**Six5 is required for spermatogenic cell survival and spermiogenesis**

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Myotonic dystrophy 1 (DM1) is a multi-system disorder characterized by endocrine defects that include testicular and tubular atrophy, oligospermia, Leydig cell hyperproliferation and increased follicle stimulating hormone (FSH) levels. DM1 results from a CTG expansion that causes transcriptional silencing of the flanking SIX5 allele. Loss of Six5 results in male sterility and a progressive decrease in testicular mass with age. We demonstrate a strict requirement of Six5 for both spermatogenic cell survival and spermiogenesis. Leydig cell hyperproliferation and increased intra-testicular testosterone levels are observed in the Six5−/− mice. Although increased FSH levels are observed in the Six5−/− and Six5+/− mice, serum testosterone levels and intra-testicular inhibin alpha and inhibin beta B levels are not altered in the Six5 mutant animals when compared with controls. Significantly, steady-state c-Kit levels are reduced in the Six5−/− testis. Thus, decreased c-Kit levels could contribute to the elevated spermatogenic cell apoptosis and Leydig cell hyperproliferation in the Six5−/− mice. The results support the hypothesis that the reduced SIX5 levels contribute to the male reproductive defects in DM1.

**INTRODUCTION**

The Six series of genes code for an evolutionarily conserved family of transcription factors characterized by the Six domain and the Six-homeo-domain, both of which are required for specific DNA binding. As ectopic expression of the Six genes has been shown to alter cell fate, these data support a critical role for the Six gene family in organogenesis (1). Altered function or decreased expression of the SIX genes is associated with several human genetic disorders. Mutations in the SIX3 gene result in holoprosencephaly (2), while haploinsufficiency of SIX6 is responsible for bilateral anophthalmia (3). Significantly, SIX5 levels have been shown to decrease in myotonic dystrophy 1 (DM1) patients (4,5). However, the pathological consequences of the decreased SIX5 expression has yet to be elucidated completely.

DM1 is an autosomal dominant multi-system disorder, associated with muscular dystrophy, cardiac conduction disorders, cataracts, mental retardation, endocrine and reproductive defects (6). The genetic mutation in DM1 is the expansion of a CTG repeat sequence found in the 3′-untranslated (3′UTR) of a protein kinase, DMPK, and located immediately 5′ of SIX5 (7–10). CTG expansion in DM1 has been shown to result in heterochromatin formation in its vicinity, which results in transcriptional silencing of the linked SIX5 allele (4,5,11). Repeat expansion in DM1 has been shown to down-regulate expression of steady-state SIX5 mRNA levels by 2–4-fold (4,5).

Reproductive abnormalities are a well-recognized finding in DM1. Progressive testicular atrophy is a prominent feature and occurs with an incidence of ~80%. Histological abnormalities include tubular atrophy, hyalinization and fibrosis of seminiferous tubules and reduced sperm numbers (6). Oligospermia and azoospermia are reported in ~73% of the DM1 patients (6,12). Disease progression leads to Leydig cell hyperproliferation and elevated basal follicle stimulating hormone (FSH) levels (6,12).

The molecular etiology of DM1 associated reproductive defects is currently unknown. In this study, we demonstrate that decreased Six5 levels result in male reproductive defects that parallel those observed in DM1 patients. Specifically, we show that Six5 is required both for the viability of spermatogenic cells and for the maturation of spermatozoa. Gamete numbers are sensitive to Six5 dosage and sperm counts in the Six5−/− mice are ~60% of controls, while no viable sperm are detected in the Six5+/− mice. Importantly, characteristic features of DM1 including a reduction in testicular mass with age, decreased seminiferous tubule diameter, Leydig cell hyperproliferation and increased FSH levels are...
observed in the Six5 mutant mice. Although loss of Six5 results in increased FSH levels, we do not observe significant differences in serum testosterone levels or in steady-state inhibit alpha or inhibit beta B protein levels in testes derived from the Six5 mutant animals. Importantly, we demonstrate that Six5 loss results in decreased steady-state c-Kit levels, which may underlie the increased spermatogenic cell apoptosis and Leydig cell hyperplasia in the Six5<sup>2/2</sup> mice. These data support the hypothesis that decreased SIX5 levels contribute to the endocrine and gonadal defects that manifest in the DM1 patients.

RESULTS

Six5 is expressed in the mouse testis

*In situ* analysis of Six5 expression in the adult (3–6 month) mouse testis (*n* = 3) demonstrates significant expression of Six5 in all cells within the seminiferous tubules including the cells of the spermatogenic series, which represent cells in various stages of spermatogenesis and spermiogenesis and the Sertoli cells. Leydig cells show low levels of Six5 (Fig. 1A–D). As Six5 is expressed both in cells within the seminiferous tubules and in Leydig cells, we studied the functional consequences of Six5 loss in testis development and gametogenesis.

Loss of Six5 results in male sterility and testicular atrophy

To test the functional consequences of Six5 loss in the etiology of DM1, we created a mouse strain, in which a neomycin cassette replaced the Six5 genomic sequence. The deleted region includes 412 bp upstream of the ATG site, Six5 coding sequences and 180 bp downstream of the termination codon. Six5 expression was not detected in homozygous animals (13). All experiments in this study were carried out on a 129/Sv inbred background. We have demonstrated previously that the Six5<sup>2/2</sup> mice are viable but develop lenticular cataracts (13). However, litters are not obtained when Six5<sup>2/2</sup>/Six5<sup>2/2</sup> or Six5<sup>2/2</sup> males/wild-type females were mated at either 3 or 6 months of age (*n* = 6 for each cross). Thus, male fertility is impaired severely by loss of Six5. The size of the Six5<sup>2/2</sup> testis is normal at birth but by 12 weeks of age the average size of the Six5<sup>2/2</sup> testis is ~30% of wild-type controls (Six5<sup>+/+</sup>) (Fig. 2). To better understand the possible defects that result in this striking loss of testicular tissue and sterility we studied the development of the testis and gametogenesis in the Six5 mutant animals.

Six5 loss results in increased spermatogenic cell apoptosis

Male gametogenesis unlike female gametogenesis begins after birth. During embryogenesis, primordial germ cells migrate to and colonize the gonadal ridge, proliferate and then undergo mitotic arrest until after birth. Ingrowth of the coelomic epithelium gives rise to testicular cords that enclose both the germ cells, which give rise to the spermatogonia, and the somatic mesenchymal cells, which develop into sertoli cells. Sertoli cells are a primary target for FSH and play a critical role in the production of male gametes (spermatogenesis), the development of the male gamete into a motile spermatozoon (spermiogenesis), as well as initiating the production of a variety of hormones (14). Gonadal development at E11 (formation of the indifferent gonad) and E14.5 when testicular cords are first visible, appears normal in the male Six5<sup>+/+</sup> and Six5<sup>2/2</sup> mice (data not shown; *n* = 2 for each genotype). These data demonstrate that early steps in testis formation are normal. Consistent with these results there is no significant difference in testis size (Fig. 3A and C, *P* = 0.43), number of seminiferous tubules (Fig. 3B and D, *P* = 0.12) or sertoli cell/spermatogonia ratios (Fig. 3E, *P* = 0.138) in the newborn Six5<sup>+/+</sup> and Six5<sup>2/2</sup> mice when compared with the Six5<sup>+/+</sup> controls (*P*-value represents a three-way comparison among the Six5<sup>+/+</sup>, Six5<sup>2/2</sup> and Six5<sup>2/2</sup> mice).

Spermatogonia continue to undergo mitotic divisions for ~10 days after birth, at which time meiosis begins. In the wild-type mice, the formation of haploid spermatids occurs between 14 and 21 dpp (15). At 14 dpp, significant differences between the Six5<sup>+/+</sup> and Six5<sup>2/2</sup> testis size are observed (*n* = 3 for each genotype). Histological observation of 14 dpp Six5<sup>2/2</sup> testis demonstrates dying cells within the seminiferous tubules. Cell death occurs by apoptosis as judged by the TUNEL assay (Fig. 4A–C). Although spermatogenic cell death is a feature of normal male gametogenesis, ~5-fold and
male Six5−/− mice demonstrate the presence of haploid cells in the Six5+/− testis (Fig. 6A and B, n = 3 for each genotype). The presence of haploid spermatids with distinct acrosomal caps was confirmed by electron microscopic observation of testes derived from 2-week-old Six5−/− mice (Fig. 6C). These data demonstrate that meiosis is completed to result in haploid cells in the Six5−/− mice. However, the haploid spermatids do not develop into mature spermatozoa in the absence of Six5, thus exhibiting a complete block in spermiogenesis (Fig. 6C). These data, therefore, demonstrate a strict requirement of Six5 for the successful completion of spermiogenesis.

The Six5+/− and Six5−/− mice demonstrate tubular atrophy, oligozoospermia and increased FSH levels

The Six5+/− and Six5−/− mice show decreased tubule diameter and increased FSH levels. Seminiferous tubule diameter decreased proportional to Six5 dose (Fig. 7B and C, P < 0.001, n = 3–4 for each genotype. P-value represents a three-way comparison among the Six5+/+, Six5−/− and Six5−/− mice). Electron microscopic observation of 14 dpp Six5−/− testis shows viable sertoli cells and some spermatogonia, however, most cells of the spermatogenic series are either lost or disarrayed when compared with the controls (Fig. 5A and B). These data demonstrate that loss of one or both Six5 alleles results in a progressive increase in spermatogenic cell apoptosis.

In the wild-type mice, the first cycle of spermatogenesis is complete at around 6 weeks of age, when terminally differentiated spermatozoa are released from the sertoli cells into the lumen of the seminiferous tubules (15). Consistent with previous observations, at 6 weeks of age, histological examination of the Six5+/+ testis showed the formation of terminal differentiated spermatozoa, with a high percent of the spermatozoa undergoing apoptosis (16) (Fig. 4D and E). An ~3-fold increase in apoptotic cells (other than terminally differentiated spermatoza) was detected in the wild-type mice at 6 weeks when compared with that observed at 2 weeks of age (Fig. 4C and F). In the Six5+/− and Six5−/− testes, apoptotic cell death increased by ~4-fold and ~16-fold when compared with the Six5+/+ mice, thus leading to sharply elevated numbers of dying cells (Fig. 4D–F, P = 0.0001, P-value represents a three-way comparison among the Six5+/+, Six5−/− and Six5−/− mice). At 12 weeks, electron microscopic studies show vacuolated dying spermatogenic cells typical of apoptosis in the Six5−/− testes (Fig. 5D). Thus, taken together these data demonstrate that Six5 is a survival factor required for spermatogenic cell viability.

Spermiogenesis is disrupted in the Six5−/− mice

Terminally differentiated spermatozoa are never observed in the Six5−/− testis at 6 weeks of age (Figs. 4D and E, 5D). Thus Six5 loss appears to block the development of terminally differentiated spermatozoa at the end of the first meiotic cycle. To determine the stage at which spermatogenesis was arrested, FACS analysis of all testicular cells, including Leydig cells, sertoli cells and the spermatogenic cell series, from Six5+/+, and Six5−/− was carried out. FACS analysis of testicular cells derived from 2 week (data not shown) and 3-month-old mice show that the Six5−/− mice demonstrate oligozoospermia. Specifically, sperm counts in the Six5+/+ mice were ~60% of the wild-type controls (Fig. 7E, n = 4 for each genotype, P = 0.001 for a three-way comparison among the Six5+/+, Six5−/− and Six5−/− mice, P = 0.03 for a two-way comparison between the Six5−/− and Six5−/− mice). Importantly, these data demonstrate that dose-dependent effects influence the number of viable gametes produced in the male Six5+/+ and Six5−/− mice.

The Six5+/− mice demonstrate Leydig cell hyperproliferation and increased intra-testicular testosterone levels

Leydig cells are located in the interstitial spaces between the seminiferous tubules. Under the influence of LH, Leydig cells produce androgenic hormones including testosterone, which are critical for the development of the male reproductive system (14,15). In the Six5−/− mice, progressive Leydig cell hypertrophy is observed such that nodular hyperplasia is seen at 3 months (n = 5). At 6 months of age, ~75% of the inter-tubular spaces are filled with Leydig cells (n = 5) and at 10 months of age, in three of four mice examined, spaces between the seminiferous tubules were filled completely with Leydig cells (Fig. 7A). Bromo-deoxyuridine labeling showed prominent labeling of Leydig cells (data not shown; n = 2). Thus, Leydig cell hypertrophy was not a consequence of progressive tubular atrophy (Fig. 7B), but was a result of increased Leydig cell proliferation.

It is of interest to note that although we observe Leydig cell hyperproliferation serum testosterone levels are not significantly higher in the Six5−/− mice.
Three testes of each genotype, Sertoli cells and spermatogonia in 32 seminiferous tubules from each genotype were counted. Sertoli cell/spermatogonia ratios from the Six5 mutant mice are shown at 20x and 40x, respectively. (C) Bar graph represents testis size as a function of the cross-sectional area (P = 0.43, n = 20 cross-sections derived from the mid-point of three testes of each genotype). (D) Bar graph represents the number of seminiferous tubules in cross-sections derived from the mid-point of the testis (P = 0.12, n = 20 cross-sections derived from the mid-point of three testes of each genotype). (E) Bar graphs represent the relative numbers of spermatogonia (P = 0.417) and Sertoli cells (P = 0.143) per seminiferous tubule. Using cross-sections derived from the mid-point of three testes of each genotype, Sertoli cells and spermatogonia in 32 seminiferous tubules from each genotype were counted. Sertoli cell/spermatogonia ratios from the Six5+/+, Six5+/- and Six5-/- testis at birth were not significantly different (P = 0.138). In all cases, P-values represent a three-way comparison among the Six5+/+, Six5+/- and Six5-/- mice.

Figure 3. The Six5 mutant mice show normal testis structure at birth. (A, B) Paraffin sections obtained from Six5+/+, Six5+/- and Six5-/- testis of the newborn mice stained with H&E are shown at 20x and 40x, respectively. (C) Bar graph represents testis size as a function of the cross-sectional area (P = 0.43, n = 20 cross-sections derived from the mid-point of three testes of each genotype). (D) Bar graph represents the number of seminiferous tubules in cross-sections derived from the mid-point of the testis (P = 0.12, n = 20 cross-sections derived from the mid-point of three testes of each genotype). (E) Bar graphs represent the relative numbers of spermatogonia (P = 0.417) and Sertoli cells (P = 0.143) per seminiferous tubule. Using cross-sections derived from the mid-point of three testes of each genotype, Sertoli cells and spermatogonia in 32 seminiferous tubules from each genotype were counted. Sertoli cell/spermatogonia ratios from the Six5+/+, Six5+/- and Six5-/- testis at birth were not significantly different (P = 0.138). In all cases, P-values represent a three-way comparison among the Six5+/+, Six5+/- and Six5-/- mice.

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Different between the Six5-/- mouse and the control animals (Fig. 7D). These data could therefore, reflect a defect in the Leydig cells, wherein testosterone produced per Leydig cell may be decreased. In this event, hyperproliferation and chronic stimulation of the Leydig cells may result in lower steady-state intra-testicular testosterone levels but normal serum testosterone levels. As lowered intra-testicular testosterone levels can contribute to increased germ cell apoptosis we measured testosterone levels in the testis (17,18). We observe that mean intra-testicular testosterone levels are significantly elevated in the Six5-/- mice (67 ngT/testis) when compared with the Six5+/- (34 ngT/testis) and Six5+/+ (36 ngT/testis) animals (n = 4 testes of each genotype derived from 5-month-old animals; P = 0.85 for a two-way comparison between the Six5+/+ and the Six5-/- mice; P = 0.22 for a two-way comparison between the Six5+/- and Six5-/- mice). As the average testis mass in the Six5-/- mice studied was ~20% of the control animals, intra-testicular testosterone levels per gram of testis tissue was approximately five times higher in the Six5-/- (1675 ngT/gm) mice when compared to the Six5+/- (283 ngT/gm) and the Six5+/+ (305 ngT/gm) mice (P = 0.91 for a two-way comparison between the Six5+/- and Six5-/- mice; P = 0.046 for a two-way comparison between the Six5+/- and Six5-/- mice). The increased intra-testicular testosterone levels per gram of testis tissue in the Six5-/- mice could reflect higher Leydig cell numbers or increased testosterone biosynthesis by the Six5-/- Leydig cells in response to LH. Thus, these data demonstrate that increased germ cell apoptosis in the Six5-/- mice does not result from decreased intra-testicular testosterone levels.

**Inhibin α and inhibin βB levels are not altered in the Six5-/- testis**

In males, inhibin B and testosterone feedback to negatively regulate FSH secretion by the pituitary gland (19). Inhibin B is a glycoprotein made up of a dimer consisting of the α subunit and the βB subunit linked by disulfide bonds (20). The expression pattern of inhibin α and inhibin βB in the adult mouse testis is controversial. Both genes are believed to be expressed by Sertoli cells and possibly also by germ cells and Leydig cells (21,22). As serum testosterone levels do not appear to be significantly different in the Six5 mutant mice and controls, we measured the steady-state levels of inhibin α and inhibin βB in testicular lysates of the Six5+/+ and Six5+/- and Six5-/- mice at 4 months of age. We do
not observe significant changes in inhibin α and inhibin βB protein levels in the Six5+/− or Six5++/− mice when compared with the Six5++/− controls [Fig. 8A–C, n = 2 for each genotype, protein quality and loading were assessed by probing the stripped filters with antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH)]. These data, therefore, could indicate that defects in the pituitary may prevent normal feedback inhibition of FSH secretion in the Six5 mutant mice.

c-Kit levels are decreased in the Six5−/− testes

As sertoli cell–germ cell signaling plays an important role in spermatogenesis we counted sertoli cells and assessed their maturation in testes derived from the Six5++/+, Six5++/− and Six5−/− mice at 10 weeks of age. Expression of GATA-1, which is a marker for terminally differentiated sertoli cells (23,24), was studied by immunohistochemical staining using anti-GATA-1 antibodies. We did not observe significant differences in GATA-1 expression in sertoli cells derived from the Six5 mutant mice when compared with the controls (Fig. 9A). However, the number of sertoli cells per seminiferous tubule was decreased in the Six5−/− mice when compared with the Six5++/+ controls (Fig. 9C, P = 0.0033, P-value represents a two-way comparison between the Six5++/+ and Six5−/− mice). Thus, although decreased sertoli cell numbers can influence the number of spermatids produced (24), the complete lack of spermiogenesis in the Six5−/− mice suggests that Six5 loss may compromise germ cell viability in ways other than that dictated primarily by the numbers of available sertoli cells in the testis.

Whether a germ cell survives or not is determined by a complex network of signals, which include paracrine signals from sertoli cells, such as desert hedgehog (Dhh), and signals delivered from the sertoli cells by direct membrane contact, an example of which is SCF (25,26). Thus in a subsequent experiment we studied the expression of Dhh, which is required for normal spermatogenesis and spermiogenesis (25), in the Six5++/+, Six5++/− and Six5−/− testes. Immunohistochemical

Figure 4. Six5 loss results in spermatogenic cell apoptosis upon initiation of meiosis. (A and D) Paraffin sections of testes obtained from the Six5++/+, Six5++/− and Six5−/− mice at 2 and 6 weeks of age stained with H&E are shown. (B and E) Shown are TUNEL analyses performed on testes paraffin sections derived from 2- and 6-week-old Six5++/+, Six5++/− and Six5−/− mice. At 2 weeks of age, cells within the seminiferous tubules (ST) in the Six5++/− and Six5−/− mice show increased apoptosis (white arrows point to cells undergoing apoptosis) when compared with the wild-type controls. (C) Bar graph represents the relative numbers of apoptotic cells found within seminiferous tubules in testes derived from 2-week-old Six5++/+, Six5++/− and Six5−/− mice (P = 0.0004; n = 100 seminiferous tubules from 10 sections derived from three testes of each genotype, P-value represents a three-way comparison among the Six5++/+, Six5++/− and Six5−/− mice). (E) At 6 weeks of age apoptotic cell death occurs in some terminally differentiated spermatozoa (red arrows) both in the wild-type and in the Six5−/− mice. Cells within the seminiferous tubules (other than the spermatooza) in the Six5++/+ and Six5−/− testes that show apoptotic death are indicated by white arrows. (F) Bar graph represents the relative number of apoptotic cells (other than spermatooza) within the seminiferous tubules in testes derived from 6-week-old Six5++/+, Six5++/− and Six5−/− mice (P = 0.0004; n = 100 seminiferous tubules from 10 sections derived from three testes of each genotype, P-value represents a three-way comparison among the Six5++/+, Six5++/− and Six5−/− mice).
staining of the sertoli cells with Dhh antibodies showed that both the Six5\(^{+/+}\) and Six5\(^{-/-}\) mice expressed Dhh in sertoli cells. In the adult mouse testis, SCF is produced by the sertoli cells while its receptor, the c-Kit tyrosine kinase, is expressed on the surface of the adjoining spermatogenic cells. SCF/c-Kit signaling is required for normal spermatogenesis both in mice and in humans (26). SCF or c-Kit mutations in mice affect survival and proliferation of primordial germ cells and spermatogonia (26). When the SCF/c-Kit interaction is blocked in vivo by using the ACK2 antibody the incidence of apoptosis in spermatogonia and spermatocytes is increased, which suggests that SCF is required for the survival of these cell types (27). Consistent with this idea all germ cell types are protected from apoptosis in vitro by the addition of SCF to the medium (28). Importantly, although SCF expression is unaltered, steady-state c-Kit levels are significantly decreased in the Six5\(^{+/+}\) testicular lysates when compared with the controls (Fig. 8C–E). These data point to intrinsic germ cell defects and demonstrate that the decreased c-Kit levels within the testis may be an important factor that contributes to elevated germ cell apoptosis in the Six5\(^{-/-}\) mice.

DISCUSSION

In this study, we demonstrate that Six5 is essential for male gametogenesis. Our data show that Six5 acts as a survival factor for germ cells and demonstrate a strict requirement for Six5 in spermiogenesis.

Normal spermatogenesis requires both pituitary gonadotrophins and testicular androgens. LH and FSH are produced by the pituitary in response to the hypothalamic gonadotrophin releasing hormone. LH acts on Leydig cells to produce testosterone. The sertoli cells, which contain both intracellular androgen receptors as well as FSH receptors, receive inputs from both testosterone and FSH to support spermatogenesis by a number of paracrine-acting factors. Testosterone and testosterone in conjunction with the inhibin B, feedback to negatively regulate LH and FSH secretion by the pituitary, respectively (29).

In the Six5\(^{+/+}\) and Six5\(^{-/-}\) mice, we observe that LH and serum testosterone levels are normal, while FSH levels are elevated (Fig. 7D). Although FSH levels are increased we do not observe changes in steady-state inhibin \(\alpha\) or inhibin \(\beta B\) protein levels in testicular lysates of the Six5\(^{+/+}\) and Six5\(^{-/-}\) mice when compared with the Six5\(^{-/-}\) mice (Fig. 8A and B). These data could indicate abnormal pituitary function, which may prevent the proper feed back inhibition of FSH secretion by testosterone and inhibin B.
Sertoli cell–germ cell signaling plays an important role in germ cell viability. Sertoli cells form tight junctions with each other as well as with the developing germ cells, nourish the germ cells and facilitate spermatogenesis by producing a number of paracrine factors (14,24). Electron microscopic observation of the Six5-/- testes demonstrates the presence of morphologically normal sertoli cells located among dying spermatogenic cells (Fig. 5B). Although we observe that the number of mature sertoli cells expressing GATA-1 is relatively smaller in the Six5-/- mice (Fig. 9C), this change does not appear to be large enough to account for the complete lack of terminally differentiated spermatozoa in the Six5-/- mice. Thus although decreased sertoli cell numbers may contribute to the lack of spermatozoa in the Six5-/- mice, it is more likely that other functional abnormalities in the sertoli cell or the germ cell may play a significant role in the block in spermiogenesis observed in the Six5-/- mice.

To test if expression of paracrine factors that act on germ cells is altered in the Six5-/- mice we studied the expression of Dhh and SCF, two sertoli cell factors that act on the adjacent germ cells to facilitate spermatogenesis (25,26). However, Dhh expression was detected in sertoli cells by immunohistochemistry and steady-state SCF levels were unaltered in the Six5 mutant mice as determined by western blot analyses of total testicular lysates (Figs 8D and 9B). We therefore assessed germ cell integrity by studying the expression of c-Kit, which is a receptor for SCF (26). Importantly, steady-state c-Kit levels are decreased in the Six5-/- testicular lysates (Fig. 8C). As decreased c-Kit levels have been shown to significantly increase spermatogonia and spermatocyte apoptosis in vivo (26), reduced c-Kit levels observed in the Six5-/- mice provide an explanation for the increased germ cell apoptosis observed in the Six5-/- mice. It is, however, important to note that although decreased c-Kit expression has been shown to increase apoptosis of spermatogonia and spermatocytes (26,27), the effect of lowered c-Kit levels on spermiogenesis is less clear. It is, therefore, conceivable that in addition to altered SCF/c-Kit signaling other...
defects may contribute to the arrest in spermiogenesis observed in the $Six5^{-/-}$ mice. LH acts on Leydig cells to produce the androgenic hormones that are required for the normal function of the seminiferous epithelium and the development of spermatogenic cells. In the $Six5$ knockout mice, we observe normal LH and serum testosterone levels (Fig. 7D). However, Leydig cell hyperproliferation and elevated intra-testicular testosterone levels per gram of testicular tissue are observed in the $Six5^{-/-}$ mice (Fig. 7A). In this regard, it is of interest to

Figure 7. $Six5^{+/+}$ and $Six5^{-/-}$ mice show progressive Leydig cell hyperproliferation, tubular atrophy, increased FSH levels and reduced sperm counts. (A) Progressive Leydig cell proliferation occurring between 6 and 40 weeks of age in the $Six5^{-/-}$ testis is shown. (B) $Six5$ dose-dependent variation in seminiferous tubule diameter at 12 weeks of age. (C) Bar graphs represent the diameter of seminiferous tubules in the $Six5^{++}$, $Six5^{-/-}$ and $Six5^{-/-}$ mice, respectively ($P < 0.001$, $n = 3–4$ for each genotype; $P$-value represents a three-way comparison among the $Six5^{++}$, $Six5^{-/-}$ and $Six5^{-/-}$ mice at 6, 12 and 24 weeks of age). (D) Bar graph represents serum hormone levels in 22–24-week-old $Six5^{++}$, $Six5^{-/-}$ and $Six5^{-/-}$ mice ($P$-values $< 0.05$ for a three-way comparison among the $Six5^{++}$, $Six5^{-/-}$ and $Six5^{-/-}$ mice are marked with an $^{*}$; $n = 5–10$ of each genotype). (E) Bar graph represents the sperm counts in 12-week-old $Six5^{++}$, $Six5^{-/-}$ and $Six5^{-/-}$ mice, respectively ($P = 0.001$ for a three-way comparison among the $Six5^{++}$, $Six5^{-/-}$ and $Six5^{-/-}$ mice; $n = 4$ of each genotype).
SCF and GAPDH in testicular extracts from the Six5 mutant flies (31). So far it has been unclear whether abnormal gamete–soma interactions have been hypothesized to underlie the aberrant gametogenesis observed in d-Six4 mutant flies (31). So far it has been unclear whether Six5 or Six4 is an ortholog of d-Six4 or if functions of d-Six4 are shared between Six4 and Six5. As no phenotypic abnormalities have been reported in the Six4 knockout mice (32), our data strongly suggests that Six5 is the ortholog of d-Six4.

In DM1 patients SIX5 levels are decreased from 2 to 4-fold as a result of CTG repeat expansion (4,5). In this study, we have studied the pathological changes associated with the loss of one Six5 allele or a complete loss of Six5 expression in the Six5+/− and Six5−/−, mice respectively. We observe that dose-dependent changes in Six5 levels significantly affect male reproduction. Specifically, the Six5+/− mice show decreased tubule diameter, increased FSH levels and oligozoospermia. In the Six5−/− animals, decreased testicular mass, smaller tubule diameters, increased FSH levels, arrested spermiogenesis and Leydig cell hyperproliferation are observed. If the Six5 levels are ~50% of the wild-type levels in the Six5+/− mice, DM1 pathology associated with a 2–4-fold decrease in SIX5 should lie between that observed in the Six5+/− and Six5−/− mice. Thus, these results support the hypothesis that a decrease in SIX5 dosage contributes to features of DM1 associated gonadal defects. The data suggest that fluctuations in SIX5 levels may affect male fertility in the general population.

MATERIALS AND METHODS

In situ hybridization

A 1.6 kb Six5-specific fragment encoding the 5'-UTR and part of exon 1 from mouse genomic DNA was amplified using primers 5'-TCCCCGTGTACAGTCGACTC-3' and 5'-GCAGCCGTATATTGTCCACAG-3' and subsequently cloned into the Eco RV site of pBluescript IIKS(+) (Stratagene) to generate pSix5a. DIG-labeled riboprobes were synthesized by in vitro transcription of the subcloned Six5 sequence. The sense Six5 riboprobe was transcribed with T3 RNA polymerase (Roche) from pSix5a digested with Eco47III and the antisense Six5 riboprobe was transcribed with T7 RNA polymerase (Roche) from pSix5a digested with HindIII. Sections of the wild-type testis were analyzed by in situ hybridization as described previously (33).

Histology and immunohistochemistry

Testes were dissected and fixed in 10% (V/V) neutral buffered formalin, progressively dehydrated with ethanol and embedded in paraffin. Deparaffinized sections (5 μm thick) were stained with hematoxylin and eosin (H&E) using standard procedures for light microscopy.

Mice (12 weeks) were injected with BrdU solution (100 μg BrdU per gram body weight) 2–3 h prior to sacrificing. Testes were dissected and fixed in Carnoy’s fixative solution for 3 h and paraffin embedded. The BrdU assay was carried out on 5 μm sections as described (34).

Embryos were obtained at E11.5 and E14.5. The yolk sac was used for genotyping, and embryos were fixed in absolute ethanol/glacial acetic acid (7:1) at 4°C. After embedding in paraffin, deparaffinized sections were rehydrated through a graded ethanol series, and stained for alkaline phosphatase activity in a buffer containing 25 mM sodium tetraborate,
3.5 mM MgCl₂, 0.1 mg/ml sodium naphtylphosphate and 0.1 mg/ml Fast Red TR salt (Sigma).

Paraformaldehyde (4%) fixed and paraffin embedded testes were sectioned at 5 μm and analyzed for detection of apoptotic cell death using the in situ cell death detection kit, fluorescein (Roche). All steps were followed according to the manufacturer’s protocol. For immunostaining anti-GATA-1 and anti-Dhh antibodies (Santa Cruz Biotechnologies) were used at dilutions of 1:200 and 1:100, respectively, as described previously (23).

FACS analyses
The testes were collected in Hank’s buffer (Sigma), and seminiferous tubules were dissociated using sequential enzymatic digestion by collagenase (1 mg/ml) and 0.1% trypsin (Gibco). Cells were resuspended in PBS and the single-cell suspension was stained with propidium iodide and subjected to FACS analysis.

Electron microscopy
Testes (2 weeks and 3 months) were fixed in 4% paraformaldehyde (4%) fixed and paraffin embedded testes were sectioned at 5 μm and analyzed for detection of apoptotic cell death using the in situ cell death detection kit, fluorescein (Roche). All steps were followed according to the manufacturer’s protocol. For immunostaining anti-GATA-1 and anti-Dhh antibodies (Santa Cruz Biotechnologies) were used at dilutions of 1:200 and 1:100, respectively, as described previously (23).

Hormone assays
Serum steroid analysis was performed for testosterone, FSH and LH by RIA at the Oregon Regional Primate Center, OR by David L. Hess. Intra-testicular testosterone levels were measured by first weighing and then homogenizing each testis in 5 ml of phosphate-buffered saline (PBS) with a polytron homogenizer. The homogenate was extracted with diethyl ether and the ether phase was dried. The steroids were resuspended in 500 μl of PBS and analyzed by RIA at Antech Diagnostics.

Statistical analysis
Distributions were compared across genotypes using the non-parametric Kruskal–Wallis test. Descriptive statistics and exact P-values were calculated using SAS software (version 8). P-values for intra-testicular testosterone levels were calculated using Student’s two sided t test.

Western blot analyses
Tissue extracts were prepared by homogenization of the dissected testes with a lysis buffer [25 mM Tris–HCl, pH 7.6, 500 mM NaCl, 0.75% NP40, 5 mM EDTA, 25% sucrose, 2 mM phenylmethysulfonyl fluoride and protease inhibitor (Sigma)]. The lysates were incubated on ice for 1 h and centrifuged. Equal amount of protein (30 μg/lane) from the supernatants were loaded on SDS–PAGE gels, electrophoresed and transferred on to Hybond P membranes (Amersham). The membranes were probed with goat anti-inhibin α and βB polyclonal antibodies, rabbit anti-c-Kit polyclonal antibodies or mouse anti-SCF monoclonal antibodies using standard techniques. To account for protein quality and loading the membranes were stripped and re-probed with goat anti-GAPDH polyclonal antibodies (all antibodies were purchased from Santa Cruz Biotechnology, Inc.).
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