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DEVELOPMENT AND DISEASE

Dax1 regulates testis cord organization during gonadal differentiation

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SUMMARY

Mutations of the *DAX1* nuclear receptor gene cause adrenal hypoplasia congenita, an X-linked disorder characterized by adrenal insufficiency and hypogonadotropic hypogonadism. Targeted deletion of *Dax1* in mice also reveals primary testicular dysgenesis, which is manifest by obstruction of the rete testis by Sertoli cells and hyperplastic Leydig cells, leading to seminiferous tubule dilation and degeneration of germ cells. Because Dax1 is expressed early in gonadal development, and because Sertoli and Leydig cells are located ectopically in the adult, we hypothesized that these testis abnormalities are the result of an early defect in testis development. In *Dax1*$^{-/Y}$ males, the gonad develops normally until 12.5 dpc. However, by 13.5 dpc, the testis cords are disorganized and incompletely formed in *Dax1*-deficient mice. The number of germ and Sertoli cells is unchanged, and the expression of Sertoli-specific markers appears to be normal. However, the number of peritubular myoid cells, which normally surround the testis cords, is reduced. BrdU labeling of peritubular myoid cells is low, consistent with decreased proliferation. The basal lamina produced by peritubular myoid and Sertoli cells is disrupted, leading to open and incompletely formed testis cords. Leydig cells, which normally reside in the peritubular space and extend from the coelomic surface to the dorsal surface of the gonad, are restricted to the coelomic surface of *Dax1*-deficient testis. We conclude that Dax1 plays a crucial role in testis differentiation by regulating the development of peritubular myoid cells and the formation of intact testis cords. The developmental abnormalities in the *Dax1*-deficient testis lay the foundation for gonadal dysgenesis and infertility in adult mice and, potentially in humans with *DAX1* mutations.

Key words: Dax1, Sex differentiation, Gonadal development, Mouse

INTRODUCTION

DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene 1) is a member of the nuclear receptor superfamily (NR0B1) (Achermann et al., 2001). DAX1 is expressed in the adrenal cortex, gonads, ventromedial hypothalamus and pituitary gonadotropes (Ikeda et al., 1996; Ikeda et al., 2001). It is characterized as an orphan receptor, as no ligand has been identified to regulate its function. Though DAX1 shares structural homology with the C terminus of other nuclear receptors, it lacks the zinc-finger DNA-binding domain that is characteristic of most nuclear receptors (Muscatelli et al., 1994). Instead of the zinc fingers, the N terminus of DAX1 contains multiple copies of a unique 66 amino acid repeat motif that appears to mediate protein-protein interactions. DAX1 acts in part by repressing the transcription of other nuclear receptors, including steroidogenic factor 1 (SF1) (Ito et al., 1997; Lalli et al., 1997), estrogen receptor (Zhang et al., 2000) and androgen receptor (Holter et al., 2002).

Mutations of DAX1 in humans cause the X-linked clinical syndrome, adrenal hypoplasia congenita (AHC) (Muscatelli et al., 1994). This syndrome is characterized by impaired development of the adult zone of the adrenal cortex, leading to adrenal insufficiency and by hypogonadotropic hypogonadism, caused by impaired production of hypothalamic GnRH and pituitary gonadotrope production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (reviewed by Achermann et al., 2001). More recently, individuals with this syndrome have been shown to have gonadal dysgenesis that is independent of the gonadotropin deficiency (Ozisik et al., 2002). Treatment with exogenous gonadotropins stimulates testosterone production but does not appear to normalize spermatogenesis in the few individuals who have been carefully studied (Reutens et al., 1999).

Targeted mutagenesis of the genes encoding orphan nuclear receptors has proven useful for clarifying their functions, especially as there are no known ligands that can be used as agonists or antagonists. Disruption of *Sf1*, for example, causes adrenal and gonadal agenesis and leads to impaired gonadotropin production, indicating that it plays a crucial role in the development of these glands, as well as regulating the expression of a variety of target genes involved in steroidogenesis and reproduction (Ingraham et al., 1994; Parker et al., 2002). Targeted mutagenesis of *Dax1* (also known as *Ahch*; *Nr0b1* – Mouse Genome Informatics) has also been performed, using a Cre-loxP strategy to circumvent the X-linked infertility in males (Yu et al., 1998). The phenotype of these mice is similar to that of individuals with the AHC syndrome, although the adrenal and pituitary abnormalities are less pronounced in mice (Yu et al., 1998; Babu et al., 2002). The testes of the *Dax1*-deficient mice were initially shown to be small with degeneration of the seminiferous epithelium and loss of germ cells (Yu et al., 1998). Subsequent studies revealed multiple abnormalities including clusters of poorly differentiated Sertoli cells within the lumen of the seminiferous tubules and efferent ducts, hyperplasia of epithelial cells within the rete testis and efferent ducts and Leydig cell hyperplasia (Jeffs et al., 2001b). The lumen of the rete testis, normally a patent passage formed by the union of seminiferous tubules and the epididymus, is obstructed by these cells, leading to dilation and pressure atrophy of seminiferous tubules. In the area around the rete testis, the basal lamina that encircles the seminiferous tubules is intermittently disrupted. Clusters of undifferentiated PTM cells surround the seminiferous tubules and Leydig cells are ectopically located within the tubules (Jeffs et al., 2001b). These features led us to hypothesize that *Dax1* deficiency causes a defect during early gonadal development.

Development of the gonad is a complex and highly orchestrated process (Tilmann and Capel, 2002; Yao et al., 2002a). The indifferent gonad arises from the ventral surface of the urogenital ridge (Kaufman, 1995). At 10.5 dpc, the primordial germ cells (PGCs) migrate from the allantois into the gonad of both sexes (Hogan, 1994). A wave of Sry expression is initiated at 10.5 dpc in the male gonad and begins the process of sexual dimorphism (Hacker et al., 1995). Soon after Sry expression, the pre-Sertoli cells at the coelomic surface proliferate and invade the male gonad where they cluster near the germ cells (Schmahl et al., 2000). Peritubular myoid (PTM) cells surround and enclose the Sertoli and germ cells, creating discrete testis cords. It is hypothesized that PTM cells are among the cells that originate from the mesonephros (Martineau et al., 1997; Tilmann and Capel, 1999). When the PTM and Sertoli cells make contact, they secrete a matrix that forms the basal lamina, separating the testis cords from the interstitial compartment (Tung et al., 1984). Delay or disruption of testis cord formation could result in gonadal dysgenesis or infertility. In this report, we examined early gonadal development in *Dax1*-deficient mice to assess whether alterations might account for the testicular dysgenesis seen in adult mice and in humans with *DAX1* mutations. We identify a marked deficiency of PTM cells, associated with incomplete formation of testis cords, providing the likely cause of the progressive pathogenesis and infertility seen in animals of reproductive age.

MATERIALS AND METHODS

Mouse and embryo dissection

Wild-type 129/SvJ (Jackson Labs) males were bred to *Dax1*$^{Del/Wt}$ females (129/SvJ) to attain both wild-type and *Dax1*$^{-/Y}$ male mice. Animals were placed together at approximately 4:00 pm and vaginal plugs were examined the following morning by 8:00 am. Females were euthanized and embryos were dissected on days 11.5, 12.5, 13.5, 14.5 and 17.5 after mating, defining the first day after plug identification as 0.5 dpc. A region of the embryo was used for genotyping by PCR. Whole-mount gonads were dissected, examined with a MZLF III dissecting microscope (Leica, Heerbrugg, Switzerland) and photographed with a MagnaFire digital camera (Optronics, Goleta, CA). All animals were housed in a barrier facility under normal light and dark conditions and fed ad libitum; all procedures were approved by the Northwestern University Animal Care and Use Committee.

Histology, immunohistochemistry, and in situ hybridization

For immunohistochemistry, embryos were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and 3 μm sagittal sections were cut using a Jung microtome (Leica, Heerbrugg, Switzerland). Tissue sections were viewed with a Zeiss Axioskop (Thornwood, NY). Standard histological techniques were used for Hematoxylin and Eosin staining. Sections were deparaffinized in xylenes and descending ethanols, followed by antigen retrieval in sodium citrate buffer. Sections were blocked in normal serum (5%) for 45 minutes and incubated with primary antibody for 4 hours at room temperature or 4°C overnight. Rabbit anti-laminin (Sigma, 1:200), goat anti-GATA-4 (Santa Cruz, 1:200), Dax1 (1:3,000) and Sf1 (K. Morohashi, 1:500) were used as primary antibodies, with specific secondary antibodies at 1:200 dilution (Jackson Immunoresearch). After washing with PBS+0.1% Triton X, sections were incubated in secondary antibody for 2 hours at room temperature, washed again and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). In situ hybridization was performed using a standard protocol (Wilkinson, 1998). Probes for Amh, Scc, Dhh and Sox9 were graciously provided by B. Capel (Duke University), Andy McMahon (Harvard University) and Peter Koopman (University of Queensland). In all cases, littermates were used as wild-type controls.

Cell death and proliferation

To determine if cells were dividing, 0.1 mg/g body weight of 5-Bromo-2′-deoxyuridine (BrdU 10 mg/ml, Sigma-Aldrich), a thymidine analog incorporated during S phase was injected i.p. into pregnant mothers. Two hours post-injection, embryos were removed, fixed in 10% formalin and embedded. After sectioning, slides were deparaffinized and treated with sodium borate (pH 8.5) to quench endogenous fluorescence. To increase penetration of the antibody, slides were treated with pepsin (10 mg/ml, Sigma-Aldrich) in 0.01 N HCl at 37°C for 1 hour, followed by incubation with DNase (100 U/ml) at 37°C for 1 hour to further increase nuclear penetration. Rat anti-BrdU:FITC antibody (Serotec, Kidlington, Oxford, UK) was applied to sections for 2 hours at room temperature. When bright-field microscopy was employed, sections were then incubated with biotinylated rabbit-anti-rat secondary antibody, followed by RTU horseradish peroxidase streptavidin and finally development with DAB peroxidase substrate (all from Vector Laboratories Burlingame, CA), Slides were then washed and covers were applied. When colocalization with BrdU was used, slides were treated by antigen retrieval instead of pepsin/DNase and the anti-BrdU antibody was applied at the time of incubation with the secondary antibody. Cell count values were made on eight wild-type and 10 Dax1KO gonads.

Cell death was determined by labeling the fragmented DNA generated by endonucleases during apoptosis. Instructions provided

in the Fluorescein-FragEL Cell Death Detection Kit (Oncogene, La Jolla, CA) were followed. Briefly, sections were deparaffinized through xylenes and ethanols, and permeabilized with proteinase K for 20 minutes. After equilibration with reaction buffer, sections were treated with the TdT labeling reaction for 1.5 hours at 37°C, washed and mounted. Sections were viewed with a Zeiss Axioskop.

RESULTS

Testis cords do not form normally in *Dax1*-deficient testis

Dax1-deficient gonads were initially characterized beginning at 13.5 dpc, a time point when the testis cords are readily discernable. In wild-type mice, a large arterial vessel is characteristic of the male gonad, and courses across the coelomic surface (asterisk, Fig. 1A) (Brennan et al., 2002). When viewed from the medial surface (Fig. 1B), testis cords are visible and are separated by thick columns of cells that define the peritubular space (arrows, Fig. 1A). The male gonad (testis) is larger than the female gonad (ovary) when compared across the coelomic-mesonephric axis (Fig. 1B versus 1F). In the *Dax1*-deficient testis, the coelomic vessel is still present on the ventral surface, but the ventral-dorsal width is intermediate between male and female gonads (Fig. 1D). Most strikingly, there are relatively few testis cords in the *Dax1*-deficient testis and they are poorly distinguished from the peritubular space (Fig. 1D). As an occasional testis cord can be identified, the *Dax1*-deficient gonad is not sex reversed.

Histological examination of wild-type and *Dax1*-deficient gonads was performed during the time that spans testis cord formation and early gonadal differentiation (11.5-17.5 dpc; Fig. 2). The normal gonad is histologically indifferent at 11.5 dpc, and there are no apparent differences between wild-type male, female or *Dax1*-deficient gonad at 11.5 dpc (Fig. 2A,B).

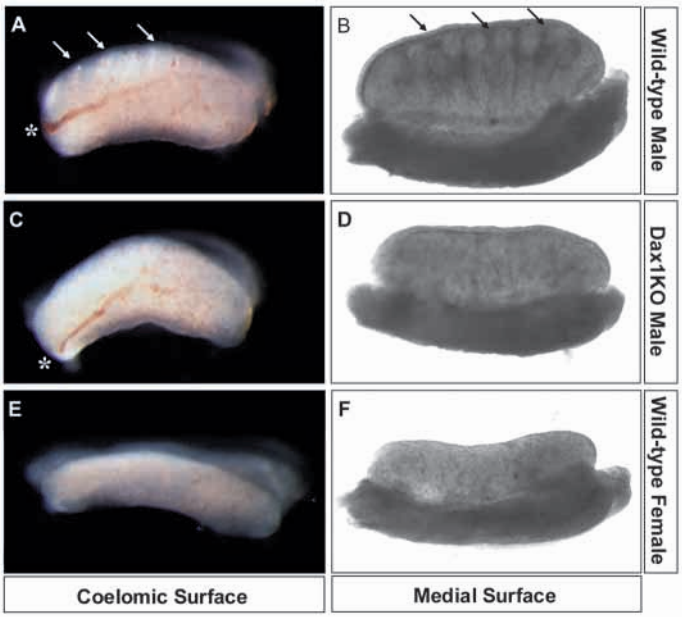

Fig. 1. Testicular cord formation is delayed in *Dax1*-deficient testis. Gross examination of gonads from wild-type male (A,B) and female (E,F), and *Dax1*-deficient testis (C,D). Gonads were dissected from 13.5 dpc embryos; littermates were used for comparison. Testis cords are readily observed in wild-type testis, whereas a decreased number of cords, which are less organized, are present in Dax1-deficient testis (arrows) middle. Although the *Dax1*-deficient testis is larger than a wild-type female gonad (bottom), it is smaller than the wild-type male gonad. Coelomic and medial surfaces of gonads are displayed in left and right columns. Gonads on the right are visualized with light reflected from a mirror below the stage in order to gain better perspective for condensed tissue. Asterisks indicate the coelomic vessel.

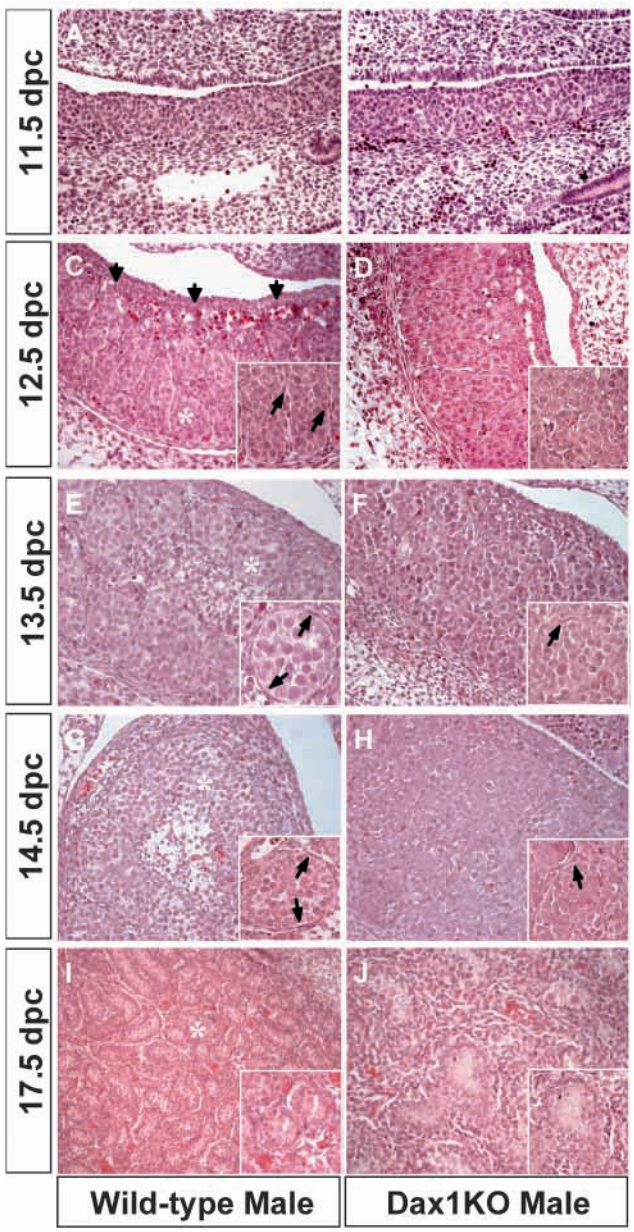

Fig. 2. Histological evidence of disrupted testis cords in *Dax1*-deficient testis. Histological comparison of *Dax1*-deficient (B,D,F,H,J) and wild-type (A,C,E,G,I) testes taken at 11.5 dpc (A,B), 12.5 dpc (C,D), 13.5 dpc (E,F), 14.5 dpc (G,H) and 17.5 dpc (I,J). Although development of the testis cords is readily discerned in wild-type testis, little organization is present in *Dax1*-deficient testis. Asterisks identify testis cords; arrows indicate peritubular myoid cells.

Thus, Dax1 is not required for gonadal development from the urogenital ridge. At 12.5 dpc, sexual dimorphism is first apparent histologically. On the ventral surface of the male gonad, the coelomic vessel is present and filled with red-blood cells (arrowheads, Fig. 2C). This large arterial vessel extends capillaries dorsally into the substance of gonad within the peritubular space. Individual testis cords are identifiable and PTM cells circumscribe their borders (asterisks, arrow; Fig. 2C). As observed in the whole-mount analyses, *Dax1*-deficient testes have a patent coelomic vessel, yet few capillaries are present the intertubular space (Fig. 2D). Within the *Dax1*-deficient testis, Sertoli and germ cells are heterogeneously clustered without discernable separation between the cords (Fig. 2D). Further inspection suggests that the testis cords lack organization because of a marked reduction in PTM cells in the Dax1KO testis.

At 13.5 dpc, the testis cords are larger and more clearly defined in the wild-type gonad (asterisk, Fig. 2E). PTM cells become differentiated and flatten, forming a concentric layer of cells that encircles the testicular cord (arrows, Fig. 2E). Differentiated Sertoli cells are present at the periphery of the tubule and their apical cytoplasm is polarized opposite to the PTM cells (Fig. 2E). In the *Dax1*-deficient testis, germ cells and Sertoli cells are seen within the dorsal surface of the gonad (Fig. 2F). These cells cluster together at 13.5 dpc, but few PTM cells are observed (arrow, Fig. 2F). Although the phenotype encompasses a spectrum of cellular disorganization, very few discrete testis cords are discernable from peritubular tissue in the *Dax1*-deficient testis.

By 14.5 dpc, the normal male testis is more sphere-shaped (Fig. 2G). The testis cords become further differentiated as the triangular-shaped Sertoli cells line the outer-edge of the cord in clear opposition to the PTM cells (arrows, Fig. 2G). By contrast, testis cords remain rudimentary in *Dax1*-deficient testis (Fig. 2H). Occasionally, scattered PTM cells are present in *Dax1*-deficient gonads at 14.5 dpc (arrow, Fig. 2H). This occurs predominately at the ventral surface, leaving the dorsal half of the gonad more disorganized (Fig. 2H). Most cells remain round and undifferentiated in *Dax1*-deficient gonads. At 17.5 dpc, testis cords are round, uniform in size and resemble early seminiferous tubules (asterisk, Fig. 2I). In the *Dax1*-deficient testis, individual tubules are larger in size and pleomorphic in shape, without clearly defined borders (Fig. 2J).

The basal lamina is discontinous or absent in Dax1 deficient testis

Beginning at 12.5 dpc, PTM and Sertoli cells cooperate to deposit a layer of basal lamina that defines the edges of individual testis cords (Tung et al., 1984). Because PTM cells are reduced in the *Dax1*-deficient testis, we assessed the formation of the basal lamina. In wild-type males at 12.5 dpc, laminin is present in the peritubular space throughout the gonad as the nascent cords are first identifiable (Fig. 3A). In the *Dax1*-deficient gonads, laminin expression is disorganized and patchy as the peritubular space is less identifiable (Fig. 3B). By 13.5 dpc, the edge of individual testis cords is clearly identifiable by a sharp, continuous border of laminin (Fig. 3C). In *Dax1*-deficient gonads, laminin is expressed heterogeneously throughout the gonad (Fig. 3D). Although rudimentary cords are periodically identifiable, only fragments

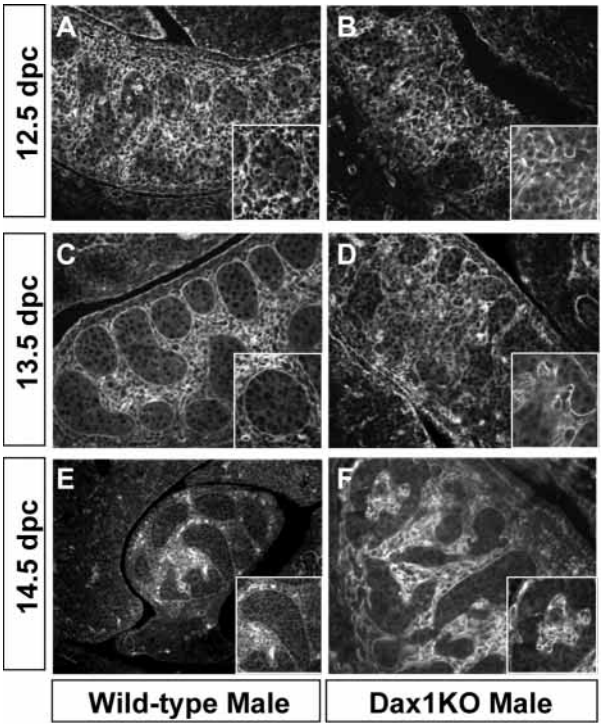

Fig. 3. The basal lamina is disrupted in Dax1 deficient testis at 12.5, 13.5 and 14.5 dpc. Immunohistochemistry with a laminin antibody reveals that laminin deposition clearly separates the testis cords from the peritubular space in wild-type testis (A,C,E); the basal lamina is disrupted or absent in *Dax1*-deficient testes (B,D,F).

of the circumferential border are positive for laminin (Fig. 3D). By 14.5 dpc, testis cord-like structures are more easily identifiable in the *Dax1*-deficient testis. However, these cords are smaller, irregular in shape, and there are frequent breaks in laminin localization (Fig. 3F). In some cases, a testis cord is observed to encircle an island of laminin (inset of Fig. 3F).

Sertoli cells are present but disorganized in *Dax1*-deficient testis

In the developing male gonad, Sertoli cells initiate formation of the testis cords (Schmahl et al., 2000). In the wild-type gonad, Sertoli cells are restricted to the testis cord, which create a scalloped pattern on the mesonephric side of the gonad when labeled by in situ hybridization for the Sertoli-specific genes *Dhh*, *Amh* or *Sox9* (Fig. 4A,C,E,G). In the *Dax1*-deficient gonad, expression of these markers is present, but staining is not discretely localized to the testis cords (Figs. 4B,D,F,H). All somatic cells in the male gonad express Gata4, but Sertoli cells express much higher levels than cells within the peritubular space (Viger et al., 1998). Sections were counterstained for laminin to clearly identify the testis cords (Gata4 positive, Laminin negative). In both wild-type and Dax1KO testis, Gata4-positive Sertoli cells are identifiable, despite differences in organization within the gonad (Fig. 4I,J).

Fetal Leydig cell development is arrested in *Dax1*-deficient males

The production of androgens by Leydig cells is critical for male sexual differentiation (Habert et al., 2001). Although the origin

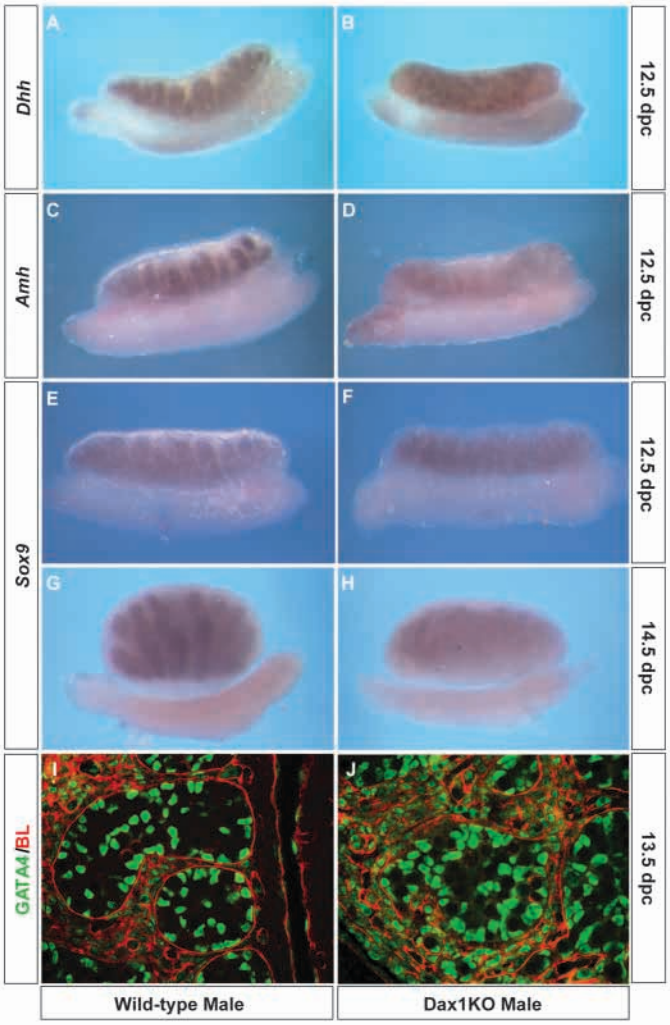

Fig. 4. Disorganization of Sertoli cells in *Dax1*-deficient testis. In situ hybridization for *Dhh* (A,B), *Amh* (C,D) or *Sox9* (E-H) reveals robust expression confined to the cords in wild-type testis, whereas expression is decreased and not clearly organized in *Dax1*-deficient testis. In situ hybridization for *Dhh* and *Amh* was performed at 12.5 dpc, and *Sox9* was analyzed at 12.5 and 14.5 dpc.
(I,J) Immunohistochemistry for Gata4 (green) and laminin (red) reveals decreased organization and differentiation of Sertoli cells at 13.5 dpc in *Dax1*-deficient testis (J) compared with wild-type males (I). Left panel, wild-type testis (A,C,E,G,I); right panel, *Dax1*-deficient testis (B,D,F,H,J).

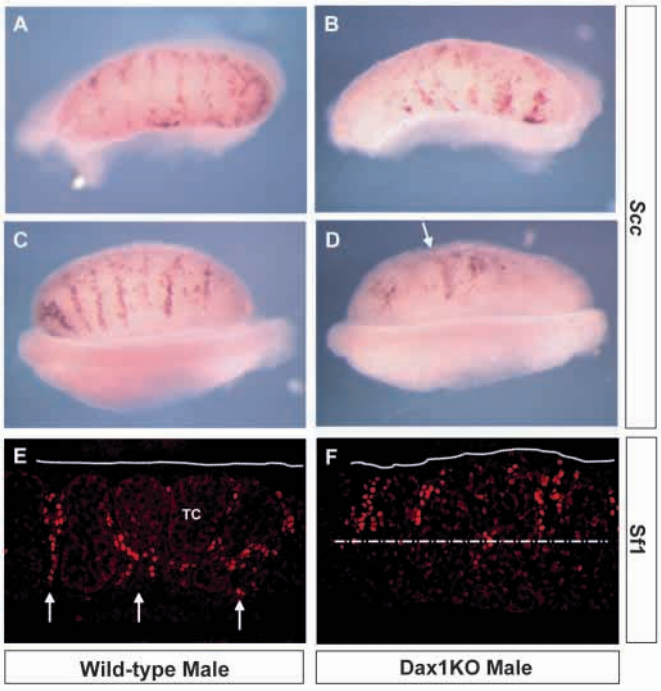

Fig. 5. Leydig cells in *Dax1*-deficient are restricted to the ventral surface. In situ hybridization for *Scc* reveals that while Leydig cells are present on the ventral surface of *Dax1*-deficient gonads (B,D) similar to wild-type males (A,C), but decreased in number and organization. Lateral examination (D) reveals that Leydig cells do not extend throughout the full ventral to dorsal thickness of the *Dax1*-deficient testis (arrow). Immunohistochemistry for Sf1 demonstrates that Sf1-positive Leydig cells are restricted to the coelomic surface in the *Dax1*-deficient testis (above the broken line, F) compared with the wild-type testis (E). Arrows identify the columns of Leydig cells in wild-type testis (E).

of these cells is unknown, immature Leydig cells are postulated to originate from within the gonad (Habert et al., 2001). One of the earliest distinguishing characteristics of immature Leydig cells is expression of side-chain cleavage (Scc), a steroidogenic enzyme required for androgen synthesis (Yao et al., 2002b; Habert et al., 2001). In the wild-type gonad, Scc-positive cells are arranged exclusively in columns between the testis cords (Fig. 5A,C). By in situ hybridization, these Scc-positive columns extend completely from the coelomic to mesonephric surface of the gonad (Fig. 5C). In the *Dax1*-deficient gonads, Scc-positive cells on the coelomic surface are located in the interstitium of structures that resemble testis cords (Fig. 5B). Overall, the number of Scc-positive cells on the coelomic surface appears similar to the wild-type male gonad. However, when viewed from the lateral aspect (Fig. 5D), very few Scc-positive cells can be identified. Sertoli cells weakly express Sf1, but its expression is strikingly upregulated in Leydig cells, probably because of the actions of Dhh (Yao et al., 2002b). These Sf1-positive Leydig cells are evident as columns that extend from the coelomic to the mesonephric surface in the normal testis in the same pattern as observed for *Scc* expression (arrows, Fig. 5E). In the *Dax1*-deficient testis, these cells are restricted to the coelomic surface and are almost completely excluded from the more dorsal part of the gonad (above the broken line in Fig. 5F).

Abnormal peritubular myoid cell development due to Dax1 deficiency

Testis cords require PTM cells for complete differentiation (Clark et al., 2000; Pierucci-Alves et al., 2001). This key myoepithelial cell-type is present exclusively in males. Histologically, few PTM cells are present in the *Dax1*-deficient gonad, suggesting a defect in PTM development (Fig. 2). DNA fragmentation was analyzed in both normal and Dax1-deficient gonads from 11.5 to 13.5 dpc to determine if PTM cells undergo accelerated apoptosis in the *Dax1*-deficient gonad. However, there was no significant difference in cell death between *Dax1*-deficient and wild-type mice (Fig. 6A-C). Rates

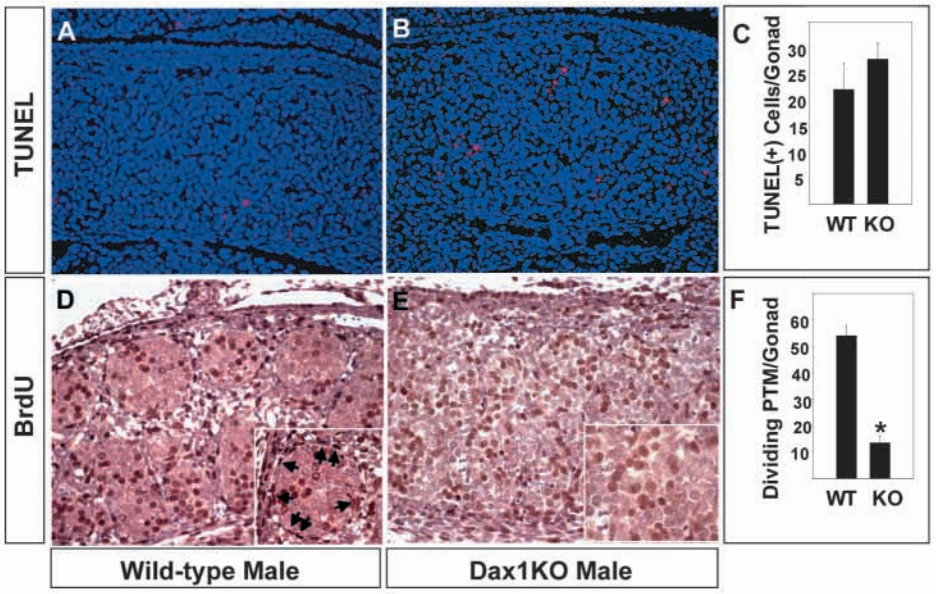

Fig. 6. Dividing peritubular myoid cells are reduced in the *Dax1*-deficient testis. TUNEL staining of the wild-type (A) and *Dax1*-deficient (B) testis at 13.5 dpc. (C) Cell counts taken from gonads at 13.5 dpc. (D,E) BrdU labeling of dividing cells (brown precipitate) in wild-type (D) and *Dax1*-deficient (E) testis at 13.5 dpc. (F) Cell counts included flat, curved epithelial cells(arrows, inset in D) in eight wild-type and ten Dax1 knockout gonads (*$P \leq 0.001$).

of proliferation were analyzed using BrdU, a thymidine analog that is incorporated during the S phase of mitosis. BrdU was injected into pregnant females to identify cells actively dividing cells. There was no difference in proliferation at 11.5 dpc, a time when Sertoli cells proliferate at the coelomic epithelium (data not shown). By 13.5 dpc in normal mice, each testis cord was surrounded by dividing PTM cells that were identified by location and cellular morphology, as specific markers are not currently available (arrows, Fig. 6D). Only a one-third as many dividing PTM cells were identified in the *Dax1*-deficient gonad compared with wild type (Fig. 6E,F). There were no differences in number of proliferating germ cells between wild-type and knockout testis.

DISCUSSION

The Dax1-deficient mouse provides an opportunity to investigate the pathogenesis of infertility in males with *DAX1* mutations. In addition, it provides a unique model for examining the role of Dax1 in gonadal development and differentiation. This study shows that Dax1 deficiency is associated with defective development of testis cords and a decreased number of PTM cells. There is, in addition, defective migration or differentiation of fetal Leydig cells, particularly towards the dorsal region of the gonad. These alterations are the likely substrate for the extensive testis pathology seen in adult *Dax1*-deficient mice (Yu et al., 1998; Jeffs et al., 2001b) and in humans with DAX1 mutations (Seminara et al., 1999). However, many of the changes seen in adult animals may be secondary events that are not direct consequences of Dax1 deficiency. For example, the dilation of seminiferous tubules and loss of germ cells may be a result of tubule obstruction and pressure atrophy (Jeffs et al., 2001b). The extensive Leydig cell hyperplasia in adults may reflect increased aromatase expression, leading to high levels of estrogen, which is mitogenic for Leydig cells. Adult mice also show dedifferentiation and proliferation of the epithelial cells that line the rete testis and efferent ducts; the embryonic progenitors of these structures have not been well characterized but also seem sensitive to Dax1 function.

The development of testis cords is a central feature of gonadal differentiation in the male, as the testis cord is the antecedent structure of the seminiferous tubule (Kaufman, 1995). Moreover, testis cords are one of the earliest structural features that discriminate testis versus ovary development from the bipotential gonad. In the *Dax1*-deficient mouse, gonadal development is apparently normal until the point of testis cord formation, suggesting that Dax1 plays a role in gonadal differentiation rather than gonadal patterning (e.g., Sf1) or sex determination (e.g., Sry). The fact that *DAX1* mutations in humans result in hypogonadism and infertility rather than sex reversal or pseudohermaphroditism is consistent with DAX1 action as a mediator of sexual differentiation.

Defective testis cord formation is most likely to reflect abnormal function of either Sertoli cells or PTM cells, which comprise the major somatic cell types of this structure. Germ cells are less likely involved as Dax1 is not expressed in germ cells, and testis cords and seminiferous tubules form normally in the absence of germ cells (Orr-Urtreger et al., 1990). There is no deficiency or overabundance of Sertoli cells at any stage of development in the *Dax1*-deficient testis. At 11.5 dpc, Sertoli cell proliferation is normal, as assessed by BrdU, and expression of the Sertoli-specific genes *Sox9*, *Dhh* and *Amh* is relatively unaffected in the *Dax1*-deficient testis. The critical difference between wild-type and Dax1-deficient testis is that Sertoli and germ cells are not arranged in testis cords, which is probably secondary to a defect in testis cord organization. Thus, although some as yet unrecognized defect may exist in *Dax1*-deficient Sertoli cells, there is no indication of a cell-autonomous abnormality in Sertoli cells. This view is supported by the fact that transgenic expression of DAX1 in Sertoli cells fails to alter testis pathology, although fertility is improved (Jeffs et al., 2001a).

The origin of fetal Leydig cells is debatable. Electron microscopic studies suggest that Leydig cells develop from a population of cells that migrate into the gonad from the mesonephros (Merchant-Larios and Moreno-Mendoza,

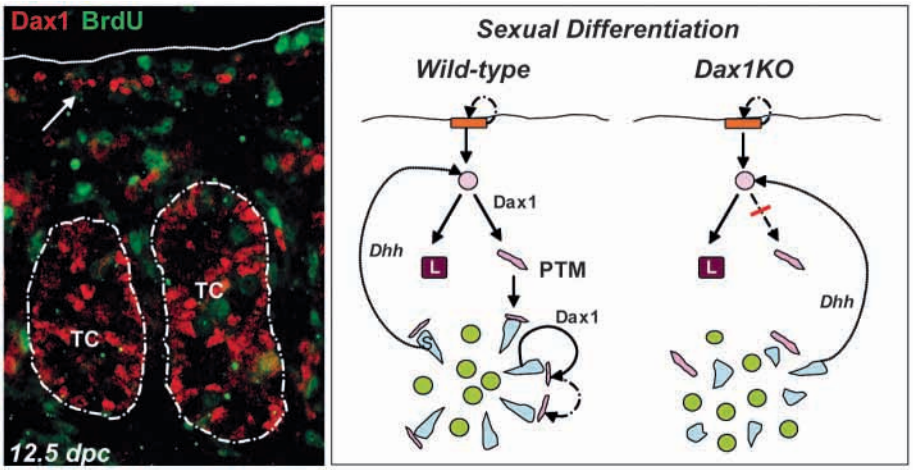

Fig. 7. Proposed mechanism of the role of Dax1 in sex differentiation. Expression of Dax1 (red) in relation to dividing cells (BrdU, green) at 12.5 dpc. Broken line reflects the coelomic surface. TC, testis cords. Arrow indicates Dax1-positive cells below the coelomic surface. Dax1 is expressed in both Sertoli cells and somatic cells below the coelomic epithelium. L, Leydig cells; S, Sertoli cell. Schematic illustrates that Dax1 appears to have a role in regulation of PTM cell development by either determining the fate of cells at the coelomic epithelium or by regulating the proliferation of PTM cells around the testis cord.

1998). The secreted morphogen Dhh is required for Leydig cell development, as *Dhh* knockout mice are deficient in fetal Leydig cells (Clark et al., 2000). The transmembrane receptor Ptch transduces the Dhh signal and is expressed by Leydig cells (Yao et al., 2002b). Using gonad-mesonephros co-culture experiments, it appears that Ptch(+) cells originate within the gonad by 11.5 dpc (Yao et al., 2002b), suggesting that Leydig cells may originate from within the gonad. Deficiency of Dax1 restricts Leydig cells to coelomic edge of the gonad. These findings suggest that at least one population of Leydig cells may arise from the coelomic epithelium and that Dax1 may contribute to the development/differentiation of this cell type. Humans with *DAX1* mutation are still able to produce testosterone when stimulated by exogenous chorionic gonadotropin, indicating that Leydig cells are present and functional in the adult. Thus, similar to Sertoli cells, Leydig cells are present and appear to function normally, but are aberrantly located in the *Dax1*-deficient testis.

Differentiation of testis cords also requires PTM cells (Clark et al., 2000). These myoepithelial cells surround the testis cord, separating it from the peritubular space and contributing to the production of the basal lamina. Examination of the adult testis from the *Dax1*-deficient mouse revealed disruption of the basal lamina and ectopic localization of Leydig cells within the seminiferous tubules (Jeffs et al., 2001b). There was also a deficiency of differentiated PTM cells, a feature that is also present during early testis development. Although there are no specific markers for developing PTM cells, the paucity of PTM appears to result from decreased proliferation rather than increased apoptosis. There are at least three possible reasons for decreased numbers of PTM cells: (1) Dax1 may play a role in PTM cell replication; (2) Dax1 may be required for progenitors of PTM cells to migrate into the gonad, where they later replicate; or (3) Dax1 may be required for the production of a mitogen that induces PTM migration and/or replication. Pending further studies needed to address these possibilities, we propose the following model (Fig. 7). Dax1 is expressed in Sertoli cells and other somatic cells immediately beneath the coelomic epithelium. As Sertoli cells appear relatively normal, it is more likely that other somatic cells, including a potential common progenitor for PTM, and Leydig cells are affected by Dax1 deficiency. A common lineage of these two cell types is likely as they are both deficient in the Dhh knockout mouse and disorganized or absent in the *Dax1*-deficient testis (Clark et al., 2000; Jeffs et al., 2001b). Thus, Dax1 may be required for the development or normal responsiveness of these progenitor cells. It is striking that Dax1 is not expressed in dividing cells (Fig. 7), suggesting that it may act to differentiate cells rather than initiate cell division. Thus, once PTM cells are generated, Dax1 might induce their differentiation.

Dax1 was initially proposed as an ovarian determining gene, an idea not supported by deletion of both Dax1 alleles in XX mice (Yu et al., 1998). However, most models of sex determination still portray Dax1 as an ‘anti-testis’ gene (Goodfellow and Camerino, 2001). This view is based on many lines of evidence. First, duplication of the *DAX1* locus in humans is associated with dysgenetic testes and male to female sex-reversal (Bardoni et al., 1994). Second, transgenic overexpression of Dax1 prevents Sry-mediated sex-reversal of XX mice (Swain et al., 1998). Our data indicates that Dax1 is necessary for normal testis differentiation. It is difficult to reconcile these findings easily with the effects of Dax1 overexpression or duplication. It is possible that the transgenic expression models do not exactly replicate the spatial or temporal pattern of Dax1 expression, thereby altering the fate of cells in a manner that precludes normal testis development. Or, Dax1 overexpression may secondarily alter other developmental pathways that interfere with gonadal development. Alternatively, Dax1 deficiency may eliminate a key progenitor cell or differentiation step without revealing Dax1 functions in other cell types or at later developmental stages. In light of our results, the view that Dax1 is an anti-testis gene oversimplifies its varied functions. It is apparent, however, that gonadal development is highly sensitive to the dose and pattern of Dax1 expression, underscoring the importance of future studies that further define its mechanism of action.

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