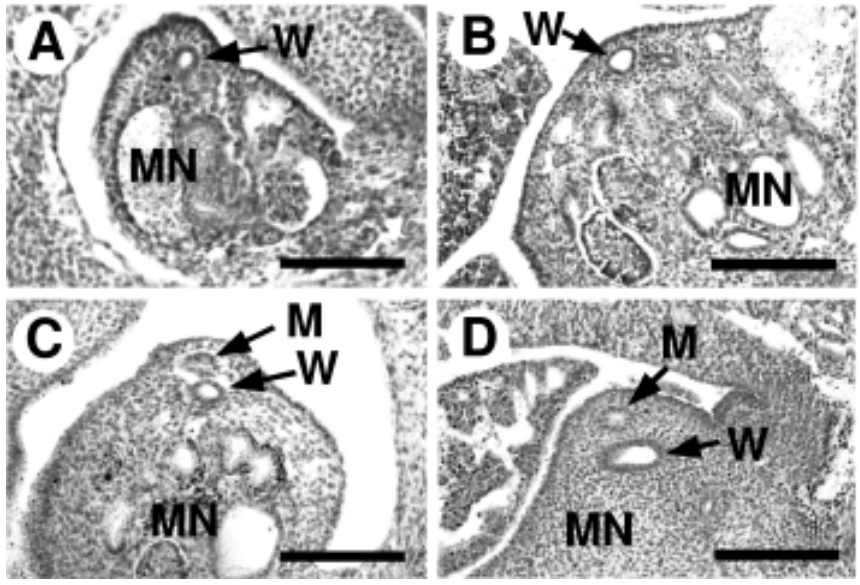

Fig. 1 (top four panels). Formation of the Wolffian and Müllerian ducts in the mesonephros of *Talpa occidentalis* males and females. *The Wolffian ducts appear in early s4 embryos* **(A)**, *whereas the Müllerian duct is still absent in the early s5a stage* **(B)**. *It appears in late s5a* **(C)** *in the cranial portion of the mesonephros and in s5c, at the level of the urogenital sinus* **(D)**. *M, Müllerian duct; W, Wolffian duct; MN, mesonephros. Scale bars represent 10 μm in all figures.*

depletion caused by this hormone (McLaren, 1990). AMH appears to be cytotoxic for meiotic cells, and oocytes are needed for correct ovarian development. This is consistent with the fact that AMH expression in testes ceases at puberty, coinciding with the onset of male meiosis. Also, AMH is produced in adult ovaries, probably by follicle cells, although its function here remains unknown (Vigier *et al.*, 1984; Bezard *et al.*, 1987).

Moles of genus *Talpa* show an exceptional sex differentiation system, as all females of the four species studied up to now (*Talpa occidentalis, T. europaea, T. romana* and *T. stankovici*) have ovotestes instead of normal ovaries (Jiménez *et al.*, 1993; Sánchez *et al.*, 1996). Female moles are thus fertile true hermaphrodites, as the ovarian portion of their ovotestes is functional due to the presence of mature oocytes during the breeding season. The ovarian portion is generally smaller than the testicular portion, which contains no germ cells. The two gonadal portions grow and regress each year in an alternating fashion. The ovarian portion may be larger than the testicular one during the breeding season, and the opposite situation invariably occurs during the resting period (Matthews, 1935; Deanesly, 1966; our personal observations). An alternating hormonal cycle accompanies this process. The testicular region of ovotestes in adult female moles actively produces testosterone during the resting period, but production is much lower during the breeding season (Jiménez *et al.*, 1993; Whitworth *et al.*, 1999). The body of female moles shows

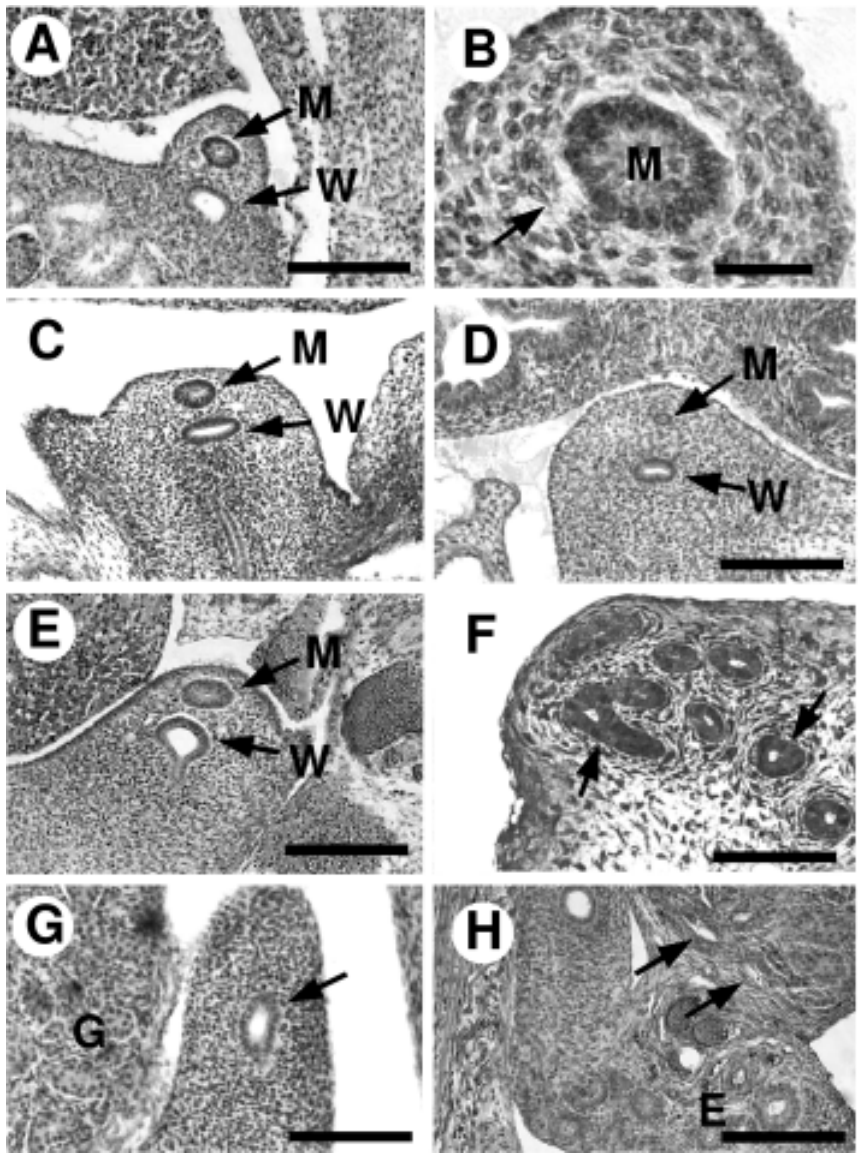

Fig. 2 (bottom eight panels). Sex differentiation and male development of the sex ducts in *T. occidentalis*. **(A,B)** *The first signs of Müllerian duct regression are seen in s5c male embryos (A), in the form of a whorl of mesenchymal cells (arrows) around the duct, as seen at higher magnification in (B). This is not present in the mesonephros of s5c female littermates* **(C). (D)** *In s6 male embryos the Müllerian ducts have almost disappeared.* **(E)** *Developing Müllerian ducts may be seen in s6 females (arrow).* **(F,G)** *Differentiating epididymary tube (arrows) (F) and* vas deferens *(arrows) (G) in s8 male foetuses.* **(H)** *Formation of the rete testis (arrow) in a newborn male. M, Müllerian duct; W, Wolffian duct; MN, mesonephros. Scale bars represent 10 μm in A and C - H, and 2 μm in B.*

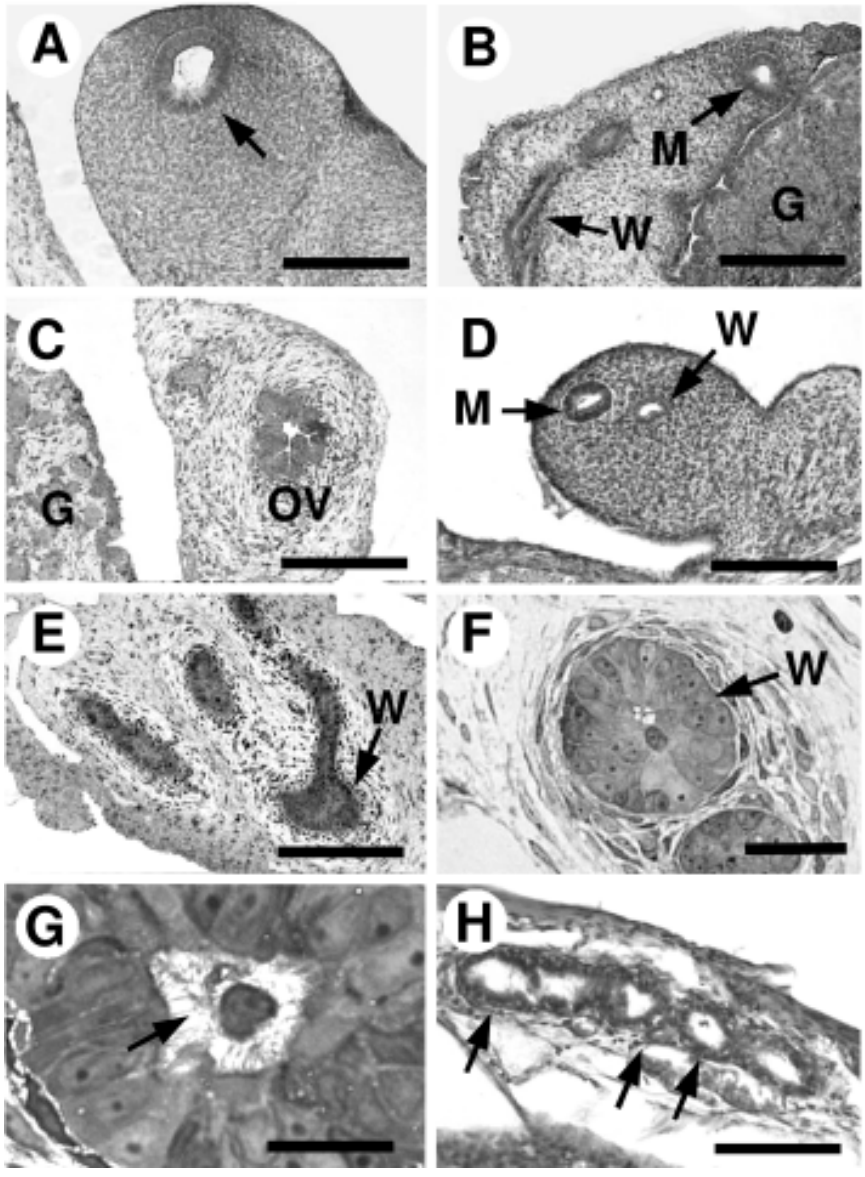

Fig. 3. Female development of the sex ducts in *T. occidentalis*. (A) *Müllerian duct in the caudal region of a newborn female, showing large diameter and clear internal lumen. Note the complete absence of any remains of the Wolffian duct.* **(B)** *The cranial region of the mesonephros in the same female as in (A); the Müllerian duct is large, but not so much as in the caudal portion, whereas the Wolffian duct appears thin and without an internal lumen.* **(C)** *Morphologically recognisable oviduct in an s11 female mole.* **(D - E)** *Degeneration process of the Wolffian ducts in female moles, as observed in s8 (D) and s11 (E) stages. Note the diameter reduction and the lack of lumen.* **(F - H)** *Developmental recovery of the Wolffian ducts in female moles: the diameter of the duct clearly increases in the s12 stage (F), the lumen opens again with epithelial cells projecting cilia in s13 (G) and a rudimentary epididymis is finally recognisable in s15b juvenile females, adjacent to the gonad (H). M, Müllerian duct; W, Wolffian duct; G, gonad; OV, oviduct. Scale bars represent 10 μm in A - E and H, 2 μm in F and 1 μm in G.*

additional signs of masculinisation other than gonads. These include a large, penile clitoris where the urethra opens at the tip, an intact perineal region without any vaginal opening during the non - breeding season (the vaginal orifice opens and closes every year) and rudimentary epididymides adjacent to the ovotestes (Matthews, 1935, Jiménez *et al.*, 1993; Whitworth *et al.*, 1999). Overall, these facts suggest that these organs were exposed to the action of testosterone during development, although it is not clear whether the hormone also originates in the gonads of developing moles or comes from the ovotestes of the mother.

Since development of the reproductive tract directly depends on gonadal function, it is informative to investigate how Müllerian and Wolffian ducts develop in animals, such as female moles, with an exceptional gonadal system. The present study describes the process of sex - duct development in both male and female moles, and its relationship with the production of two essential gonadal hormones: AMH and testosterone. A study of the sequence of a portion of the *AMH* gene from moles is also reported.

Results

Sex - duct development in moles

Wolffian ducts, already observable along the mesonephros in the earliest embryos we analysed (14 days post coitum, *dpc*, early s4 stage, CRL=6mm; Fig. 1A), were consistently observable afterwards (Fig. 1 B - D). Müllerian ducts appeared during late s5a stage of development (17 dpc; Fig. 1 B,C), and grew in a cranial - to - caudal progression. No sex differences were observable in sex ducts one day later (s5b stage; Fig. 1D).

Sex differences in sex ducts of moles are first observed in s5c embryos (19 dpc), when males showed the first signs of Müllerian - duct regression. Several concentric layers of mesenchymal cells were seen oriented parallel to the basement membrane of the Müllerian duct, thus forming a whorl around it (Fig. 2 A,B). By this time, this was found only in the mesonephric region adjacent to the gonad. No such phenomenon occurred in female embryos of the same stage (Fig. 2C). Müllerian - duct regression proceeded rapidly afterwards, as by s6 (19 - 21 dpc) the anterior portion of these ducts had almost disappeared (Fig. 2D), whereas they persisted in the urogenital sinus. Females of the same stage showed developing, size - increasing Müllerian ducts (Fig. 2E). No remains of the Müllerian ducts existed in s7 male embryos (21 - 23 dpc). Meanwhile, the Wolffian ducts grew rapidly and by s8 (24 - 28 dpc) were differentiating into morphologically recognisable epididymides (Fig. 2F) and vas deferens (Fig. 2G). A rete testis was already formed in the testes of newborn males, adjacent to the epididymis. (Fig. 2H).

In females, Müllerian and Wolffian ducts followed a developmental pathway which differed only partially from that of males. Müllerian ducts grew gradually throughout the entire pre - and postnatal development, so that by s9 they were very thick and had a clear lumen in the caudal portion of the duct (Fig. 3A), from which the utero - vaginal canal was being formed, but were less

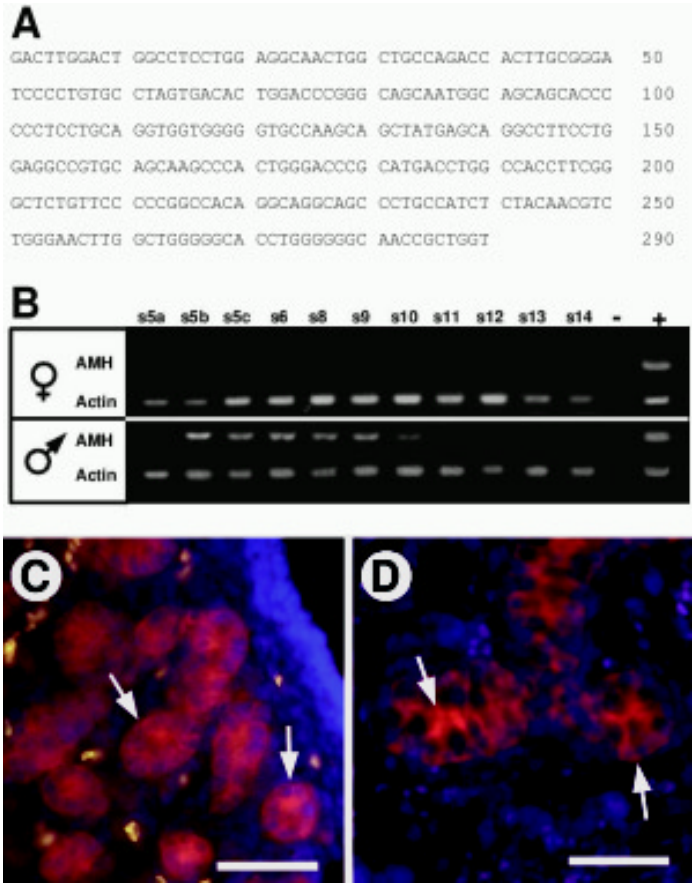

Fig. 4. The *AMH* gene of *T. occidentalis*. (A) *Nucleotide sequence of a 290 base - pair fragment of the mole* AMH *gene.* **(B)** *Time course of expression of the* AMH *gene in developing male and female mole gonads; no expression was detected in females of any developmental stage, whereas expression was evident in males from s5b (testis differentiation) until s10 (shortly after birth) stages. Amplification of the* β - actin *gene served as control of the RNA quality of the analysed samples. + and - represent the positive (genomic DNA) and negative (no sample) control RT - PCR samples which were reacted simultaneously with those of the analysed samples.* **(C,D)** *Immunohistochemical analysis using an AMH - specific antibody (red fluorescence) demonstrate that the AMH protein concentrates in the cytoplasm of Sertoli cells (arrows) in testes of either stage, including s5c (C) and s9 (D) male moles. Scale bars represent 10 μm in C and D.*

developed in the cranial portion (Fig. 3B), from which the oviduct derives. Oviducts and uterus were clearly recognisable in s11 females (Fig. 3C). In the Wolffian ducts, degeneration was evident in s8 females (just before birth) with clear diameter reduction (Fig. 3D). In newborn females (s9 stage), the caudal portion of the Wolffian ducts had completely disappeared, with only a thick Mullerian duct present in this region (Fig. 3A), whereas the cranial segment still persisted, adjacent to a less developed Müllerian duct (Fig. 3B). However, the Wolffian - duct diameter began slowly to increase again several days after birth, so that developmental recovery became evident between the s11 and s12 stages (10 - 20 dpp; Fig. 3 E,F). These ducts apear surrounded by a whorl of mesenchymal cells, and the process continues in s13, when the Wolffian ducts of female moles recovered the internal lumen. These regenerated Wolffian ducts began in this stage to show epididymary features such as the re - opened lumen becoming ciliated (Fig. 3G). As a result of this process, a rudimentary epididymis is formed adjacent to the testicular portion of the female ovotestes, which may be seen in fully grown female moles at the s15b stage (Fig. 3H). This epididymis continues to grow throughout the juvenile and adult life of female moles, so that it is larger and more complex in older adult females (not shown; see Sánchez *et al.*, 1996).

The mole AMH gene

Figure 4A shows the sequence of a 290 bp fragment of the *AMH* gene from *Talpa occidentalis* [1]. Compared with other mammalian species (Table 1), this sequence showed high homology and similarity percentages with primates (*Homo*) and arcthiodactyls (*Sus* and *Bos*), but homology was low when compared with rodents (*Rattus* and *Mus*).

The time course of expression of the *AMH* gene in developing moles is shown in Fig. 4B. In males, expression was first detected in s5b embryos, coinciding with the onset of testis differentiation, and it continued until shortly after birth (s9 stage). In s10 males (5 dpp), either little or no amplification was detected in different RT - PCR reactions, suggesting that *AMH* expression slowly declines after birth but never persists in s11 moles. No *AMH* expression was detected throughout gonad development in female moles. Immunostaining with an anti - AMH antibody (Fig. 4 C,D) showed that the AMH protein concentrates in the cytoplasm of Sertoli cells of the developing testis of moles.

Serum testosterone

Table 2 and Fig. 5 show the time course of serum - testosterone concentrations during postnatal development of male and female moles. Males showed a peak of serum testosterone in s10, but the

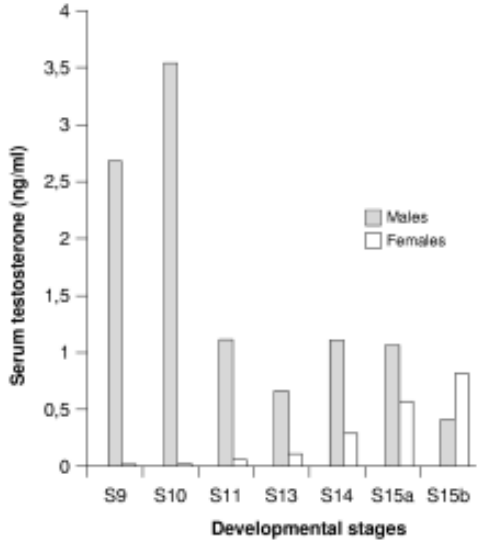

Fig. 5. Variations in serum - testosterone concentrations *during postnatal development of male and female moles.*

[1]EMBL accession number: AJ550376

TABLE 1

PERCENTAGES OF IDENTITY AND SIMILARITY BETWEEN THE MOLE *T. OCCIDENTALIS* AND FIVE MAMMALIAN SPECIES FOR A 290 BP FRAGMENT OF THE *AMH* GENE

	DNA		Protein	
	Identity	Similarity	Identity	Similarity
Homo	75.9	75.9	66.0	76.3
Rattus	48.0	48.0	53.4	62.5
Mus	47.5	47.5	54.3	64.9
Sus	72.6	62.5	65.7	70.7
Bos	72.4	72.4	60.0	66.3

TABLE 2

SERUM - TESTOSTERONE CONCENTRATIONS IN MALE AND FEMALE MOLES THROUGHOUT POSTNATAL DEVELOPMENT

Developmental stage (dpp)[a]		Serum testosterone concentrations (ng/ml)[b]			
		Males		Females	
s9	(0 - 5)	2.681	(1)	<0.020	(1)
s10	(5 - 10)	3.539±1.047	(3)	0.025±0.007	(2)
s11	(10 - 15)	1.115	(1)	0.06	(1)
s13	(15 - 20)	0.663±0.135	(3)	0.110±0.020	(3)
s14	(20 - 25)	1.110	(1)	0.288	(1)
s15a	(25 - 30)	1.062	(1)	0.572	(1)
s15b	(30 -)	0.414±0.253	(3)	0.814	(1)

[a]days post partum (dpp) corresponding to each developmental stage (in parenthesis).
[b]mean ± standard deviation and sample size (in parenthesis) are given.

concentration declined afterwards. Contrarily, in females serum testosterone was practically undetectable during the s9 and s10 stages, and began to increase from s11 on. As a consequence, by the s15b stage, the serum - testosterone concentration was higher in females than in males.

Discussion

Diverse information concerning sex - duct development is currently available for some vertebrates, including reptiles (Austin, 1989), birds (Forsberg and Olivecrona, 1963) and mammals. In the latter group, two marsupial species (Burns, 1945; Whitworth *et al.*, 1997) and several eutherian orders, including primates (Taguchi *et al.*, 1984; Wartenberg, 1985), rodents (Dyche, 1979; Eusterschulte *et al.*, 1992, among others), and carnivores (Meyers - Wallen *et al.*, 1991), have been investigated. However, no species of the order Insectivora has been analysed to date, so that the present paper reports the first study on sex - duct development in a species of this taxonomical group.

In the mole, Müllerian ducts are formed according to a pattern that resembles that described for other mammals (see Byscov and Hoyer, 1994), including marsupials (Renfree *et al.*, 1996), growing in a cranial - to - caudal progression. It is well known that Müllerian - duct development may proceed in the absence of ovaries (Jost, 1947), and this is the case in female moles, as Müllerian ducts grow and differentiate for about one month before any ovarian tissue is morphologically defined in female moles (unpublished). This implies that sex differences accumulating during this period are probably due to the action of the AMH produced by the male testes.

Müllerian - duct regression in moles is very fast, as the first signs of degeneration may be observed at the s5c stage, this being only one day after the onset of *AMH* expression in s5b male embryos. In the mole, regression proceeds in a cranial - to - caudal direction, although this feature varies between vertebrate species (see Whitworth *et al.*, 1997). Contrarily, the initiation of the Müllerian - duct regression with the formation of a whorl of mesenchymal cells around the duct cells, seems to be a highly conserved process, as it has been observed in several vertebrate taxa, including reptiles (see Austin, 1989), primates (Wartenberg, 1985), marsupials (Whitworth *et al.*, 1997), and insectivores (present study). The timing of Müllerian - duct regression in moles is consistent with the expression pattern of the mole *AMH* gene. In moles, this hormone appears to exert a powerful sway over Müllerian ducts as the first effects appear rapidly and regression is complete only 5 days later (s7 embryos). However, *AMH* gene continues to be expressed for about one week more in this species, until shortly after birth. This situation is similar to that described for the mouse or human, but contrasts with that of other mammals, such as the rat (see Josso *et al.*, 1977) or the tammar wallaby (a marsupial; Whitworth *et al.*, 1997), where Müllerian - duct regression continues in the absence of the hormone.

In male mice, AMH is abundantly produced by Sertoli cells from the onset of testis differentiation until puberty (Musterberg and Lovell - Badge, 1991), when production ceases as a direct consequence of the increased testosterone levels at this time (Al - Attar *et al.*, 1997). Consistently, we have shown that the mole *AMH* expression ceases in newborn males, which show high levels of serum testosterone.

Although *AMH* is expressed at low levels in the granulosa cells of adult female ovaries, where its possible function remains speculative (Vigier *et al.*, 1984; Takahashi *et al.*, 1986; Bezard *et al.*, 1987; Musterberg and Lovell - Badge, 1991), expression is never concomitant with ovarian development, a fact which is consistent with the hypothesis that AMH is cytotoxic for germ cells entering meiosis (McLaren, 1990). This would also explain why *AMH* expression in Sertoli cells ceases in males at puberty. Granulosa cells synthesise AMH from birth in the human female (Lee *et al.*, 1996) and from a few days after birth in the mouse (Musterberg and Lovell - Badge, 1991), but we detected no AMH production throughout the whole postnatal development of the female mole. These differences may be understood if we consider that in moles ovarian development is considerably delayed, starting several days after birth (s10 pups), when a part of the primordial germ cells of the female gonad begin to enter meiosis (our unpublished data).

According to the expression profile reported here for the *AMH* gene of moles, it may be concluded that this hormone is not involved in the formation of a portion of testicular - like tissue in the gonads of female moles, and is therefore not responsible for the anomalies in sex determination found in these mammals (Jiménez *et al.*, 1993; Sánchez *et al.*, 1996). *AMH* expression is usually associated with Sertoli cells and hence to testicular - tissue differentiation, so that the absence of expression in female moles would suggest that neither of these events takes place in these animals. Consequently, no ovotestis would differentiate and no sex reversal would occur in female moles. This hypothesis, which has been supported by other authors (see Beolchini *et al.*, 2000), is nevertheless contradicted by morphological, embryological, hormonal and molecular data related to the development of mole gonads. However, this is clearly not the subject of the present

paper, although data on this question will be published elsewhere, and other aspects of this research, including the precise characterization of Sertoli and Leydig cells in male and female moles, are currently under way, so that an exhaustive discussion on these subject is not possible for the moment.

Our results indicate that the development of the Müllerian ducts in female moles is normal compared with other mammals. In fact it gives rise to a functional female reproductive tract, including utero - vaginal canal, uterus and oviducts (Jiménez *et al.*, 1988, 1993). This is consistent with the hormonal environment in which Müllerian ducts develop, as neither AMH nor testosterone are present during most of that time. However, this is not the case for Wolffian ducts in female moles. Our results demonstrate that degenerated remains of these Wolffian ducts show clear signs of developmental recovery several days after birth, thus resulting in the formation of small, underdeveloped epididymides adjacent to female gonads. It is noteworthy that this fact coincides in time with two other significant events: 1) the appearance of morphologically recognizable Leydig - like cells in the medular region of the female gonad (unpublished), and 2) the presence for the first time of circulating testosterone in female moles (this paper). These data strongly suggest that these Leydig - like cells are in fact functional, testosterone - producing Leydig cells, and that this testosterone is responsible for the late development of the epididymis in female moles. It is also probably responsible for the enlarged clitoris and the closed utero - vaginal canal shown by juvenile and non - breeding adult female moles (Mathews, 1935; Jiménez *et al.*, 1993; Whitworth *et al.*, 1999). Contrary to the absence of AMH expression, the presence of functional Leydig cells strongly suggest that development of the medular region in embryonic female gonads results in a portion of disgenic testicular tissue, and that these gonads are in fact ovotestes.

The testosterone produced by female gonads seems not to perturb ovarian tissue differentiation, which takes place postnatally as the serum - testosterone concentration increases. According to our data, the situation in moles differs from that of other mammals with clearly masculinised females, the hyaena *Crocuta crocuta*, where the presence of enlarged clitoris may not be associated with the production of testosterone by the fetal gonads but with that of the maternal placenta (Licht *et al.*, 1998).

The serum - testosterone levels described here for juvenile moles (s15b stage, see Table 1), are lower than those reported by Jiménez *et al.* (1993) for, apparently, the same type of animals. This discrepancy is very probably due to the fact that the two samples of individuals classified as juvenile moles, were different in age (juvenile moles range from one - month - old individuals just after weaning, which occurs between January and March, to moles about 8 - 9 months old before puberty, which takes place during September - October, in Southern Spain for *T. occidentalis*). In the present study, we have analysed very young juvenile individuals captured shortly after weaning (not more than two months old in all cases), whereas in our 1993 paper we studied much older juvenile moles, with larger ovotestes in the case of females, or near puberty in the case of males. We have shown here that serum testosterone levels varied with age in postnatal developing moles, so that differences in age may explain contrasts in hormone levels. These differences might also arise at random, due to low sample sizes. In fact, testosterone levels depend on gonadal size (see Jiménez *et al.*, 1993; Whitworth *et al.*, 1999) and the latter is highly variable among juvenile individuals (Sánchez *et al.*, 1996).

We have shown that serum - testosterone concentrations of postnatal developing moles change over time in both male and female pups. It is notable that hormone concentrations increase in females and decrease in males, a fact that should be interpreted in the light of the reproductive timing of these animals. Moles reproduce seasonally, with a breeding period (from November to April for *T. occidentalis* in Southern Spain; see Jiménez *et al.*, 1990) and a resting period that alternate every year. Moles born and grown during a given breeding season (weaning occurs about one month after birth) do not enter puberty until the following breeding season, so that it may be considered that after weaning they begin their first resting period (unpublished data). Taking into account that serum - testosterone concentrations during the non - breeding period are high in females and low in males, in comparison to those found during the breeding period (Jiménez *et al.*, 1993; Whitworth *et al.*, 1999), the tends for testosterone levels to rise in females and fall in males during postnatal development are consistent with the final situation.

In conclusion, the expression pattern of the *AMH* gene is normal, evidencing that this hormone is not involved in mole sex reversal, whereas gonadal testosterone seems to be responsible for masculinisation of the female body, without perturbing ovarian tissue differentiation. Thus, the present study on sex - duct development in the mole species *T. occidentalis* has revealed some exceptional features that may be associated with the unique gonadal differentiation system described for these animals.

Materials and Methods

Material analysed

In this study, we used a series of 95 embryos, foetuses and pups of the mole species *Talpa occidentalis*, collected in Granada (southern Spain) since 1990. Developmental staging of the individuals studied was based on CRL (Crown - Rump Length) and body mass values and on the morphology of major external structures. We established eight prenatal (s1 - s8) and seven postnatal (s9 - s15) stages in the development of *T. occidentalis*. Only those stages in which gonads are present (s4 - s15) were analysed here. A new postnatal stage was defined for every five days post partum (dpp). Stage s5 was in turn divided into three substages (s5a,b,c) for more accurate staging of this critical period of sex development in moles. Also, s15 was divided into two substages, in order to differentiate full - grown nestling moles (s15a) from emancipated juvenile moles (s15b).

Sexing of mole embryos

As sex cannot be determined in early embryonic stages (s4 - s6) on the basis of morphological features, we used our quick method for preparation of amniotic cells, where the sex chromatin body can be visualized. (Jiménez *et al.*, 2000).

Histology

Several embryos and foetuses were fixed *in toto* by immersion in a mix of 70% ethanol, 40% formaldehyde and glacial acetic acid, in proportions 90:5:5, respectively. The embryos were dehydrated in ethanol series, embedded in paraffin (Paraplast), serially sectioned (7 - 10 μm thick) and stained with haematoxylin - eosin, according to standard procedures. Embedding in epoxy resin was also used for light - microscope analysis in other cases. The entire reproductive tract was dissected out and fixed in Karnowski fixative (2.5% glutaraldeyde, 1% formaldeyde in 0.1M cacodylate buffer) for 45 - 60 min. Then the pieces were dehydrated and embedded following standard procedures, including post - fixation in OsO4. Semi - thin sections (0.8 μm thick) were cut with a «Reichert Ultracut» ultramicrotome and stained with toluidine blue.

Serum testosterone

The concentrations of serum testosterone were measured by radioimmunoassay (RIA) with reagents provided by Sorin Biomedica Diagnostic (Vercelli, Italy), following standard procedures. Duplicate measurements were made for all animals in the same RIA. The coefficient of variation was 7%. When the serum testosterone values were near the method sensitivity, they were further confirmed by performing an additional electrochemiluminescence immunoassay. The sensitivity was 0.069 nmol/l. Serum samples were obtained from just - killed foetuses and pups.

Molecular cloning and sequencing

A fragment of the *AMH* gene from *Talpa occidentalis* was amplified by PCR, using the following primers: sense 5' - CGG GGT ACC GAA GTG GCC TCA TCT TCC GAG AA - 3' and antisense 5' - CGC GAG CTC CTT CCT CCA GGT GTA GGA CC - 3'. The resulting 332 bp PCR product was ligated to a pKS vector and used to transform competent DH5α cells. Five positive clones were finally sequenced (Genaxis, Nimes, France).

Gene - expression analysis

The time course of expression of the *AMH* gene was studied by performing RT - PCR reactions. For this, mRNA samples were purified from single gonads dissected out from mole embryos, foetuses and pups covering all representative stages of development. Gonads were homogenized in up to 200 µl of lysis buffer, and 50 µl of the homogenates were exposed to a biotin - labelled oligo - dT capture probe and placed into 200 µl streptavidin - coated tubes («mRNA Capture» kit, Roche). After three washes with the provided buffer, one - step RT - PCR reactions were performed in the same tubes by using the «Titan one - tube» kit (Roche). The following primers were used: sense 5' - GCC TCA TCT TCC GAG AAG ACT TG - 3', antisense 5' - CTT CCT CCA GGT GTA GGA CC - 3'. The quality of the mRNA samples was assessed by performing parallel RT - PCR reactions for the β - actin gene with the primers: 5' - TGG ATG ATG ATA TTG CTG C - 3' and 5' - ATC TTC TCC ATA TCA TCC CA - 3'. These primers lead to the amplification of a 573 bp DNA fragment from genomic DNA, or a 253 bp fragment from cDNA, thus enabling detection of genomic DNA contamination of the samples.

In situ *immunofluorescence*

The reproductive tract of several *AMH* - expressing individuals was dissected out and fixed overnight in phosphate - buffered 4% paraformaldehyde at pH 7.2. These pieces were dehydrated, embedded in paraffin wax and sectioned as described above. After being dewaxed, rehydrated and washed in PBT (phosphate buffered saline with 0.1% Tween 20), preparations were immersed in 0.01M sodium citrate and treated in a microwave oven (800 watts) for about 5 min. This sodium citrate solution with the preparations was left to cool at room temperature before being washed in PBT. Blocking reaction was performed in PBT with 10% bovine - serum albumin (BSA). Preparations were exposed to a 1:400 dilution of the AMH - antibody (Santa Cruz Biotechnology, *sc 6886*), prepared in PBT with 1% BSA, and incubated overnight at 4°C. Preparations were washed again and exposed to a 1:400 dilution of an anti - rabbit secondary antibody, conjugated with Alexa fluor 594 (Molecular Probes). After a final washing, preparations were mounted in DAPI - Vectashields mounting medium, and observed in a fluorescence microscope.

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