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The spatio-temporal pattern of testis organogenesis in mammals - insights from the mole

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ABSTRACT Some cellular events are crucial in testis organogenesis, including Sertoli and Leydig cell differentiation, mesonephric cell migration and testis cord formation. These processes are controlled by transcription factors, paracrine signalling and hormones. Using the mole species *Talpa occidentalis* as an alternative animal model, we report the expression patterns of nine genes during testis differentiation and analyse their implications in the above-mentioned cellular processes. We show that: 1) Sertoli cell differentiation occurs very early and precedes mesonephric cell migration, indicating that the latter is not needed for the endocrine cytodifferentiation of Sertoli cells; 2) the time of Leydig cell differentiation is consistent with the participation of PDGFR-α in promoting the migration and/or proliferation of Leydig cell precursors, and with that of WNT4 signalling in inhibiting Leydig cell differentiation and 3) the formation of the *tunica albuginea* involves intragonadal cell migration/movement. These results demonstrate that testicular organogenesis in the mole differs from that in the mouse in some particular aspects, thus providing evidence that the spatio-temporal pattern of testis development is not highly conserved during mammalian evolution.

KEY WORDS: *Sex determination, gonad, Sertoli cell, Leydig cell, cell migration, SOX9, AMH, Talpa*

Introduction

Mammalian sex differentiation takes place in two steps. In the first, the undifferentiated gonadal primordia, which are present in both males and females, develop either as testes, or as ovaries. In marsupials and placental mammals, this process depends on the presence of the Y-linked gene *SRY* (sex-determining region of the Y chromosome; Sinclair *et al*., 1990). *SRY* encodes a putative transcription factor which acts as the genetic switch for male development by triggering a gene cascade where the genes of the male pathway (*SF1*, *SOX9*, *FGF9*, *DMRT1*, *DAX1*, *AMH*) are upregulated, whereas those of the female pathway (*WNT4*, *FST*) are downregulated (see Ross and Capel, 2005, for a review). Expression of *SRY* at the time of sex determination results in the formation of testes in males (XY). Its absence in females (XX) results in ovarian differentiation at some point later on in development. In the second step, once the gonads are differentiated as testes, the androgens produced by the testis directs the differentiation of the urogenital tract of the male embryo, thus resulting in the formation of the male secondary sex features, including external genitalia. In the absence of testicular androgens, a female phenotype develops (Jost, 1947).

The genitourinary system of mammals derives from the intermediate mesoderm. In mice, gonadal primordia appear 10 days post coitum (dpc) as paired thickenings of the coelomic epithelium in the ventrolateral surface of each mesonephros, a structure functioning as a primitive kidney during embryogenesis. This thickening is referred to as genital ridge and is composed of

Abbreviations used in this paper: AMH, Anti-Müllerian Hormone; CRL, crown-rump length; DMRT1, dsx- and mab3-related transcription factor 1; DPC, days post coitum; ELISA, Enzyme-Linked Immuno-Sorbent Assay; kD, Kilo Dalton; MT1-MMP, Matrix Type 1 Membrane Metalloproteinase; P450scc, P450 side chain cleavage; PBS, phosphate-buffered saline; PDGFRα, Platelet Derived Growth Factor receptor α; PGC, primordial germ cell; SF1, Steroidogenic Factor 1; SOX9, Sry-like HMG-Box protein 9; SRY, gene on the sex region of the Y chromosome; WNT4, wingless-related MMTV integration site family member 4.

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somatic cells (mesothelial, mesenchymal and endothelial cells) and primordial germ cells (PGCs) coming from the extraembryonic mesoderm through the gut mesentery (McLaren, 2003). Once PGCs have colonized these undifferentiated gonads, there is a high proliferation of the somatic cells, mainly of the mesothelial and mesenchimal type. In this manner, the cells of the gonadal primordia become closely packed between the coelomic epithelium and the mesonephric mesenchyme.

In mammals, testes differentiate at an earlier stage than ovaries. Accordingly, it was hypothesised that the male pathway is an active process that anticipates ovarian fate in the undifferentiated gonad. In mice, although *Sry* expression can be detected by 10.5 dpc, the most important events of the testis development take place between 11.5 and 12.5 dpc, which is when the first morphological signs of gonadal sex differentiation appear. One of them is the colonization of the XY gonad by undifferentiated cells coming from the adjacent mesonephros (Martineau *et al.*, 1997). This cell migration involves mainly endothelial cells, which organize the testicular vascular system (Brennan *et al.*, 2002). It is also known that a few steroidogenic Leydig cell precursors do enter the gonad by migration from the mesonephros (Jeays-Ward *et al.*, 2003). Peritubular myoid and perivascular cells are assumed also to take part in the migration process (Martineau *et al.*, 1997), although this assumption has yet to be demonstrated via molecular markers. Mesonephric cell migration and a high proliferation of epithelial somatic cells take place simultaneously. These cells begin to differentiate as Sertoli cells, the main somatic cell type of the testis, which aggregate around PGCs forming testicular cords (Merchant-Larios and Taketo, 1991). It has been suggested that mesonephric cell migration is necessary for Sertoli cell differentiation and testicular cord formation (Tillman and Capel, 1999; see also Brennan and Capel, 2004 for a review).

As a result of Sertoli cell differentiation, some interstitial somatic cells differentiate as peritubular myoid cells, which surround testis cords and cooperate with Sertoli cells to deposit a basal lamina around them (Tung and Fritz, 1987). Other interstitial cells differentiate as either fibroblasts, endothelial cells, or Leydig cells (Merchant-Larios and Taketo, 1991). The masculinization of the mouse embryo body begins from 12.5 dpc and is determined by three testicular hormones: Leydig cells produce testosterone and Insl3, which induce the differentiation of the Wolffian ducts within male reproductive tracts and the descent of testes, respectively (Nef and Parada, 1999), whereas Sertoli cells produce anti-Müllerian hormone (AMH; also referred to as Müllerian inhibiting substance, MIS) which promotes the regression of the Müllerian ducts, the precursors of the female reproductive organs (Vigier *et al.*, 1987).

Some mole species of family Talpidae show exceptional reproductive features. Female moles represent the only case of generalized XX true hermaphroditism described so far in mammals, since all of them are fertile despite that they have bilateral ovotestes (gonads with both ovarian and testicular tissue) instead of normal ovaries (Jiménez *et al.*, 1993; Sánchez *et al.*, 1996, Rubenstein *et al.*, 2003). Barrionuevo *et al.* (2004a) studied the gonad development in the Iberian mole, *Talpa occidentalis*, showing that the testicular portion of the ovotestes in XX moles develops in a testis-like pattern. Genital ridges are detectable at the s4 stage of mole development (corresponding to 14-16 dpc; Barrionuevo *et al.*, 2004b; the entire gestation length in this species is 28 days), and testicular cords are first observed at the s5b stage (18 dpc) in both males and females. This study also showed that the development of the XY testicular tissue in males is morphologically similar to that found in other mammals. The vascular system of the testis and the first myoid and Leydig cells appear at stage s6 (19-21 dpc), and the tunica albuginea is formed between s6 and the last prenatal stage s8 (24-28 dpc).

In this paper, we report on the study of the expression of several genes currently known to be involved in mammalian sex determination and differentiation during crucial stages of testis development in males of the mole *T. occidentalis*. Our results show that, contrary to the situation described in mice, Sertoli cell differentiation is probably independent from mesonephric cell migration in moles, and occurs substantially before testis cords become histologically detectable. Testis-cord formation in the mole occurs much more slowly than in the mouse, allowing for a more precise establishment of the sequence of events accompanying this process, including Leydig cell differentiation and the formation of the tunica albuginea.

Results

Specificity of the anti-WNT4 antibody produced in this study

Most antibodies used in this study are commercially available, and their specificity has been investigated previously by the corresponding manufacturers. However, the WNT4 antibody produced in our laboratory needed to be tested for proper specificity

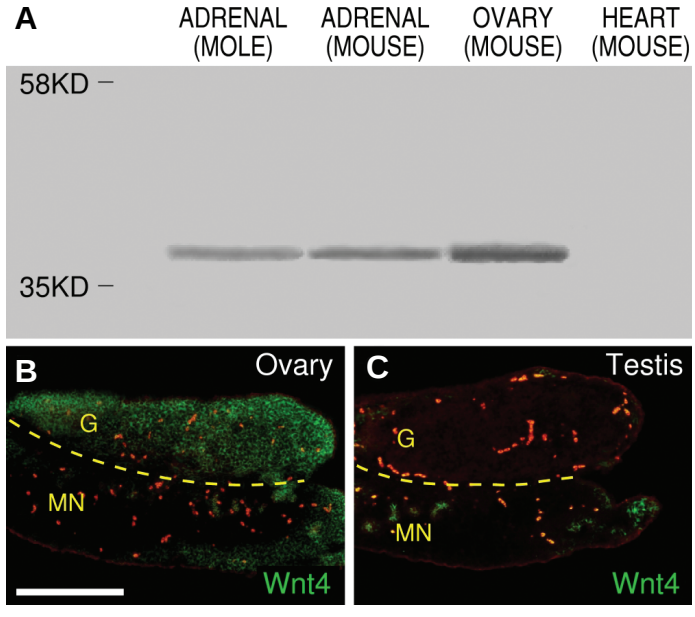

Fig. 1. Specificity of the mole anti-WNT4 antibody developed in this study. (A) *Western blot analysis of WNT4 showing a ~39 kD band detectable in the samples of adrenal gland from both mouse and mole, and of mouse ovary. The negative control, mouse heart, did not provide any cross-reaction.* **(B,C)** *Immunostaining of mouse 12.5 dpc gonads using the novel anti-WNT4 serum: mouse ovary shows high expression of Wnt4* **(B)**, *whereas mouse testis does not* **(C)**, *as described for this species. G, gonad; M, mesonephros. The dotted line marks the gonad-mesonephros border. Mole erythrocytes are autofluorescent, showing reddish fluorescence in both (B,C). Scale bar represents 200 μm in (B,C).*

against the WNT4 protein. For this, western-blotting analyses and immunofluorescence were peformed on mouse gonads (Fig 1). The western blotting (Fig. 1A) identified a single band in samples of adrenal gland from both the mouse and the mole, as well as in mouse ovary (tissues where this gene is known to be expressed). However, no band was present in the mouse heart sample, which was used as negative control. This band was ~39 kD in size, the molecular weight reported for the WNT4 protein. Furthermore, the immunofluorescence technique showed that the mole WNT4 antibody detected abundant immunoreactive cells in the gonads of female but not in those of male mice (Fig. 2 B,C), this coinciding with the expression pattern known for this gene in this species. The 293bp fragment of the mole *WNT4* gene that was amplified to produce this antibody (EMBL accession number: AM698098) was 91.3% and 90.2% identical to those of the human and mouse genes, respectively, whereas the translated amino-acidic sequence was 100% identical to those of these two species.

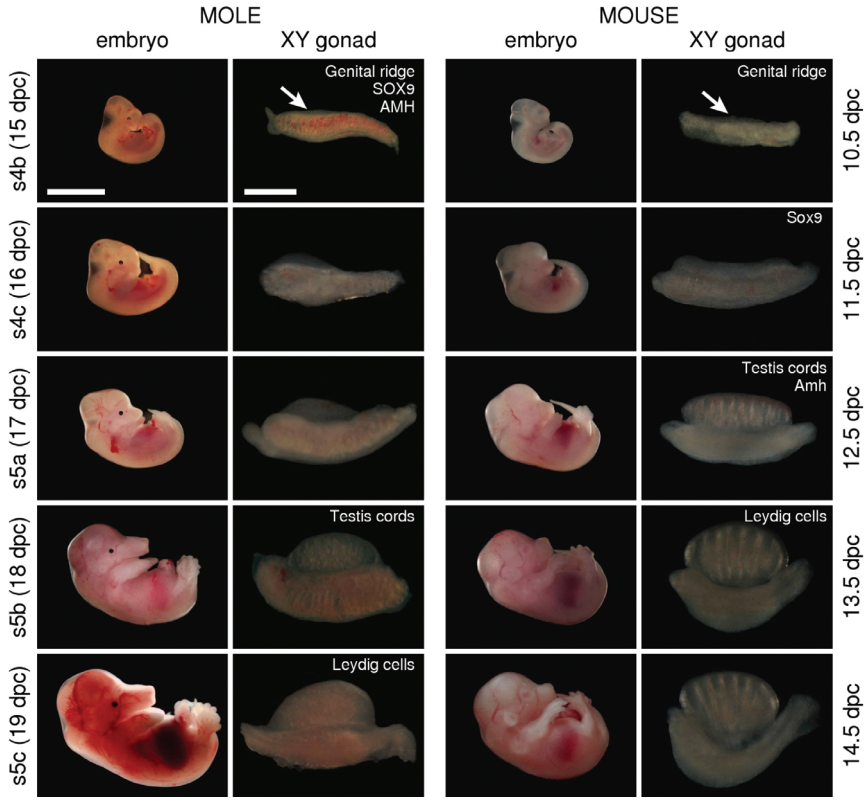

Fig. 2. Correspondences between developmental stages of the mouse and the mole during a five-day period around the time of sex differentiation. *Images of the embryos and the gonad-mesonephros complexes are shown for each stage in both species. The main developmental events occurring in particular stages in the two species are also indicated in the corresponding stages. In the mole, stages from s4b to s5c, with their corresponding gestational age (in days post coitum), are mainly those described by Barrionuevo* et al. *(2004), although new sub-stages in the s4 stage were described for this study; the genital ridge appears in s4b embryos (arrow), and the testis cords are first observable three days later, at the s5b stage. In the mouse, the genital ridge appears in 10.5 dpc embryos, and the testis cords at 12.5 dpc; mouse testis cords are much more conspicuous than those of the mole testis at this point and at subsequent stages. The scale bars represent 5 mm in the embryo columns and 0.5 mm in the gonad columns.*

Correspondences between stages of gonad development in the mouse and the mole

For comparisons of gonad-development patterns of mice and moles, the equivalences between developmental stages in both species needs to be established during the period in which the gonads emerge and differentiate. Two key events of testicular development, the appearance of the genital ridge and the formation of the testis cords, were taken as reference stages to establish the parallelism between these two species. As shown in Fig. 2, the formation of the genital ridge in the mole begins at the s4b stage (15 dpc), in the mouse at 10.5 dpc, whereas the testis cords appear at the s5b stage (18 dpc) and 12.5 dpc, respectively. Hence, the time elapsed between these two events was three days in the mole, and two days in the mouse. Only one day after the formation of the testis cords (s5c moles and 13.5 dpc mice), the testis appeared slightly more developed in the mouse than in the mole, and the difference was more evident after one more day. The mouse testis grew faster and became larger than the mole testis during the same developmental period. The mouse testis cords were clearly more conspicuous than those of the mole in these low-magnification images of fresh gonads.

Very early expression of Sertoli cell markers in the mole testis

The moment of Sertoli cell differentiation in male moles was studied by establishing the time-course expression of four Sertolian molecular markers: *SF1*, *SOX9*, *AMH* and *DMRT1*, as shown in Fig. 3. Most cells of the s4b XY gonad appear strongly immunoreactive against the SF1 antibody. At s5a, cells inside the developing cords showed stronger fluorescence than did those outside of them, but the opposite was true in s5b and later stages (Fig 3, first row). *SOX9* expression is evident in most cells of the medullary region of the gonadal primordium in XY gonads of the s4b and s4c stages (Fig. 3, second row), coinciding with the formation of the genital ridge. The expression persists later on in the developing testis cords, which are discernible for the first time in s5a gonads in SOX9-immunostained sections, and become evident from s5b on. The expression pattern of the *DMRT1* gene closely parallels that of *SOX9* (Fig.3, third row). The expression of *AMH* in male moles (Fig. 3, fourth row) begins in a few cells located in the most central region of the gonad at stages as early as s4b; this expression is generalized to most cells of the developing testis cords two days later (s5a stage), and is maintained in cells inside the testis cords at s5b and s5c testes. WNT4 protein was first detected at the s4b stage in the medullary region of XY gonads (Fig. 3, fifth row), but the expression was not as strong as in XX gonads (not shown). The expression decreases during the following stages (s5a and s5b), and is not detectable at s5c. The scarce protein that remains in s5b testes permitted us to

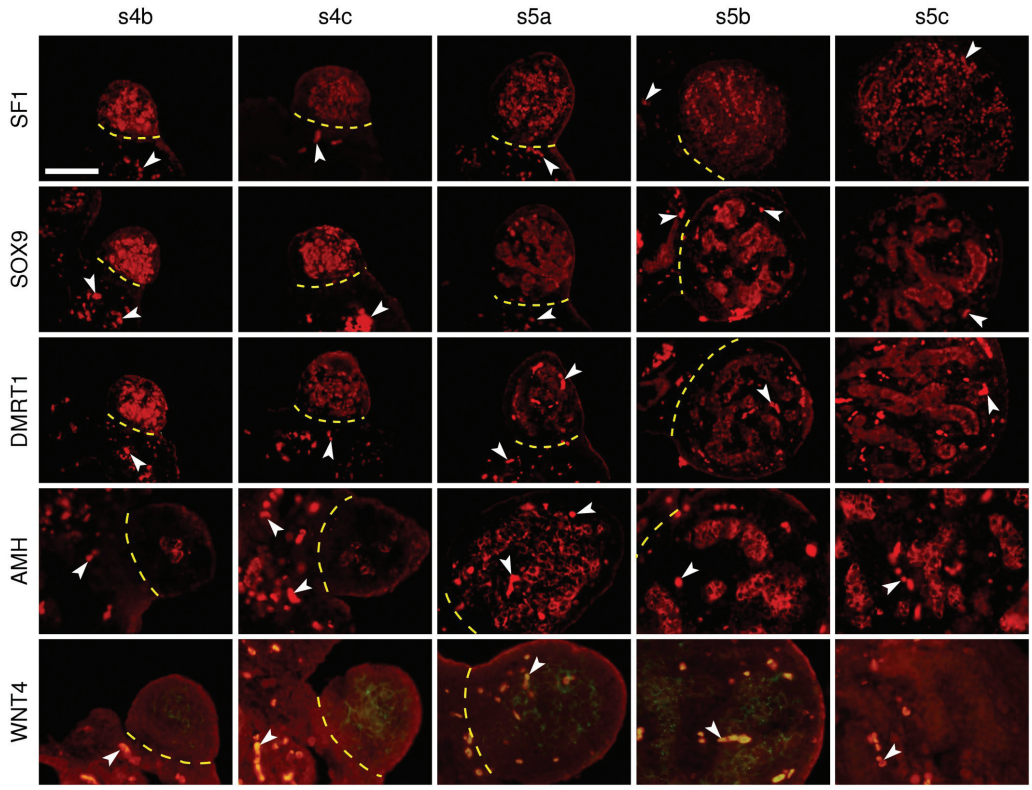

Fig. 3. Expression of Sertoli cell marker genes during early development of mole testes. **(SF1)** *Most gonadal cells contain SF1 protein at all stages.* **(SOX9)** *This gene is expressed in practically all cells of the gonadal medulla at the s4b and s4c stages, as well as in cells organizing testis cords in s5a gonads and in the cells inside the differentiated testis cords in s5b and s5c gonads.* **(DMRT1)** *The expression pattern of this gene is identical to that of* SOX9. **(AMH)** *Its expression is also similar to that of* SOX9 *during all stages except at s4b and s4c, where only a few cells appear immunoreactive (located at the centre of the gonad in s4b).* **(WNT4)** *The expression of this gene is weak from s4b to s5b on and disappears entirely in s5c gonads; the s5b image shows that the WNT4 protein is present inside the testis cords. Positive cells appear red-fluorescent in the SF1, SOX9, DMRT1, and AMH rows (photomicrographs taken using red single bandpass fluorescence mirror unit), and green in WNT4 row (photomicrographs taken using a red/green dual bandpass fluorescence mirror unit). Yellow dotted lines mark the gonad-mesonephros border line. Scale bar represent 150 μm in the AMH and WNT4 row, and 100 μm in all the rest. Arrowheads point erythrocytes, which appear autofluorescent in all immunofluorescence pictures.*

see that the immunoreactive cells are located inside the testis cords and are probably Sertoli cells.

Expression of cell migration markers during testis differentiation in male moles

PDGFR-α shows a transient expression during testis differentiation in male moles (Fig. 4, first row). Some protein may be detected at the s4b stage but maximal immunoreactivity was found in s5a gonads. The expression clearly decreases one day later (s5b stage) and is completely absent at s5c. The remaining PDGFR-α protein observed in s5b gonads indicates that it is contained in the interstitial cells, outside the testis cords. MT1-MMP metalloproteinase was detected in both gonads and mesonephros of s4b embryos, but few immunoreactive cells were observed in the borderline between these two organs at this stage (Fig. 4, second row). However, large quantities of strongly positive cells were observed in this region during the s5a and s5b stages. At s5c no MT1-MMP protein may be detected in the gonadal medulla, but a strong expression persists at the cortical region, just beneath the coelomic epithelium.

Formation of the testis cords in male moles

The organization of the testis cords in male moles was studied by detecting laminin, a protein that accumulates in the basal lamina of the testis cords, and AMH, a marker for Sertoli cells (Fig. 5). Laminin deposits appear in s4b gonads around the first Sertoli cells expressing *AMH*, although cord structures are not yet visible at this stage. Cords are still disorganized at s4c, although some cord-like structures were recognizable in s5a gonads; AMH-positive cells were located inside these differentiating testis cords. Well differentiated, elongated, true testis cords were first observed in s5b, and were evident in s5c embryos, where a well formed basal lamina encloses groups of several *AMH*-expressing Sertoli cells. Cord anastomosis is frequent at early stages and decreases as testis differentiation proceeds, and therefore it is absent in s5c gonads.

Leydig cell differentiation in male moles

The timing of Leydig cell differentiation in male moles was studied by immunofluorescence, using an antibody specific for the Leydig cell marker cytochrome P450scc (side-chain cleavage), which is present in the cytoplasm of Leydig cells, as shown in Figure 6A. The first Leydig cells differentiate at the s5c stage, where a few of them appear scattered in the interstitial areas of the testis. No Leydig cells were detectable in testes of the previous stage s5b. The number of Leydig cells increases during the next two stages, s6 and s7, where large patches of them originate, and persist until puberty (not shown). Leydig cells are also strongly immunoreactive against the SF1 antibody (Fig. 3, first row; s5c stage), whereas Sertoli cells, located inside the testis cords, appear clearly less immunoreactive (Fig. 3, first row). Double immunostaining with AMH, a Sertoli cell marker, and SF1, which marks both Sertoli and steroidogenic cell precursors, evidences that Leydig cell precursors are present in the testes of s5a and s5b

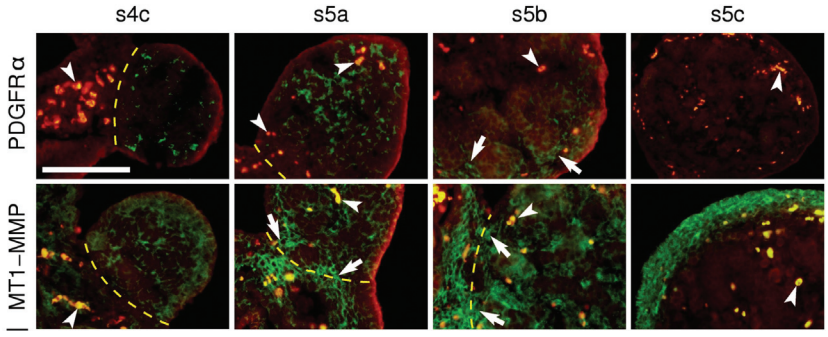

Fig. 4. Expression of genes involved in cell migration during the first stages of mole testis development. **(Pdgfr-α)** *This gene is expressed from s4c to s5b, but not at the s5c stage, reaching its peak at s5a; the remaining expression in s5b gonads evidences that it occurs in the interstitial cells, outside the testis cords (arrows).* **(MT1-MMP)** *Cells expressing this gene may be seen in both gonad and mesonephros at all stages analysed, but their distribution is stage-dependent: masses of strongly immunoreactive cells stretching out across the gonad-mesonephros borderline were observed at the s5a and s5b stages (arrows), but not at s4c; expression in deep gonadal regions occurs in the interstitial cells of s5a and s5b testes; at s5c,* MT1-MMP *expression is restricted to the peripheral layer of testicular cells forming the tunica albuginea. Yellow dotted lines mark the gonad-mesonephros borderline. Photomicrographs were taken using a red/green dual bandpass fluorescence mirror unit. Scale bar represents 200 μm in the PDGFRα-s5c picture and 100 μm in the rest. Arrowheads point out autofluorescent erythrocytes.*

embryos (Fig. 6B), as they express *SF1,* but not *AMH*. This figure clearly shows that the number of Leydig cell precursors increases in the transition between these two stages. However, no Leydig cell precursors seem to exist at previous stages (s4b and c), when all cells immunoreactive for SF1 also express *SOX9,* and are thus Sertoli cells (Fig. 3, first to third rows).

Discussion

Sertoli cells differentiate very early in the mole as compared to the mouse

In the mouse, Sertoli cells differentiate from epithelial-type cells of the undifferentiated gonadal primordium, in a two-step process. In the first step, the testis- determining gene *Sry* is activated in these cells at 10.5 dpc (for a review, see Polanco and Koopman, 2007), and the pro-testis gene *Sox9* express afterwards so that before 11.5 dpc these cells have become pre-Sertoli cells (Sekido *et al.*, 2004; Wilhelm *et al.*, 2005). In the second step, fully functional Sertoli cells differentiate coinciding with the production of Amh and the formation of testis cords between 11.5 and 12.5 dpc (see Behringer *et al.*, 1990; Brennan and Capel, 2004). Hence, differentiated Sertoli cells become visible two days after the onset of the formation of the mouse genital ridge. In the mole, however, fully differentiated Sertoli cells begin to appear in the just-formed, morphologically undifferentiated gonadal primordium (s4b stage), where a few cells may be seen expressing both *SOX9* and *AMH*. In a previous study (Zurita *et al.*, 2003), we were unable to detect *AMH* transcripts in s4 and s5a mole testes by RT-PCR, probably due to the scarcity of material from these stages in that study. The expression patterns of the other three genes investigated in the present study, *SF1, DMRT1*, and *WNT4*, coincide with those described for these genes in the mouse Sertoli cells, and support the Sertolian nature of those cells in the mole gonad. The first cells expressing *SF1*, *SOX9* and *AMH* genes in s4b embryos are located in the central region of the gonad, whereas the cells located outside this region do express *SF1* and *SOX9,* but not *AMH* (thus they are pre-Sertoli cells). Two days later, at the s5a stage, most gonadal cells expressed all three genes. This indicates that, in the mole, the expression of *SF1* and *SOX9* precedes that of *AMH*, corroborating the hypothesis that SOX9 interacts with SF1 to upregulate the expression of *AMH*, as has been suggested for mammals (Arango *et al.*, 1999), but not for the chicken (Oreal *et al.*, 1998) or the alligator (Western *et al.*, 1999) where *AMH* activates before *SOX9*. Our results also suggest that Sertoli cell differentiation occurs in the mole according to a centrifugal wave that proceeds rapidly through the dorsoventral axis of the gonad, between the s4b and s5a stages. A similar situation has been described for the mouse testis, where *Sry* also activates following a dynamic wave through the anteroposterior axis of the gonad, moving from the centre to the poles (Bullejos and Koopman, 2001). It is not known, however, whether the mouse *Amh* activates according to the same pattern, or whether mole Sertoli cells follow a centre-to-poles differentiation wave.

Testicular cords of the mole testis organize slowly and after Sertoli cell differentiation

Our results in the mole show that cord formation takes place

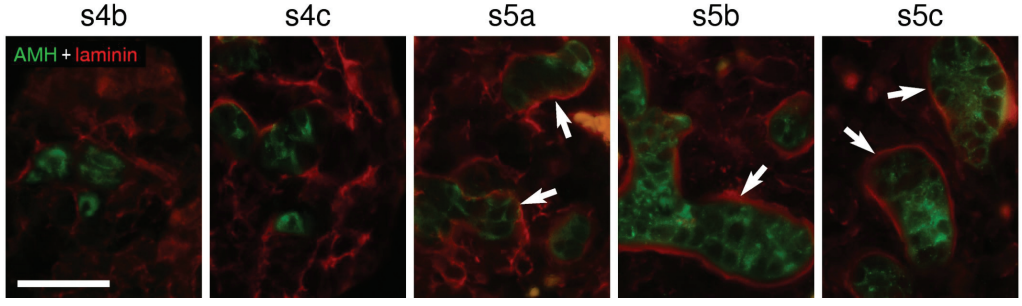

Fig. 5. Formation of the testis cords in male moles. *Merged images of laminin+AMH double sequential immunostaining of mole gonads in five developmental stages from s4b to s5c are shown. The first laminin deposits appear as early as the s4b stage, located around the first few differentiated Sertoli cells. Testis cord-like structures are not detectable at s4c, and first appear at s5a. Closed, well-formed testis cords are first observed in s5b embryos, when all Sertoli cells (AMH-positive) appear enclosed inside a testis cord, and appear more clearly defined at s5c stage. Fluorescence colours are red for laminin and green for AMH. Arrows point out cord-like structures and testis cords in s5a and s5b-c, respectively. Scale bar represents 40 μm.*

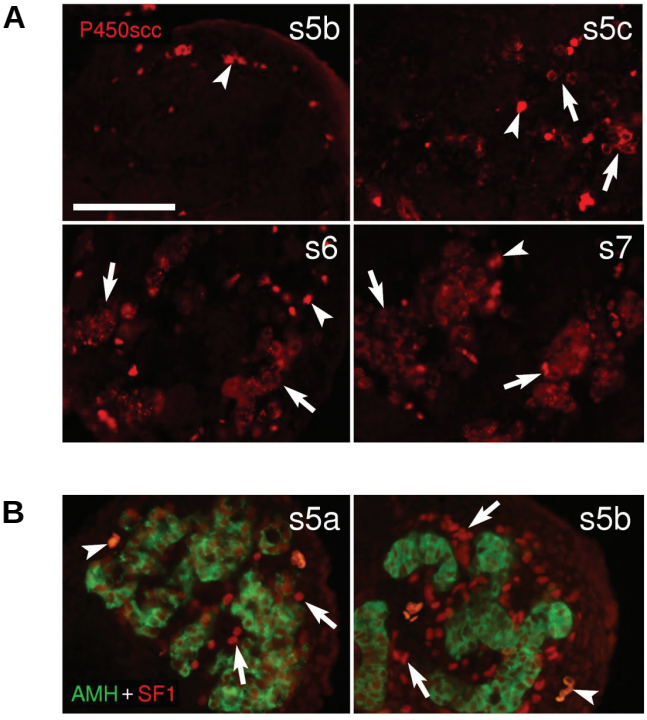

Fig. 6. Differentiation of Leydig cells in the mole testis. **(A)** *Immunostaining for the cytochrome P450scc shows that the first Leydig cells appear at the s5c stage (arrows), as no interstitial cell is immunoreactive in the previous stage, s5b. The number of differentiated Leydig cells increases during the two following stages s6 and s7, where large patches of them are formed (arrows).* **(B)** *Merged images of double, sequential immunostaining for the Sertoli cell marker AMH (cytoplasmic green fluorescence) and the Sertoli- and steroidogenic cell marker SF1 (nuclear red fluorescence), evidences that Leydig cell precursors (expressing* SF1 *but not* AMH; *arrows) are present in the male gonad of s5a and s5b mole embryos. Red fluorescence is stronger in Leydig cell precursors than in Sertoli cells. The number of Leydig cells increases between s5b and s5c. Scale bar represents 100 μm. Arrowheads point to autofluorescent erythrocytes in all pictures.*

slowly, throughout a three- day period, from the s4b stage, where the first laminin deposits appear, to the s5b stage, where testis cords are histologically recognizable for the first time, as described previously (Barrionuevo *et al.*, 2004a). Immunofluorescence images of Sertoli cell markers in the mole *(SF1*, *SOX9*, *AMH* and *DMRT1)* reveal structures resembling cords in s5a testes, although at this stage, testis cords are not yet histologically defined. Similarly, laminin immunostaining shows that the organization of some cords is quite advanced by the s5a stage (Fig. 5). These data indicate that, even though testis cords are not histologically recognizable before the s5b stage, functional compartments do already exist in the gonads of s5a mole embryos, but not at earlier stages. This proves consistent with the hypothesis that cords differentiate in the murine testis before they become morphologically conspicuous, and that it is the interposition of less dense tissue composed of migrating and proliferating mesenchymal cells that allows for the identification of those cords (Merchant-Larios and Taketo, 1991; Merchant-Larios *et al.*, 1993).

The formation of testis cords is crucial for the development and functioning of the male gonad. In the mouse embryo, it has been ascertained that both Sertoli cell differentiation and testis cord formation occur in just one day, between 11.5 and 12.5 dpc, and that these two events are the results of mesonephric cell migration (Tilmann and Capel., 1999). Magre and Jost (1984) suggested that Sertoli cells can differentiate in the absence of testis cords, and Yao and Capel (2002) have provided evidence to further corroborate this presumption. In the mole, on the other hand, Sertoli cell differentiation is very fast, whereas testis cord formation is very slow in comparison with the mouse, thus rendering it unlikely that these two events share a common origin. Rather, the sequence of events observed in the mole clearly suggests that testis cord formation requires the presence of previously differentiated Sertoli cells.

Sertoli cell differentiation does not depend on mesonephric cell migration

Cell migration is a key event in gonad development. Although there is intragonadal cell movement at different stages of testis development (see MT1-MMP immunostaining at s4b and s5c stages in Fig. 4), the migration of mesonephric cells to the gonad prior to the formation of the testis cords is particularly important (Buehr *et al.*, 1993). In the mouse, it has been suggested that the differentiation of Sertoli cells, the formation of the testis cords and the establishment of the testis-specific vascular system require such migration (Tilmann and Capel, 1999; Brennan *et al.*, 2002). Accordingly, a prerequisite for this hypothesis is that mesonephric cell migration must precede all these developmental events.

Due to the specific nature of our study material, which consists in gonads of mole embryos obtained from pregnant female moles captured in the wild, this material is very scarce, and therefore *in vitro* organ culture experiments, which might provide tangible evidence for mesonephric cell migration, are unlikely to be a feasible project with this species. Thus, although the presence of structures such as a profuse vasculature (our unpublished data), and such cell types as peritubular myoid cells (Barrionuevo *et al.*, 2004), known to rely upon mesonephric cell migration, lead to the assumption that it also occurs in the mole, we are unable to find direct evidence pinpointing the time when this cell migration takes place. However, it is possible to know when migration is absent. Here we have investigated the presence of MT1-MMP, a migrating cell marker (see Gálvez *et al.*, 2001), and PDGFRα, a key component of a signalling pathway involved in XY gonad-specific cell proliferation, mesonephric cell migration and Leydig cell differentiation in the mouse (Brennan *et al.*, 2003; Ricci *et al.*, 2004; see Fig. 4). The expression of *MT1-MMP* does not indicate by itself that migration occurs, as it may also reflect local, non-migratory, cell movement, but the absence of these markers constitutes clear and direct evidence that no migration has taken place. Thus, we provide evidence that no cells migrate from the mesonephros to the gonads in mole embryos at the s4c or previous stages, as *PDGFRα* expression is very low, and *MT1-MMP* expresses inside the gonad, but not in the mesonephros-gonad border or in adjacent mesonephric areas (see Fig. 4). Hence, considering that cells in the gonads of s4b and s4c mole embryos do express both *SOX9* and *AMH* Sertolian markers, we conclude that mesonephric cell migra-

tion does not precede Sertoli cell differentiation and that it is not necessary for the endocrine differentiation of Sertoli cells.

PDGFR-α-independent intragonadal cell movement/migration accompanies the formation of the* tunica albuginea *of the testis

The *tunica albuginea testis* is the major component of the capsule of mammalian testes. It is composed mainly of fibroblasts interspersed with collagen fibres (see Setchell *et al*., 1994), and contains contractile cells (Middendorff *et al*., 2002). Different aspects of the function of the tunica albuginea have been investigated, including 1) promotion of sperm export to the epididymis, 2) contribution to maintaining the intratesticular pressure and 3) control of the testicular blood flow (for a review, see Setchell *et al*., 1994), but little is known about its development. In the mouse, the tunica albuginea begins to form at 12.5 dpc, once the testis cords have organized (Karl and Capel, 1998). In the mole, morphological differentiation of the tunica albuginea occurs between s6 and s8 stages (Barrionuevo *et al*., 2004a), but in the present paper we report that the process begins one day earlier, at the s5c stage. This process involves mainly the production of collagen fibres and the differentiation of the cells forming the basement membrane layer underlying the coelomic epithelium, which flatten and dispose parallel to the surface epithelium. These shape changes probably require active cell movement, which is consistent with our finding that these cells show a marked expression of the migration marker *MT1-MMP*. Unlike mesonephric cell migration, these cell movements start just as *PDFGR-α* is repressed in the testis, which indicates that intragonadal cell migration/movement accompanying the tunica albuginea differentiation is not mediated by *PDFGR-α* signalling.

Mole embryonic Leydig cells differentiate once* WNT4 *and* PDGFR-α *are repressed

The differentiation of mole embryonic Leydig cells begins at the s5c stage, when *P450scc* expression indicates that testosterone is being produced. However, we have shown that their cell precursors were already present in the testis interstitial regions two days before, at stages s5a and s5b, but not in previous stages. In the mouse, it has been demonstrated that Leydig cell precursors are present in the gonads of 11.5 dpc embryos at least one day before the differentiation of these cells at 12.5 dpc (Brennan *et al*., 2003). This raises the question as to why Leydig cell differentiation does not occur as soon as the precursors are present both in moles and in mice. Recent papers have reported that the presence of Leydig cells in the gonad is controlled by at least four genes involved in different signalling pathways: *PDFGR-α, DHH, ARX and WNT4.* The first three genes promote the formation of Leydig cells in testes (Kitamura *et al*., 2002; Yao *et al*., 2002; Brennan *et al*., 2003), whereas the latter gene inhibits their formation in ovaries (Jeays-Ward *et al*., 2003).

Brennan *et al*. (2003) have shown that Leydig cell development requires *Pdfgr-α*, and endorse the hypothesis that this gene is involved in a signalling pathway which promotes the expansion of Leydig cell precursors. However, mouse data did not provide concluding evidence as to whether *Pdfgr-α* is also involved in Leydig cell differentiation. The expression pattern of the mole *PDFGR-α* gene supports the hypothesis that this gene is involved in the maintenance and/or proliferation of Leydig cell precursors, but clearly rules out the possibility that it is responsible for Leydig cell differentiation, as *PDFGR-α* expression coincides at the s5a and s5b stages with the presence of Leydig cell precursors, but ceases just before Leydig cell differentiation takes place at s5c. The fact that *PDFGR-α* is also involved in cell migration, is consistent with the hypothesis that the precursors of Leydig cells migrate from the mesonephros to the gonad, together with those of peritubular myoid cells and endothelial cells.

Vainio *et al.,*1999) identified WNT4 as part of the signalling pathway responsible for the absence of Leydig cells in female gonads, and suggested that this gene suppresses the differentiation of the Leydig cell precursors already present in the gonad. Contrarily, based on their observations in transgenic XY mice expressing Wnt4, Jeays-Ward *et al*. (2003) have shown that one function of this gene is to suppress the migration of a few steroidogenic adrenal cells within the gonad. In the present paper we show that *WNT4* expresses in the developing mole testis from the s4b to s5b stages. This expression pattern is consistent with the function of WNT4 in suppressing the differentiation of pre-existing Leydig cell precursors, as Leydig cell differentiation occurs just when *WNT4* expression ceases, but not with the preclusion of mesonephric cell migration, as most cell migration probably occurs during the s5a and s5b stages, which coincides with the expression timing of *WNT4*. Nevertheless, in mouse female gonads, WNT4 represses mesonephric cell migration through follistatin (Yao *et al*., 2004); therefore, the expression of *WNT4* in mole gonads at stages where cell migration is taking place is not surprising, as no follistatin has been detected in those gonads (our unpublished data). It also bears pointing out that the *WNT4* expression observed in the gonads of male moles is not as intense as that found in females (unpublished). In this sense, it is necessary to consider that the expression level detected in males might have no functional effect. Quantitative RT-PCR analyses currently being undertaken in our lab may shed light on this issue.

Concluding remarks

The laboratory mouse permits the use of the most powerful research tools in modern biology, including transgenesis and targeted mutagenesis. The logical consequence is that in the better part of the ongoing research in mammalian developmental biology, the laboratory mouse is used as an animal model. However, in some instances, the results of this research cannot be extrapolated to the rest of mammals -including humans- since the mouse itself is the exception in these cases. A case in point is the genetic system controlling sex determination: the mouse *Sry* gene is unique among mammals with regard to its structure and expression pattern (Capel *et al*., 1993). Hence, other mammalian species must be investigated to test the degree of evolutionary conservation of the developmental processes discovered in the mouse. One of the main difficulties found in research on gonad development in the mouse is that many complex genetic and cellular events take place almost simultaneously between 11.5 and 12.5 dpc, making it difficult to establish the sequence of those events, which is necessary to postulate cause-effect relationships. In other mammals, this very same process is much slower, which facilitates the work. This is the case for testis organogenesis in the mole, which we have depicted in this paper. Our results from studying the mole provide evidence that the

TABLE 1

ANALYSED MATERIAL CLASSIFIED ACCORDING TO DEVELOPMENTAL STAGES

Developmental stage	Age (dpc)	No. of litters	No. of male embryos analysed
s4b	15	3	5
s4c	16	2	4
s5a	17	4	7
s5b	18	4	7
s5c	19	3	5
s6	19-21	2	3
s7	21-23	2	3

spatio-temporal pattern of testis development is not perfectly conserved in mammals, since we found differences with respect to the mouse testis organogenesis. This fact is even more significant when we consider that, apart from the mouse, the mole is probably the one of the best-known mammalian species in terms of the genetic control of testis development, implying that more peculiarities would be found if more species were investigated.

Materials and Methods

Material analysed

A total of 34 male embryos of the mole species *T. occidentalis* were analysed. As moles do not breed in captivity, embryonic gonad collection required the dissection of wild pregnant female moles. They were captured in poplar groves around Chauchina and Santa Fé (Granada province, southern Spain), as described previously (Barrionuevo *et al.*, 2004a). Captures were made under annual permission granted by the Andalusian Environmental Council. Handling followed the guidelines and approval of the 'Ethical Committee for Animal Experimentation' of the University of Granada. Table 1 summarizes the material analysed according to the developmental stages described by Barrionuevo *et al.* (2004a and b) for this species. However, in this study, we have distinguished three new substages in the s4 stage (s4a-c), in accordance with our new findings in these embryos. At the s4a stage, mole embryos show no gonad development, at s4b the gonad emerges, and at s4c it remains morphologically undifferentiated.

Five litters of Swiss mouse embryos, from 10.5 to 14.5 dpc, provided by the Laboratory Animal Production Unit of the University of Granada, were used to establish the equivalences between the developmental stages of the mole and the mouse throughout the gestation period in which gonad differentiation occurs in both species, and to perform immunofluorescence analyses in order to test the specificity of the antibody produced in our laboratory (see below).

Developmental staging and sexing of the embryos

After capture, pregnant females were detected by abdominal palpation, and the developmental stages of the embryos were thus estimated by the size of the uterine swellings. Accurate determination of the developmental stage of each litter was performed after dissection, based on the values of crown-rump length (CRL) and body mass, and the morphology of major external structures, according to Barrionuevo *et al.* (2004b). As embryos of the s4a-c and s5a stages are not sexually differentiated, identification of individual sex based on morphological criteria is not possible. These embryos were sexed by performing sex-chromatin preparations of amniotic cells, which were analysed for the presence (females) or absence (males) of the Barr's body. Sex-chromatin preparations were made as in Jiménez *et al.* (2000).

Immunofluorescence

After the embryonic gonads were dissected out, one was fixed in paraformaldehyde solution (4%) overnight. The other gonad from each embryo was immediately frozen in liquid nitrogen for further purification of RNA samples. After having been washed in PBS, fixed samples were dehydrated, embedded in paraffin wax and sectioned, following standard procedures. Gene-expression analyses were performed by immunofluorescence. De-waxed preparations were exposed to specific primary antibodies for several h, then to fluorochrome-conjugated secondary antibodies, as summarized in Table 2. In some instances, double, sequential immunostaining was performed with two different primary antibodies, and the resulting images were then merged using the Gimp program. Two complete sets of gonads at all developmental stages were used with each primary antibody in order to test the reproducibility of the results. For the more controversial results, we repeated the immunofluorescence technique, using up to five different gonads, when available.

Molecular cloning, sequencing and antibody production

Commercially available antibodies for the human or mouse protein WNT4 were not immunoreactive against the corresponding protein from the mole. Therefore, we needed to produce a new mole-specific WNT4 antiserum. A fragment of the mole *WNT4* gene was PCR amplified and sequenced. For the PCR amplification, forward (5'-TTG TGG ATG TGC GGG AGA G-3') and reverse (5'-TGT GTG CGG CTT GAA CTG TG-3') primers were selected from among evolutionarily conserved sequences. The 293 base pair (bp) PCR product was sequenced in an automatic Applied Biosystem 3100-Avant Genetic Analyser. The sequence obtained was translated *in silico,* and the "antigenic" software from the

TABLE 2

PRIMARY ANTIBODIES USED IN THIS STUDY

Gene[1] product	*Description/Utility*	*antibody source*	*references[2]*
AMH	Anti-Müllerian hormone. Sertoli cell marker.	goat polyclonal, raised against human protein	Santa Cruz Biotechnology Ref. sc-6886
DMRT1	Zinc-finger-like DNA-binding motif protein. Sertoli and germ cell marker.	rabbit polyclonal raised against mouse protein	Dr. Sylvana Guioli
Laminin	Basal lamina protein. Testis cord formation marker.	rabbit polyclonal, raised against mouse protein	SIGMA Ref. L9393
MT1-MMP	Membrane type 1-matrix metalloproteinase. Migrating cell marker.	mouse monoclonal, raised against human protein	Dr. Alicia G. Arrollo
P450 scc	Androgen metabolism cytochrome. Leydig cell marker.	goat polyclonal, raised against human protein	Santa Cruz Biotechnology Ref. sc-18043
PDGFRα	α receptor of the platelet-derived growth factor. Indirect migration signalling.	rabbit polyclonal, raised against human protein	Santa Cruz Biotechnology Ref. sc-338
SF1	Steroidogenic factor. Sertoli and Leydig cell marker.	rabbit polyclonal, raised against mouse protein	Dr. Ken Morohashi
SOX9	Transcription factor SOX. Sertoli cell marker.	rabbit polyclonal, raised against human protein	Santa Cruz Biotechnology Ref. sc-20095
WNT4	Signalling molecule involved in the ovarian development.	rabbit polyclonal, raised against mole protein	Developed by the authors

1) Gene product names: AMH, Anti-Müllerian Hormone; DMRT1, dsx- and mab3-related transcription factor 1; MT1-MMP, Matrix Type 1 Membrane Metalloproteinase, P450scc, P450 side chain cleavage; PDGFRα, Platelet Derived Growth Factor receptor α; SF1, Steroidogenic Factor 1; SOX9, Sry-like HMG-Box protein 9; WNT4, wingless-related MMTV integration site family member 4.

2) Dr. Sylvana Guioli, National Institute for Medical Research, MRC, London, UK. Dr. Alicia G. Arrollo, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain. Dr. Ken Morohashi, National Institute for Basic Biology, Okazaki, Japan.

EMBO package (Rice *et al*., 2000) was used to identify potential antigenic sites. The peptide sequence RVECKCHGVSGSCEV from the mole WNT4 protein was synthesised and used to immunize rabbits. One New Zealand rabbit, 60 days post partum (dpp), was immunized with the mole WNT4 peptide, following standard procedures. The resulting serum was titrated by performing periodical ELISA tests, and the rabbit was bled when titres reached values above 1/450. This antibody was tested by western blotting and immunofluorescence, following standard procedures.

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