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Abstract
The homeodomain CUX1 protein exists as multiple isoforms that arise from proteolytic processing of a 200-kDa protein or an alternate splicing or from the use of an alternate promoter. The 200-kDa CUX1 protein is highly expressed in the developing kidney, where it functions to regulate cell proliferation. Transgenic mice ectopically expressing the 200-kDa CUX1 protein develop renal hyperplasia associated with reduced expression of the cyclin kinase inhibitor p27. A 55-kDa CUX1 isoform is expressed exclusively in the testes. We determined the pattern and timing of CUX1 protein expression in developing testes. CUX1 expression was continuous in Sertoli cells from prepubertal testes but became cyclic when spermatids appeared. In testes from mature mice, CUX1 was highly expressed only in round spermatids at stages IV–V of spermatogenesis, in both spermatids and Sertoli cells at stages VI–X of spermatogenesis, and only in Sertoli cells at stage XI of spermatogenesis. While most of the seminiferous tubules in wild-type mice were between stages VI and X of spermatogenesis, there was a significant reduction in the percentage of seminiferous tubules between stages VI and X in Cux1 transgenic mice and a significant increase in the percentage of seminiferous tubules in stages IV–V and XI. Moreover, CUX1 was not expressed in proliferating cells in testes from either wild-type or transgenic mice. Thus, unlike the somatic form of CUX1, which has a role in cell proliferation, the testis-specific form of CUX1 is not involved in cell division and appears to play a role in signaling between Sertoli cells and spermatids.

Keywords
Sertoli cells, spermatid, spermatogenesis, testis

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Asynchronous Expression of the Homeodomain Protein CUX1 in Sertoli Cells and Spermatids During Spermatogenesis in Mice¹

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ABSTRACT

The homeodomain CUX1 protein exists as multiple isoforms that arise from proteolytic processing of a 200-kDa protein or an alternate splicing or from the use of an alternate promoter. The 200-kDa CUX1 protein is highly expressed in the developing kidney, where it functions to regulate cell proliferation. Transgenic mice ectopically expressing the 200-kDa CUX1 protein develop renal hyperplasia associated with reduced expression of the cyclin kinase inhibitor p27. A 55-kDa CUX1 isoform is expressed exclusively in the testes. We determined the pattern and timing of CUX1 protein expression in developing testes. CUX1 expression was continuous in Sertoli cells from prepubertal testes but became cyclical when spermatids appeared. In testes from mature mice, CUX1 was highly expressed only in round spermatids at stages IV–V of spermatogenesis, in both spermatids and Sertoli cells at stages VI–X of spermatogenesis, and only in Sertoli cells at stage XI of spermatogenesis. While most of the seminiferous tubules in wild-type mice were between stages VI and X of spermatogenesis, there was a significant reduction in the percentage of seminiferous tubules between stages VI and X in Cux1 transgenic mice and a significant increase in the percentage of seminiferous tubules in stages IV–V and XI. Moreover, CUX1 was not expressed in proliferating cells in testes from either wild-type or transgenic mice. Thus, unlike the somatic form of CUX1, which has a role in cell proliferation, the testis-specific form of CUX1 is not involved in cell division and appears to play a role in signaling between Sertoli cells and spermatids.

Sertoli cells, spermatid, spermatogenesis, testis

INTRODUCTION

The molecular mechanisms regulating mammalian spermatogenesis are not well understood. The process of spermatogenesis is facilitated by Sertoli cells, which surround developing germ cells and provide the appropriate milieu. Sertoli cells function to create a niche for spermatogenesis, which includes establishing the blood–testis barrier (BTB), regulating the transition of spermatocytes through meiosis I and II to form spermatids, and regulating the process of spermiation to form mature sperm [1, 2]. These functions are facilitated by extensive cell–cell interactions between adjacent Sertoli cells and between Sertoli cells and germ cells [1, 3]. Cell–cell interactions, which include hemidesmosomes [1–4], tight junctions [5–7], adherens junctions [1, 8], and gap junctions [9, 10], undergo extensive restructuring to allow the transit of primary spermatocytes across the BTB and the release of spermatozoa into the tubular lumen [1, 11–13]. There are numerous cell adhesion molecules involved in forming the interactions between Sertoli cells and germ cells. These molecules include immunoglobulin (Ig) superfamily members, such as cell adhesion molecule 1 (CADM1) [14–18], poliovirus receptor (PVR) [19], Nectin-2 [20], Nectin-3 [21], junctional adhesion molecule JAM2 (also known as JAM-B [18, 22]), and JAM3 (also known as JAM-C [23–24]), and non-Ig molecules, such as cadherins and integrins [25–29]. During mouse spermatogenesis, there is extensive junctional reorganization corresponding to the release of mature sperm into the lumen of the seminiferous tubule. At the same time, there is reorganization of the BTB, allowing the passage of primary spermatocytes from the basal to the apical compartment [11]. This process is tightly regulated to ensure that these junctional reorganizations occur simultaneously, thus maintaining the seminiferous epithelial cycle [11].

The relationship between Sertoli cells and germ cells is tightly regulated. Each Sertoli cell supports a fixed number of germ cells [30, 31]. Thus, the number of Sertoli cells determines the size of the testes and sperm production [30, 31]. The number of Sertoli cells in the adult testis is determined early in development, and Sertoli cell proliferation is dependent on the expression of the cyclin kinase inhibitors p21 (CDKN1A) and p27 (CDKN1B) [32]. Moreover, mice carrying targeted mutations in CDKN1A and CDKN1B exhibit increased numbers of Sertoli cells and enlarged testes [32].

CUX1 is a murine homologue of the Drosophila homeodomain cut protein and contains four DNA binding domains: three cut repeats and the homeodomain [33–39]. The murine cut homologues function as cell cycle-dependent transcription factors, and CUX1 is part of the network controlling G₁–S transition, where it represses the expression of CDKN1A [40]. CUX1 also represses expression of CDKN1B, and transgenic mice constitutively expressing CUX1 develop renal hyperplasia, resulting from the aberrant repression of CDKN1B [41].
These mice exhibit enlargement of several other organs, including testes [41]. In addition to regulating the cell cycle, CUX1 has been shown to regulate expression of E-cadherin and N-cadherin and to stabilize SRC [42–43].

In addition to the CUX1 protein containing three cut repeats and a homeodomain called p200, there are several truncated isoforms that have been identified. These isoforms include a testis-specific isoform containing one cut repeat and the homeodomain called p55. In situ hybridization showed that mRNA for p55 was most abundant in round spermatids [44]. Additional isoforms include p75, a protein similar in structure to p55, derived from the use of an alternate promoter in intron 20 [45]; the cut alternately spliced protein (CASP), a Golgi protein that contains amino-terminal sequences but none of the cut repeats or homeodomains [46]; and several other isoforms (p80, p90, p110, and p150) that appear to arise via proteolytic processing [47, 48]. While p200 functions exclusively as a transcriptional repressor, the transcriptional mechanism used by the other CUX1 isoforms is more complex. Isoforms p75, p90, and p110 can function either as repressors or activators of transcription, depending on the promoter context, while p80 functions exclusively as an activator. The p150 isoform does not bind DNA but instead acts as a dominant negative in the mammary gland during lactation [48]. In the present study, we examined the expression of CUX1 protein in prepubertal and adult testes from wild-type and Cux1 transgenic mice to correlate CUX1 expression with the stages of spermatogenesis and determine the effects of deregulation of CUX1 expression on spermatogenesis.

MATERIALS AND METHODS

Animals

Cux1 transgenic mice expressing full-length Cux1 cDNA under the control of the cytomegalovirus (CMV) immediate-early gene promoter were generated previously [41]. Genotyping was performed by Southern blot analysis of tail DNA after digestion with appropriate restriction nucleases or by PCR analysis using a 5′ primer specific for the CMV promoter and a 3′ primer specific for the Cux1 cDNA. Age-matched wild-type littersmates were used as controls. Mice were maintained in accordance with the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Immunohistochemistry Analysis

Immunohistochemistry analysis was performed as previously described [49]. Briefly, isolated testes were immersion-fixed in 4% paraformaldehyde and blocked in paraffin. Three-micrometer-thick sections were deparaffinized with xylene and hydrated with concentration-graded ethanol. Sections were washed in PBS containing 1% Tween 20 (PBST) and blocked in 10% normal goat serum at room temperature for 1 h. Rabbit anti-CUX1 (product no. sc-13024; Santa Cruz Biotechnology), mouse anti-PCNA (product no. p-8825; Sigma), rabbit anti-WT1 (product no. sc-192; Santa Cruz Biotechnology), or rat anti-germ cell nuclear antigen (GCNA) primary antibody [50] was applied to sections and incubated at room temperature for 1 h at final concentrations of 1:100, 1:3000, 1:100, and undiluted, respectively. Biotinylated goat anti-rabbit (1:400) antibody was used to detect CUX1 and WT1 antibody. A horse antimouse-Texas Red-conjugated secondary antibody (Vector) was used to detect PCNA (1:400). A goat anti-rat-Texas Red-conjugated secondary antibody (Vector) was used to detect GCNA (1:400). Sections were then washed in PBST and incubated with either fluorescein isothiocyanate-conjugated avidin (Vector) or with avidin-biotin-peroxidase complex (ABC Elite; Vector) and diaminobenzidine (DAB). In some cases, following DAB incubation, sections and incubated at room temperature for 1 h at final concentrations of 1:100, 1:3000, 1:100, and undiluted, respectively. Biotinylated goat anti-rabbit (1:400) antibody was used to detect CUX1 and WT1 antibody. A horse antimouse-Texas Red-conjugated secondary antibody (Vector) was used to detect PCNA (1:400). A goat anti-rat-Texas Red-conjugated secondary antibody (Vector) was used to detect GCNA (1:400). Sections were then washed in PBST and incubated with either fluorescein isothiocyanate-conjugated avidin (Vector) or with avidin-biotin-peroxidase complex (ABC Elite; Vector) and diaminobenzidine (DAB). In some cases, following DAB incubation, sections were counterstained with periodic acid-Schiff (PAS) stain (American Master Tech). All sections were washed and then either mounted with Vectashield medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector) or dehydrated with graded ethanol, mounted with Permount (Fisher), and covered with glass coverslips. Images were captured using Nomarski differential interference contrast with a Leica DMR model microscope equipped with an Optronics MagnaFire digital camera or by using an Olympus BH-2 microscope equipped with an Olympus DP71 digital camera.

Morphometry Analysis

The tubule’s minimum cross-sectional distance was taken as the diameter, to account for obliquely cut sections [51]. The cross-sectional area (A) of the seminiferous tubule was calculated by dividing the average diameter for each tubule to obtain the radius and then multiplying r times the radius squared (A = πr²). The density of Sertoli cells in seminiferous tubules was calculated by dividing the number of WT1-positive cells by the estimated circumference of the tubule (average of minimum and maximum diameter × π) from over 100 seminiferous tubules in testes sections from at least two animals of each genotype [52–55]. To quantify the percentage of seminiferous tubules expressing CUX1 in Sertoli cells only, in Sertoli cells and spermatids, in spermatids only, or in neither Sertoli cells nor spermatids, the number of tubules exhibiting these patterns were counted in three testis sections from three animals of each genotype. All morphometry analysis was performed in a blinded fashion. Data are presented as means ± SD. The cross-sectional area, the density of Sertoli cells, and the percentage of seminiferous tubules expressing CUX1 were analyzed by Student t-test. Differences were considered significant at a P value of <0.05. Interstitial cell counts were obtained from hematoxylin-eosin-stained 5-μm sections of testis sections from four Postnatal Day-14 (P14) and P35 transgenic and wild-type mice. Photomicrographs were taken at 200× original magnification, and the number of interstitial cell nuclei in 10 medium power fields from each sample was counted. Data were analyzed by Student t-test.

Western Blot Analysis

Nuclear extracts (30 μg) were separated by electrophoresis on 4–15% gradient polyacrylamide gels and transferred to nitrocellulose membranes, which were then blocked in 5% milk-PBST, as previously described [49]. Membranes were probed with rabbit anti-CUX1 (product no. sc-13024; Santa Cruz Biotechnology) primary antibody at a dilution of 1:50, followed by washes with PBST and application of horseradish peroxidase (1:10000) secondary antibody. Following additional washes with PBST, bound antibody was detected by chemiluminescence (Pierce) according to the manufacturer’s directions.

Testosterone and Follicle-Stimulating Hormone Assays

Blood was collected from 4-week-old wild-type (n = 4) and Cux1 transgenic (n = 3) mice and from 6-month-old wild-type (n = 4) and Cux1 transgenic (n = 3) mice prior to being euthanized, and isolated serum was delivered to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for analysis of serum testosterone and follicle-stimulating hormone (FSH). Results were compared using the Student t-test for paired samples.

TUNEL Assay

Sections were processed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) with a TUNEL apoptosis detection kit (Millipore) according to the manufacturer’s directions. Sections were mounted with Vectashield medium with DAPI (Vector) and covered with glass coverslips. Images were captured with a Leica DMR model microscope equipped with an Optronics MagnaFire digital camera.

RESULTS

We previously described a testis-specific Cux1 mRNA transcript of 2.4 kb encoding a 55-kDa protein that was primarily expressed in postmeiotic round spermatids [44]. We also showed that a 13-kb Cux1 transcript corresponding to the full-length 200-kDa protein was expressed in testes from mice homozygous for the atrichosis (at/at) mutation, which lacks germ cells [44]. This somatic isoform is expressed in many other tissues, including the kidney. Transgenic mice constitutively expressing the full-length isoform of CUX1 develop multiorgan hyperplasia, including an increase in the size of the testes [41]. To determine whether the expression of the testis CUX1 protein is altered in transgenic mice, we evaluated CUX1 protein expression in postpubertal testes isolated from wild-type and transgenic mice. Using an antibody directed to the C terminus of CUX1, which is shared between somatic- and testis-specific isoforms of the CUX1 protein, we evaluated
CUX1 protein expression in testes from wild-type and Cux1 transgenic mice. In testes from sexually mature (35 days postpartum [p.p.]) wild-type mice, we observed CUX1 protein expression in spermatids and in Sertoli cells. Moreover, expression in both cell types varied between tubules, giving four different patterns consisting of one, both, or neither cell type expressing CUX1 (Fig. 1). Using periodic acid-Schiff (PAS) staining following immunohistochemical labeling for the CUX1 protein, we correlated the four CUX1 expression patterns with spermatid morphology [56, 57]. We found very little expression of the CUX1 protein in either Sertoli cells or spermatids at stages I–III (Fig. 2A). This finding was consistent with that of our previous studies evaluating Cux1 mRNA in postpubertal testes by in situ hybridization [44]. At stages IV–V, we observed expression of the CUX1 protein only in round spermatids (Fig. 2B). Again, this pattern of protein expression paralleled that of the mRNA expression previously reported [44]. Beginning at stage VI and continuing through stage VIII, is, in some cases, oriented toward the basal membrane of the tubule, CUX1 is highly expressed in both round spermatids (arrows) and Sertoli cells (arrowheads). D) At stages IX–X, indicated by elongation of the spermatid nuclei, CUX1 continues to be highly expressed in both elongating spermatids (arrows) and Sertoli cells (arrowheads). E) At stage XI, indicated by compacted and darkly stained spermatid heads (arrows), CUX1 is expressed only in Sertoli cells (arrowheads). Original magnification ×400.
we observed intense expression of CUX1 in both round spermatids and Sertoli cells (Fig. 2C).

Although we previously reported Cux1 mRNA expression in round spermatids in stage VI–VIII tubules, we had not observed Cux1 mRNA expression in Sertoli cells [44]. At stages IX–X, we observed CUX1 protein in both elongating spermatids and Sertoli cells (Fig. 2D). The expression of CUX1 protein in elongating spermatids contrasts with that of the previous study, in which Cux1 mRNA was not observed in elongating spermatids at stage IX or X [44]. Finally, at stage XI, the CUX1 protein was detected only in Sertoli cells, with no expression in the spermatids (Fig. 2E). This is consistent with the absence of Cux1 mRNA in spermatids in seminiferous tubules at stages IX–XI, as described previously [44]. Differences in Cux1 mRNA expression levels previously reported and in the pattern of CUX1 protein expression suggest that the translated protein remains in maturing spermatids after mRNA is degraded.

To determine when the differential expression of CUX1 begins during testis development, we evaluated CUX1 expression with prepubertal testes (Fig. 3). In testes sections from 7-day-old mice, CUX1 protein was uniformly expressed in most cells of the seminiferous tubules (Fig. 3A). This uniform pattern of expression continued at 14 days p.p. (Fig. 3B). At 21 days p.p., a differential pattern of CUX1 expression was observed, revealing the four different patterns of expression observed in adult mice (Fig. 3C). This pattern of expression was also observed at 28 and 35 days p.p. (Fig. 3D and Fig. 1).

To determine whether CUX1 was also expressed in spermatogonia, we colabeled testis sections from 21- and 35-day-old
mice with antibodies directed against CUX1 and GCNA, an antigen expressed in all diploid spermatogonia, as well as in spermatocytes and round spermatids [50]. Figure 4 shows CUX1 and GCNA were expressed in mutually exclusive patterns at the periphery of the seminiferous tubules of both 21-day p.p. and 35-day p.p. mice, indicating that CUX1 is not expressed in spermatogonia. To correlate the differential patterns of CUX1 expression with the sizes of the CUX1 proteins, we performed Western blot analysis with testes isolated from 7-day-old mice, when CUX1 is uniformly expressed in seminiferous tubules, and with testes isolated from 28-day-old mice, after a differential pattern of CUX1 expression is observed. At 7 days p.p., the most abundant CUX1 protein is the p200 somatic form, with no detectable expression of p55 (Fig. 5A). In contrast, at 28 days p.p., the most abundant CUX1 proteins are p55 and p75, with relatively lower levels of p200 (Fig. 5B).

We previously showed that testes isolated from Cux1 transgenic mice were increased in size by ~25% at 6 weeks of age, although differences in size between the Cux1 transgenic and wild-type mice were less evident as the mice aged [41]. To determine whether spermatogenesis is altered in mice constitutively expressing the somatic isoform of CUX1, we evaluated CUX1 expression in testes isolated from wild-type and Cux1 transgenic mice. Testes sections from both wild-type and Cux1 transgenic mice showed the same differential patterns of expression between Sertoli cells and spermatids during spermatogenesis (Fig. 6). To determine whether the increase in overall testis size resulted from an increased size of seminiferous tubules, we measured the cross-sectional areas of individual seminiferous tubules from three wild-type and three Cux1 transgenic mice. A comparison between the cross-sectional areas of individual seminiferous tubules from wild-type and that of transgenic mice revealed no significant differences (wild-type, $3.34 \times 10^5 \mu m^2$ vs. transgenic, $3.56 \times 10^5 \mu m^2$; $P = 0.57, n = 144$). Full-length CUX1 protein regulates the cell cycle by repressing the cyclin kinase inhibitors CDKN1A1 and CDKN1B [40, 41]. We have previously shown that renal hyperplasia in Cux1 transgenic mice resulted from repression of CDKN1B [41]. Moreover, mice carrying targeted deletions of Cdkn1b and Cdkn1a show an increase in the number of Sertoli cells resulting from increased proliferation [32]. As CUX1 functions to regulate the cell cycle by repressing CDKN1A and CDKN1B during kidney development, one possibility is that the increase in testis size resulted from an increased number of Sertoli cells. To determine whether Sertoli cell proliferation was increased in Cux1 transgenic testes, we evaluated the density of Sertoli cells in the seminiferous tubules of 35-day p.p. wild-type and Cux1

FIG. 5. CUX1 (p55) is not expressed until after the appearance of round spermatids. Western blotting of nuclear extracts (30 µg) isolated from testes of 7-day p.p. (P7) and 28-day p.p. (P28) mice. A) Testes from P7 mice express high levels of the CUX1 (p200) protein, corresponding to the appearance of round spermatids. Expression of the CUX1 (p75) protein is also detected in P28 testes. The expression of CUX1 (p200) is much lower in P28 testes, corresponding to asynchronous expression in Sertoli cells.

FIG. 6. Asynchronous CUX1 expression in testes from both wild-type and Cux1 transgenic mice is not associated with cell proliferation. Testes sections from 35-day p.p. wild-type (A, C, E, G, I) mice and Cux1 transgenic (B, D, F, H, J) mice were colabeled with antibodies directed against CUX1 (green) and PCNA (red) to identify proliferating cells. Sections were then counterstained with DAPI. Seminiferous tubules corresponding to the indicated stages of spermatogenesis are outlined. CUX1 and PCNA did not colocalize at any stage of spermatogenesis in either wild-type or transgenic mice, indicating that CUX1 expression in either Sertoli cells or spermatids does not regulate cell proliferation. Original magnification ×630.
transgenic mice. Testis sections were labeled with WT1 antibody to identify Sertoli cells, and the number of WT1-positive cells/circumferences of seminiferous tubules were determined. We found no difference in numbers of Sertoli cells between the wild-type and the transgenic mice (wild-type, 0.047 ± 0.008 SD vs. transgenic, 0.044 ± 0.007 SD cells per micrometer of circumference; n = 144).

In the kidneys of Cux1 transgenic mice, ectopic expression of CUX1 results in increased cell proliferation, concomitant with reduced expression of the cyclin kinase inhibitor CDKN1B [41]. To determine whether CUX1 regulates cell proliferation during testis development, we colabeled testis sections from wild-type and Cux1 transgenic mice with antibodies directed against CUX1 and PCNA. As expected, CUX1 and PCNA did not colocalize in the postmeiotic round spermatids of either wild-type or Cux1 transgenic mice (Fig. 6). To determine whether the ectopic expression of CUX1 had an affect on the pattern of CUX1 expression in adult testes, we evaluated the percentage of seminiferous tubules exhibiting each of the four patterns of expression. We found a significant shift in the percentage of seminiferous tubules in three of the four patterns of CUX1 expression (Fig. 7). In Cux1 transgenic mice, there was a significant increase in the number of tubule profiles that showed Sertoli or spermatid expression only and a significant decrease in the number of tubule profiles that showed both Sertoli and spermatid expression of CUX1. While there was an increase in the number of tubules that did not show any expression of CUX1, the increase did not reach significance and represented only a small percentage of tubules in either wild-type or transgenic mice.

Because Sertoli cells do not proliferate in mature testes, we compared patterns of CUX1 expression during testis development between wild-type and Cux1 transgenic mice. CUX1 and PCNA did not colocalize at any stage of development during spermatogenesis, either in wild-type or Cux1 transgenic mice (Figs. 6 and 8 and Supplemental Figs. S1 and S2, available online at www.biolreprod.org). Thus, unlike in the developing kidney, CUX1 does not appear to play a role in promoting cell proliferation in the developing testis. At the earliest stages of testis development (7, 10, and 14 days p.p.), patterns of CUX1 and PCNA expression were unchanged between wild-type and Cux1 transgenic mice (Supplemental Fig. S1). In contrast, while testes from Day-21 p.p. wild-type mice showed the switch to the differential pattern of CUX1 expression (Fig. 8A),
testes from Day-21 p.p. transgenic mice showed a more uniform CUX1 expression pattern, with no expression in spermatids and high expression in cells at the periphery of the tubules, consistent with the Sertoli cell expression (Fig. 8B). By Day 28 and Day 35, the expression patterns of CUX1 of wild-type mice were similar to that of transgenic mice, with both showing the differential pattern of CUX1 expression (Fig. 8C, D, and Supplemental Fig. S2). This suggests that CUX1 expression in Sertoli cells functions to regulate the switch to the differential expression pattern and that ectopic expression of CUX1 delays this switch.

The hormone testosterone is required for spermatogenesis, and changes in testosterone levels can dramatically affect the development of sperm cells [58]. Accordingly, we evaluated serum testosterone levels in wild-type and Cux1 transgenic mice at 4 weeks and 6 months of age were measured by radioimmunoassay. Serum testosterone was significantly increased in 6-month-old Cux1 transgenic mice compared to wild-type mice (A). Error bars indicate standard deviation. *, *P = 0.0198.

FIG. 9. Increased testosterone in Cux1 transgenic mice. Total serum testosterone (A) or FSH (B) levels from wild-type and Cux1 transgenic mice at 4 weeks and 6 months of age were measured by radioimmunoassay. Serum testosterone was significantly increased in 6-month-old Cux1 transgenic mice compared to wild-type mice (A). Error bars indicate standard deviation. *, *P = 0.0198.

testosterone, we counted the number of interstitial cells in testis sections from Day-14 and Day-35 p.p. wild-type and Cux1 transgenic mice (Table 1). There was a significant increase in the number of interstitial cells in Cux1 transgenic mice at Day 14 p.p. compared to that in wild-type mice. In contrast, there was no difference between the number of interstitial cells at 35 days p.p. in wild-type mice and that in Cux1 transgenic mice.

Finally, apoptosis occurs normally in spermatogenesis. To determine whether apoptosis was disrupted in Cux1 transgenic mice, we performed TUNEL assays of 35-day p.p. wild-type and Cux1 transgenic mice. No differences in the number of TUNEL-positive cells were observed between wild-type and Cux1 transgenic mice (Fig. 10).

**DISCUSSION**

The murine Cux1 homeobox gene is related to the Drosophila cut gene. We have previously identified a testis-specific isoform of CUX1 that is expressed beginning at P21 in postmeiotic round spermatids [44]. The 2.4-kb mRNA encoding the testis-specific 55-kDa CUX1 isoform likely results from the use of an alternate promoter. An alternate promoter in intron 20 has been identified in the generation of the CUX1 (p75) protein. Since p55 and p75 have similar structures, it is possible that the same promoter is used to generate both transcripts. Moreover, the amino acid sequence of the 55-kDa protein is identical to the corresponding region of the larger CUX1 protein. This larger protein is translated from a 13-kb transcript that is highly expressed in many different tissues during development. In the kidney, the 200-kDa CUX1 protein is most highly expressed in the nephrogenic zone, where cells are actively proliferating, but is sharply downregulated coincident with exit from the cell cycle [38, 41].

To determine whether downregulation of the larger protein is required for normal kidney development, we constitutively expressed cDNA expressing the 200-kDa CUX1 protein in transgenic mice, using the CMV immediate-early gene promoter. The Cux1 transgenic mice exhibited enlarged organs, including the testis, and in the kidney, this resulted from aberrant repression of the cyclin kinase inhibitor CDKN1B [41]. Recently, we have determined that CUX1 functions to repress CDKN1B during kidney development in cooperation with the groucho homologue GRG4 (official symbol TLE4) [60]. To determine whether the increase in testis size that we observed in Cux1 transgenic mice resulted from repression of CDKN1B and/or whether expression of the testis-specific CUX1 protein was disrupted, we evaluated expression of the CUX1 protein in testes isolated from wild-type and Cux1 transgenic mice.

The number of Sertoli cells in the adult testis determines the size of the testis and the sperm production [30–32]. Moreover, each Sertoli cell supports a relatively fixed number of germ cells [30]. Mice carrying a targeted Cdkn1b deletion show an increase in testis size that results from an increase in Sertoli cell proliferation [32]. Because CUX1 functions to repress the Cdkn1b gene expression in developing kidneys and Cux1 transgenic mice develop renal hyperplasia from the aberrant

<table>
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<th>Day</th>
<th>Wild type</th>
<th>Transgenic</th>
<th>Relative increase (%)</th>
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<tr>
<td>P14</td>
<td>203 (11)</td>
<td>243 (13)*</td>
<td>20</td>
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<tr>
<td>P35</td>
<td>163 (6)</td>
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* Data are expressed as group means of the number of interstitial cells per field; standard error is indicated in parentheses (*P = 0.046).
repression of CDKN1B, one possibility for the increased size of testes in the Cux1 transgenic mice was an increase in the number of Sertoli cells. However, a comparison of the numbers of Sertoli cells in transgenic versus those in wild-type mice showed no differences. Moreover, CUX1 expression did not colocalize with PCNA in cells in the testes in either wild-type or transgenic mice, even at the stages of development when Sertoli cells proliferate, suggesting that CUX1 does not function to regulate the cell cycle during spermatogenesis.

Using an antibody directed against the C-terminal end of CUX1, which recognizes both the 200-kDa and the 55-kDa CUX1 isoforms, we observed CUX1 expression in both Sertoli cells and spermatids in sexually mature wild-type mice. However, unlike other genes expressed in Sertoli cells, such as the Wt1 [61] and Rbl2 p130 [62] genes, the Cux1 gene gave a phasic and stage-specific pattern of expression in spermatids and Sertoli cells of mature testes. This expression pattern varied between seminiferous tubules, with one, both, or neither cell type expressing CUX1. Using published criteria based on spermatid morphology to classify the seminiferous tubules [56–57], we found these patterns of CUX1 expression corresponded to stages I–III of spermatogenesis (neither spermatid nor Sertoli cell expression), stages IV–V of spermatogenesis (spermatid expression only), stages VI–VIII (both round spermatids and Sertoli cell expression), stages IX–X (both elongating spermatids and Sertoli cell expression), and stage XI (Sertoli cell expression only). Prior to P21, CUX1 expression was continuous in Sertoli cells and not expressed in developing germ cells, consistent with the expression of mRNA previously reported. At P21, CUX1 was observed in spermatids, and this expression was associated with the change to the phasic, stage-specific pattern of expression. The change in CUX1 expression in Sertoli cells, from continuous in prepubertal mice to phasic when spermatids are first present at P21, suggests that there is negative feedback from the spermatids to the Sertoli cells, suppressing CUX1 expression in the latter. Alternatively, the downregulation of CUX1 expression in Sertoli cells may be required for expression in round spermatids. In contrast to that of wild-type mice, CUX1 expression was continuous in the Sertoli cells of testes isolated from P21 Cux1 transgenic mice, which did not show the switch to asynchronous expression until P28. This suggests that forced expression of CUX1 in the Sertoli cells slowed the initial spermatogenic cycle or that it temporarily prevented the expression of CUX1 protein in round spermatids.

At stage VIII of spermatogenesis, there is a reorganization of the BTB that permits the release of mature sperm into the lumens of the seminiferous tubules [1, 3, 11–13]. The BTB is concurrently reorganized, permitting the movement of primary spermatocytes into the apical compartment. During stage VIII of mouse spermatogenesis, there is intense CUX1 expression in both round spermatids and Sertoli cells. In testes from wild-type mice, more than 60% of the seminiferous tubules showed CUX1 expression in both spermatids and Sertoli cells, consistent with their being between stages VI and X. However, in the testes from Cux1 transgenic mice, there was a significant reduction in the percentage of tubules showing CUX1 expression in both spermatids and Sertoli cells, with a significant increase in the percentage of tubules showing CUX1 expression in only spermatids or Sertoli cells. This suggests either that progression through stages VI–X of spermatogenesis is accelerated in the transgenic testes or that the rate of progression through the other stages is reduced.

CUX1, in cooperation with transcription factors snail and slug, has recently been shown to regulate the expression of E-cadherin and N-cadherin [42]. Moreover, CUX1 was able to repress the expression of E-cadherin and activate the expression of N-cadherin. Thus, one possibility is that CUX1 normally functions in Sertoli cells to regulate the expression of N-cadherin, an integral membrane protein of the BTB. However, the reorganization of junctions that occurs during spermatogenesis results primarily from endocytosis and recycling of membrane proteins that form the junctions [11, 63, 64]. CUX1 has also been shown to stabilize SRC through repression of CDKN1B.
upregulation of C-terminal Src kinase (CSK) [43]. In spermatogenesis, SRC functions to phosphorylate N-cadherin and β-catenin, leading to the release of mature sperm [58, 65, 66]. Moreover, SRC is most abundant at stages VI–VIII of spermatogenesis, and both SRC and CSK are found to associate with both Sertoli cells and spermatids at this stage [65]. Thus, upregulation of CUX1 in Sertoli cells beginning at stage VI may be involved in stabilizing SRC, triggering the release of sperm in stage VIII. One possibility is that increased expression of CUX1 in transgenic mice results in increased CSK and SRC and an altered rate of progression through spermatogenesis. In addition, testosterone activation of Sertoli cells results in increased SRC phosphorylation [67]. Thus, another possibility is that the increase in testosterone in the Cux1 transgenic mice increases SRC activity, resulting in an altered rate of progression through spermatogenesis. The importance of SRC in spermatogenesis has been demonstrated in vivo using the SRC inhibitor PP1 [65]. Inhibition of SRC resulted in a disruption of interactions between Sertoli cells and round spermatids and spermatocytes, leading to the loss of these germ cells. In contrast, there was no observable change in the morphology of seminiferous epithelium or apparent affect on fertility of the Cux1 transgenic mice. Moreover, the targeted deletion of the cut repeat 3 and homeodomain of CUX1 is postnatally lethal for the C3H/He or C57BL/6 Sv background strain but is viable and fertile for the outbred OF1 background, despite the absence of the CUX1 protein in the testis [68]. These results suggest that SRC activity is not dependent on CUX1.

We also observed a significant increase in the number of interstitial cells in prepubertal transgenic mice. This increase could account for the overall increase in testis size in the Cux1 transgenic mice and for the increase in testosterone levels. While the number of interstitial cells was similar in microscopic fields of postpubertal testes of wild-type and transgenic mice, the overall increase in testis size suggests an overall increase in the number of interstitial cells. The mechanism underlying the increase in interstitial cells in the Cux1 transgenic mice is unclear, as we did not observe ectopic expression of CUX1 at any stage examined. One possibility is that the number of Leydig stem cells is increased in the Cux1 transgenic mice. Further studies will be required to determine if this is the case.

The asynchronous expression of CUX1 and alterations in the percentage of seminiferous tubules in spermatogenic stages in the Cux1 transgenic mice suggest that there is cross-talk between the Sertoli cells and spermatids that affects CUX1 expression in both the spermatids and the Sertoli cells. The process of spermatogenesis is tightly regulated, with feedback controls to maintain adequate numbers of spermatogonia and adequate sperm production. Sertoli cell production of GDNF suppresses commitment of spermatogonia to meiosis and spermiogenesis, thus maintaining numbers of spermatogonia [69–72]. Sertoli cell expression of GDNF is regulated by both autocrine and paracrine mechanisms, primarily by FSH, growth factors, and cytokines, at least one of which, tumor necrosis factor (TNF), is expressed by round spermatids [73–75]. Thus, it is possible that expression of CUX1 beginning in round spermatids is involved in signaling between the spermatids and Sertoli cells. One possibility is that the increased level of testosterone in the Cux1 transgenic mice disrupts the normal regulation of SRC, leading to alterations in the percentage of seminiferous tubules in spermatogenic stages. However, numerous genes are expressed in haploid germ cells during spermiogenesis. Indeed, of ~600 testis-specific protein-encoding genes, ~350 are expressed exclusively in spermiogenesis [76–79]. While a number of transcriptional targets of the 200-kDa CUX1 protein have been identified, there are no known targets of the testis-specific 55-kDa CUX1 protein. It will be interesting to discover whether p55 functions as both a transcriptional activator and a repressor; its structural similarity to the p75 protein supports this possibility. Future studies directed at identifying the targets of this protein in spermatids will be essential to determine its function in spermiogenesis.

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