BIOLOGY OF REPRODUCTION **76,** 804–812 (2007)
Published online before print 17 January 2007.
DOI 10.1095/biolreprod.106.059212

Proliferation of Adult Sertoli Cells Following Conditional Knockout of the Gap Junctional Protein GJA1 (Connexin 43) in Mice[1]

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ABSTRACT

GJA1 (also known and referred to here as connexin 43 and abbreviated CX43) is the predominant testicular gap junction protein, and CX43 may regulate Sertoli cell maturation and spermatogenesis. We hypothesized that lack of CX43 would inhibit Sertoli cell differentiation and extend proliferation. To test this, a Sertoli cell-specific *Cx43* knockout (SC-*Cx43* KO) mouse was generated using Cre-lox technology. Immunohistochemistry indicated that CX43 was not expressed in the Sertoli cells of SC-*Cx43* KO mice, but was normal in organs such as the heart. Testicular weight was reduced by 41% and 76% in SC-*Cx43* KO mice at 20 and 60 days, respectively, vs. wild-type (wt) mice. Seminiferous tubules of SC-*Cx43* KO mice contained only Sertoli cells and actively proliferating early spermatogonia. Sertoli cells normally cease proliferation at 2 wk of age in mice and become terminally differentiated. However, proliferating Sertoli cells were present in SC-*Cx43* KO but not wt mice at 20 and 60 days of age. Thyroid hormone receptor alpha (THRA) is high in proliferating Sertoli cells, then declines sharply in adulthood. *Thra* mRNA expression was increased in 20-day SC-*Cx43* KO vs. wt mice, and it showed a trend toward an increase in 60-day mice, indicating that loss of CX43 in Sertoli cells inhibited their maturation. In conclusion, we have generated mice lacking CX43 in Sertoli cells but not other tissues. Our data indicate that CX43 in Sertoli cells is essential for spermatogenesis but not spermatogonial maintenance/proliferation. SC-*Cx43* KO mice showed continued Sertoli cell proliferation and delayed maturation in adulthood, indicating that CX43 plays key roles in Sertoli cell development.

cytokines, gamete biology, Sertoli cell differentiation and proliferation, spermatogenesis, spermatogonia, testis

INTRODUCTION

Gap junctions are intercellular channels that connect the cytoplasm of adjoining cells, allowing intercellular passage of small (<1.5 kDa) molecules and regulating essential processes during development and differentiation. In the seminiferous epithelium, gap junctional coupling occurs between Sertoli cells and adjacent Sertoli cells, spermatogonia, and spermatocytes [1]. Each gap junction channel is composed of two hemichannels or connexons, and each connexon is formed by aggregation of six protein subunits known as connexins [2].

GJA1 (also known and referred to here as connexin 43 and abbreviated CX43) is the most abundant and ubiquitously distributed gap junction protein in the testis [3]. Various knockout mouse models have demonstrated the importance of CX43 in spermatogenesis. *Cx43* knockout (*Cx43* KO) mice were not viable postnatally due to cardiac malformation, and the testes were hypotrophic because of a severe germ cell deficiency [4]. When testes from *Cx43* KO fetuses were grafted under the renal capsules of adult males, the seminiferous epithelium showed a Sertoli cell-only phenotype with only a few germ cells, which indicated that CX43 is required for postnatal germ cell expansion [5]. The requirement for CX43 is tissue specific; when *Cx43* was replaced with *Gja5* (also known as *Cx40*) using a knockin model, the cardiac malformation responsible for lethality of the global knockout was reversed, and the animals were viable. However, seminiferous epithelial abnormalities seen in *Cx43* KO mice persisted in the *Cx40* knockin [6], illustrating an essential and nonredundant role of CX43 in the testis, despite the ability of other connexins to effectively substitute for CX43 in heart. CX43 also may play a critical role in human spermatogenesis, as indicated by the recent report that CX43 was reduced in infertile men with secretory azoospermia [7].

CX43 also has been implicated in regulation of cell growth and differentiation in both normal and tumor cells [8]. For example, forced CX43 expression in lung and liver carcinoma cells in vitro [9] decreases their proliferation. This may be due partially to its role in gap junctions, but CX43 also plays a role in growth regulation that is independent of its role in gap junctions. This is illustrated by the finding that blockade of gap junction function does not abolish the ability of CX43 to regulate cell proliferation, and a mutant form of *Cx43* that lacks gap junction activity is as effective as native *Cx43* in suppressing cell growth [10]. This role of CX43 may reflect effects on the cell cycle machinery. *Cx43* expression in neoplastic cells in vitro results in increased expression of the cyclin-dependent kinase inhibitor CDKN1B (also known as $p27^{Kip1}$), which may mediate growth inhibitory effects of

[1]Supported by the Billie A. Field Endowment, University of Illinois (P.S.C.), National Institutes of Health grants HL64757 (G.I.F.) and HL081336 (D.E.G.), and a Grant-in-Aid from the American Heart Association (D.E.G.). The work at the University of Illinois was conducted in a facility constructed with support from Research Facilities Improvement Program grant number C06 RR16515 from the National Center for Research Resources, National Institutes of Health.
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Received: 29 November 2006.
First decision: 20 December 2006.
Accepted: 15 January 2007.

ISSN: 0006-3363. http://www.biolreprod.org

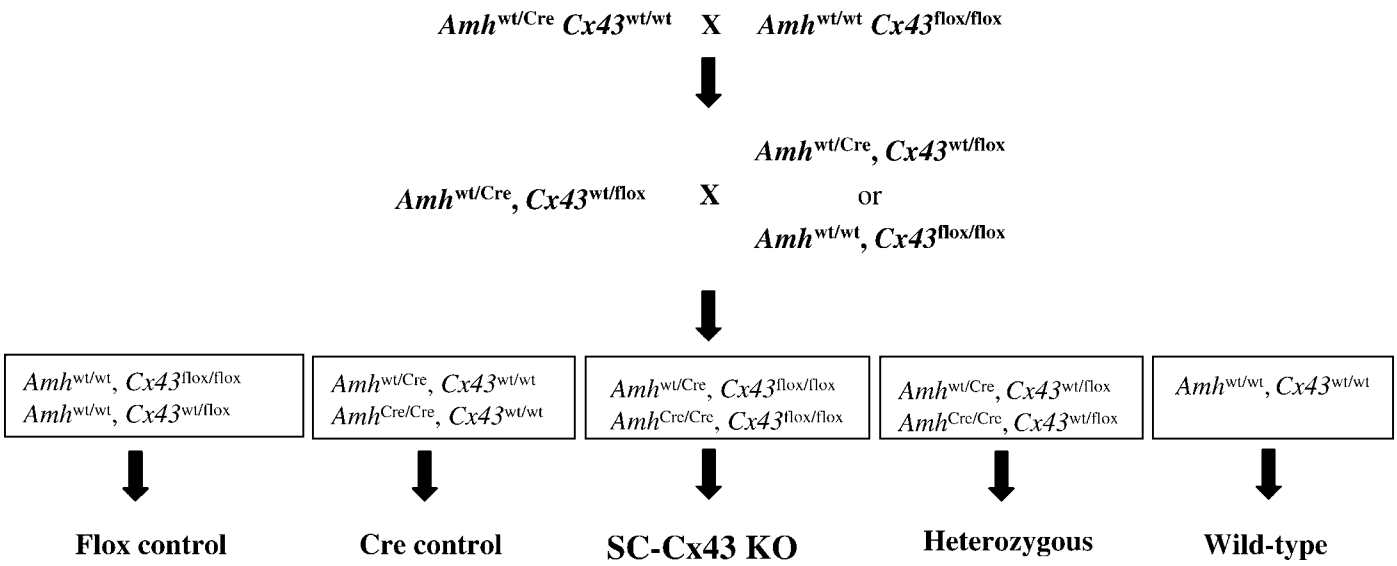

FIG. 1. Generation of SC-*Cx43* KO mice. Transgenic *Amh*-Cre (C57BL/6) male mice expressing Cre-R under the control of the *Amh* gene promoter were bred with C57BL/6 female mice containing two floxed copies of *Cx43*. Mice heterozygous both for *Amh*-Cre and floxed *Cx43* then were mated with either mice heterozygous for *Amh*-Cre and floxed *Cx43* obtained from the first mating or mice that had been bred an additional generation to obtain animals that were homozygous for floxed *Cx43* to yield SC-*Cx43* KO, Cre and flox controls, and heterozygous and wt mice.

CX43 [9]. Furthermore, in human osteosarcoma cells, transfection of *Cx43* inhibited the ubiquitination and subsequent degradation of S-phase kinase-associated protein 2 (SKP2) [11], another cell cycle protein that is the major regulator of CDKN1B. Thus, CX43 actions on cell proliferation may involve actions on cell cycle proteins.

We and others have previously shown that thyroid hormone is a major regulator of Sertoli cell proliferation and maturation, and that thyroid hormone effects may involve CDKN1B and SKP2 ([12–14]; A. Oki et al., unpublished data). Furthermore, CX43 in neonatal rats was localized predominantly in the cytoplasm of Sertoli cells, where it is believed to be inactive. However, during the pubertal period in which Sertoli cells undergo terminal differentiation, CX43 becomes localized to the plasma membrane, where it is presumably active. This process was inhibited by neonatal hypothyroidism [15], suggesting that CX43 in Sertoli cells could be involved in mediating the inhibitory role of thyroid hormone on Sertoli cell proliferation. In addition, a recent study using the 42GPA9 Sertoli cell line demonstrated that thyroid hormone increases expression of CX43 and reduces proliferation in this cell line, again suggesting that CX43 regulates Sertoli cell proliferation and may mediate thyroid hormone effects on this process [16].

Based on emerging literature showing that CX43 is important in Sertoli cells and that CX43 has regulatory effects on proliferation in cell lines, we hypothesized that CX43 could be a major regulator of Sertoli cell proliferation. However, the neonatal lethality of the *Cx43* KO precludes a simple analysis of Sertoli cell number in adults to test this hypothesis. An alternative method for addressing this question is to make a conditional knockout lacking *Cx43* expression only in Sertoli cells. Conditional *Cx43* KO mice lacking CX43 in the heart and other organs have been developed [17–22]. These mice are produced by mating mice expressing Cre recombinase (Cre-R) under the control of a cardiac-specific promoter with mice in which the *Cx43* gene is flanked by loxP sites, or "floxed." A loxP site is a 34-base pair DNA recognition sequence of the Cre-R enzyme. The Cre-R enzyme recognizes and cuts out the intervening DNA between two loxP sites. The Cre/Lox excision activity is highly efficient for producing tissue-specific recombination events in mice.

Lecureuil et al. [23] have developed mice expressing Cre-R in Sertoli cells under the control of the anti-Mullerian hormone (*Amh*) promoter. This mouse, in conjunction with mice containing floxed *Cx43* genes [19, 20], was used here to produce a conditional *Cx43* KO lacking CX43 only in Sertoli cells. Our results show that loss of Sertoli cell CX43 dramatically alters Sertoli cell proliferation and maturation, indicating that CX43 is a major regulator of Sertoli cell development.

MATERIALS AND METHODS

Animals and Animal Care

Transgenic mice expressing Cre-R under the control of the *Amh* gene promoter were obtained from INRA (National Institute for Agronomic Research), Paris, France [23]. Female homozygous *Cx43* floxed mice, in which the *Cx43* coding region is flanked by two loxP sites in both *Cx43* alleles, were developed as described previously [19, 20].

Mice were housed at 25°C with 12L:12D cycles and were given water and a standard rodent diet ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of SC-Cx43 KO Mice

Transgenic *Amh*-Cre (C57BL/6) male mice expressing Cre-R under the control of the *Amh* gene promoter were bred with C57BL/6 female mice containing two floxed copies of *Cx43* (Fig. 1). Mice heterozygous both for the *Amh*-Cre and the floxed *Cx43* then were mated with either mice heterozygous for *Amh*-Cre and floxed *Cx43* obtained from the initial mating or mice that had been bred an additional generation to obtain animals that were lacking *Amh*-Cre and homozygous for floxed *Cx43* (Fig. 1). These matings yielded SC-*Cx43* KO mice, along with other genotypes. Based on previous reports that one copy of the *Amh*-Cre was sufficient to excise floxed androgen receptor gene and create a conditional androgen receptor knockout in Sertoli cells [24], we anticipated that expression of *Amh* promoter-driven Cre-R would efficiently and selectively delete the floxed *Cx43* gene in Sertoli cells even when only one copy of *Amh*-Cre was present.

Genotyping

Animals were genotyped using tail genomic DNA with the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. SC-*Cx43* KO mice ($Amh^{wt/Cre}$, $Cx43^{flox/flox}$ or $Amh^{Cre/Cre}$, $Cx43^{flox/flox}$) expressed

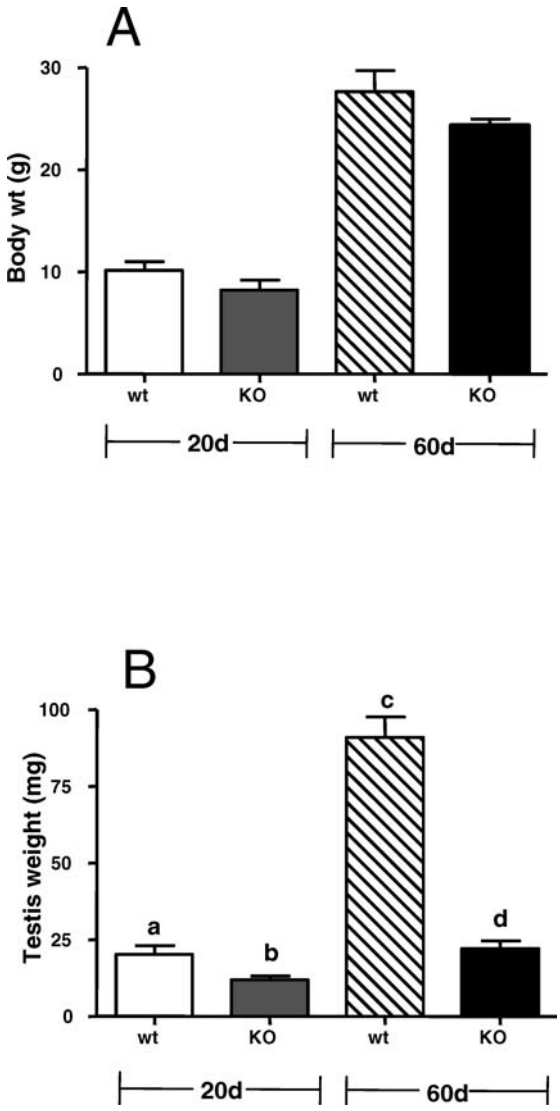

FIG. 2. Body and testis weights of wt and SC-*Cx43* KO mice. **A**) Body weights of wt and SC-*Cx43* KO mice were comparable at 20 days and 60 days. **B**) Testis weights were significantly reduced in 20-day-old and 60-day-old SC-*Cx43* KO mice compared with wt controls. All data are expressed as mean ± SEM (n = 5 per genotype). Values that do not share a common superscript are significantly different from the similarly aged control.

two floxed *Cx43* and one or two Cre alleles, whereas control animals for Cre ($Amh^{Cre/Cre}$, $Cx43^{wt/wt}$ or $Amh^{wt/Cre}$, $Cx43^{wt/wt}$) expressed one or two Cre alleles but not floxed alleles, and control animals to evaluate the effects of the floxed *Cx43* gene alone ($Amh^{wt/wt}$, $Cx43^{flox/flox}$ or $Amh^{wt/wt}$, $Cx43^{wt/flox}$) expressed no Cre but one or two floxed alleles. Heterozygous mice ($Amh^{wt/Cre}$, $Cx43^{wt/flox}$ or $Amh^{Cre/Cre}$, $Cx43^{wt/flox}$) expressed both Cre and floxed alleles (one or more Cre alleles with one floxed allele), and wt mice ($Amh^{wt/wt}$, $Cx43^{wt/wt}$) expressed neither *Amh*-Cre nor floxed *Cx43* alleles.

Experimental Groups

Body and testis weights were measured for all genotypes (Fig. 1) at 20 and 60 days of age. For all other experiments, only 20- and 60-day-old wt and SC-*Cx43* KO mice were used.

Immunohistochemistry

Testes of 20-day-old mice were fixed by immersion in 10% neutral-buffered formalin (NBF), whereas those from 60-day-old mice were fixed by whole-body vascular perfusion using 10% NBF. Fixed testes were embedded in paraffin using standard techniques. Embedded testes were sectioned at 4 μm, deparaffinized, and rehydrated. To facilitate antigen detection of all proteins except CX43, slides were placed in boiling 10 mM sodium citrate buffer (pH 6.0) for 10 min and then cooled to ambient temperature. Endogenous peroxidase activity was quenched by incubation with 0.3% H_2O_2 for 20 min. Binding of primary antibody was localized using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) for each antibody and DAB Substrate Kit (Vector Laboratories), according to the manufacturer's instructions. Negative controls were processed without the corresponding primary antibody.

Immunohistochemistry for CX43

Heart and testis of adult wt and SC-*Cx43* KO mice were immersion fixed in Bouin fluid overnight, dehydrated in 70% ethanol, and embedded in paraffin. Tissue sections were deparaffinized in HistoClear (Fisher Scientific, Ottawa, ON, Canada) and rehydrated by immersion in a series of graded ethanols. Immunohistochemistry was done using the DAKO Catalyzed Signal Amplification System (DAKO, Carpenteria, CA) according to the manufacturer's instructions. Tissue sections were incubated overnight at 4°C with anti-CX43 antisera (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). This antibody has previously been reported to specifically recognize CX43 in testis [15]. CX43 expression was detected using an anti-rabbit horseradish peroxidase-conjugated secondary antiserum (DAKO), according to the manufacturer's instructions. Sections were counterstained with methylene blue. Tissue sections incubated with normal rabbit serum were used as a negative control.

Immunohistochemistry for Wilms Tumor 1 and MKI67

To assess Sertoli and germ cell proliferation in wt and SC-*Cx43* KO mice (n = 4 per genotype), serial testes sections were stained for a Sertoli cell marker, Wilms tumor 1 (WT1), using a rabbit polyclonal IgG to human WT1 (Santa Cruz Biotechnology) and a cell proliferation marker, MKI67, using a mouse anti-human monoclonal IgG to human MKI67 (BD Transduction Laboratories, Lexington, KY). Following immunostaining, tissues were counterstained with Mayer hematoxylin (Sigma-Aldrich, St. Louis, MO). The percentage of proliferating Sertoli cells was determined by MKI67 staining [25] in approximately 500 Sertoli cells identified by positive WT1 staining per testis. We used WT1 staining to calculate total Sertoli cell number per testis based on the count of round objects in sections of known thickness, using the morphometric methods previously described [26].

Isolation of Sertoli Cell RNA

Sertoli cells were isolated from wt and SC-*Cx43* KO mice at 20 and 60 days of age by sequential enzymatic digestion with 0.1% collagenase, 0.1% hyaluronidase with trypsin, and trypsin inhibitor with 0.5% BSA, as previously described [14]. Total RNA was isolated from Sertoli cell samples using the Qiagen RNeasy Mini RNA Isolation kit (Qiagen), according to the manufacturer's instructions. RNA samples were quantified with a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). All RNA samples isolated had an A260/280 ratio of 1.9–2.2. Random RNA samples also were examined for integrity by 1% agarose gel electrophoresis and were stained with 0.5 μg/ml ethidium bromide. RNA samples were stored at −20°C after isolation.

Real-Time PCR

First-strand cDNA was synthesized from total RNA (1 μg) using Superscript reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). Real-time PCR was performed using the ABI Prism 7000 Sequence detection system with validated ABI Taqman gene expression assays (Applied Biosystems, Foster City, CA). The expression value of thyroid hormone receptor alpha *(Thra)* mRNA was normalized to the amount of an internal control gene (18S ribosomal RNA) to calculate a relative amount of RNA in each sample. The expression value of *Thra* gene in the wt sample was arbitrarily defined as 1 unit. All assays were carried out in triplicate for the *Thra* mRNA, and the normalized expression values for all wt samples and SC-*Cx43* KO samples were averaged. A relative quantitative fold change was determined using the ΔΔCt method (ABI Chemistry Guide no. 4330019). The ABI Taqman gene expression assay used for *Thra* transcript was MA00617505-A_1.

Statistical Analysis

For body and testis weights, data were analyzed with one-way ANOVA. All other data were analyzed using Student *t*-test and are presented as mean ± SEM. Differences were considered significant at $P < 0.05$. Statistical analysis was carried out using Graph Pad Prism 4.0 (Graph Pad Software Inc., San Diego, CA).

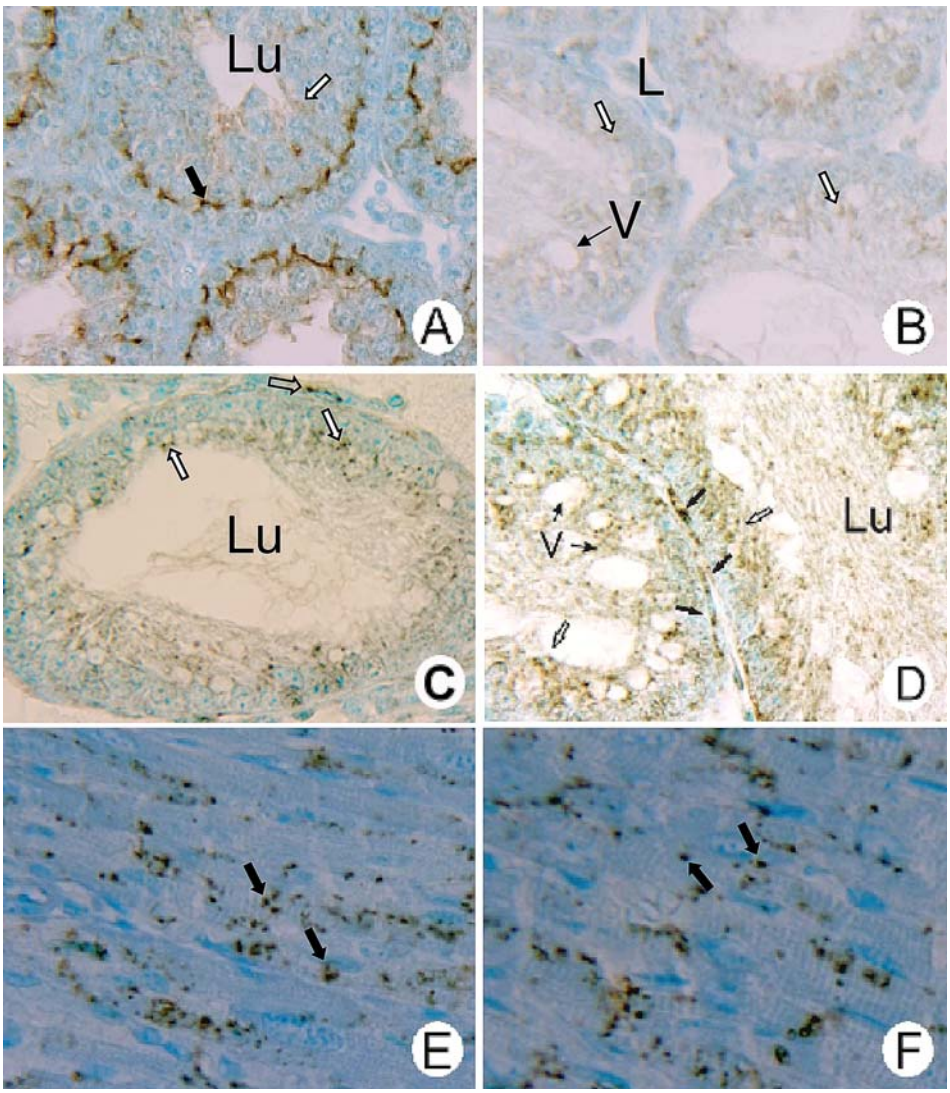

FIG. 3. Immunolocalization of CX43 in testis and heart of adult wt and SC-*Cx43* KO mice. **A**) Cx43 (black arrows) in wt mice was localized to Sertoli cells at the basal region of the seminiferous tubule. CX43 also was localized more apically in the Sertoli cells (white arrows). **B** and **C**) In SC-*Cx43* KO mice, CX43 immunostaining was absent between adjacent Sertoli cells. However, CX43 immunoreactivity was still seen in germ cells (white arrows). Most tubules contained vacuoles (V) that appeared to be surrounded by a plasma membrane. Immunoreactivity between Leydig cells (open arrow) also was present. **D**) CX43 also was observed in myoid cells (black arrows). Heart was used as a positive control (**E** and **F**). **E**) In wt mice, CX43 was observed between cardiac myocytes (black arrows). In SC-*Cx43* KO mice, CX43 was present and also appeared to be localized to cardiac myocytes. Intensity of CX43 immunoreaction in the heart sections of SC-*Cx43* KO mice was comparable to that in wt mice. Lu, lumen of seminiferous tubule; L, Leydig cell. Original magnification ×240 (**A**–**D**) and ×400 (**E** and **F**).

RESULTS

Effect of Loss of CX43 in Sertoli Cells on Body and Testis Weights

Body weights in wt and SC-*Cx43* KO mice were not significantly different at 20 and 60 days of age (Fig. 2A). Conversely, testis weights (Fig. 2B) were significantly lower at both ages in SC-*Cx43* KO mice (11.4 ± 1.2 and 22.1 ± 2.5 mg, respectively) compared with wt mice (20.3 ± 2.9 and 91.0 ± 6.7 mg, respectively). Testis weights were reduced 41% ($P < 0.05$) and 76% ($P < 0.00001$) in SC-*Cx43* KO mice at 20 and 60 days of age, respectively, compared with age-matched wt mice. Mice heterozygous or homozygous for *Amh*-Cre and with one or no floxed *Cx43* alleles did not show any significant changes in body or testes weights compared with wt mice at 20 and 60 days of age (data not shown). Similarly, mice heterozygous or homozygous for floxed *Cx43* but without *Amh*-Cre also had body and testes weights that were not significantly different from wt controls (data not shown).

CX43 Expression in Testis and Heart of Conditional Cx43 *Knockouts*

CX43 was strongly expressed in seminiferous epithelium of wt mice (Fig. 3A). Conversely, CX43 staining was absent in Sertoli cells of SC-*Cx43* KO testis (Fig. 3, B–D). In addition, CX43 expression was strong and comparable in hearts of both wt (Fig. 3E) and SC-*Cx43* KO (Fig. 3F) mice, indicating that loss of Sertoli cell CX43 seen in SC-*Cx43* KO mice was not accompanied by loss of CX43 in other organs. Excision of *Cx43* was detected by PCR in Sertoli cells from SC-*Cx43* KO mice, but not in heart from these animals (data not shown), confirming the immunohistochemistry results.

*Testicular Histology of SC-*Cx43 *KO*

Normal spermatogenesis was initiated in wt mice at Day 20, and full spermatogenesis was seen at Day 60 (Fig. 4, A and C). Spermatogenesis was absent at 20 and 60 days in SC-*Cx43* KO mice (Fig. 4, B and D). At both ages, seminiferous tubules had a Sertoli cell-only phenotype with a few early-stage germ cells. Seminiferous tubules of SC-*Cx43* KO mice were vacuolated, and this was more pronounced at 60 days (Fig. 4, D and E). SC-*Cx43* KO testis contained extensive interstitial cells, and this was more prominent at 60 days (Fig. 4E). However, this may be due to decreased size of seminiferous tubules and testes in SC-*Cx43* KO mice rather than hyperplastic changes in the interstitium.

Sloughing of Sertoli cells from the basement membrane also was observed in SC-*Cx43* KO mice. Sloughed cells formed

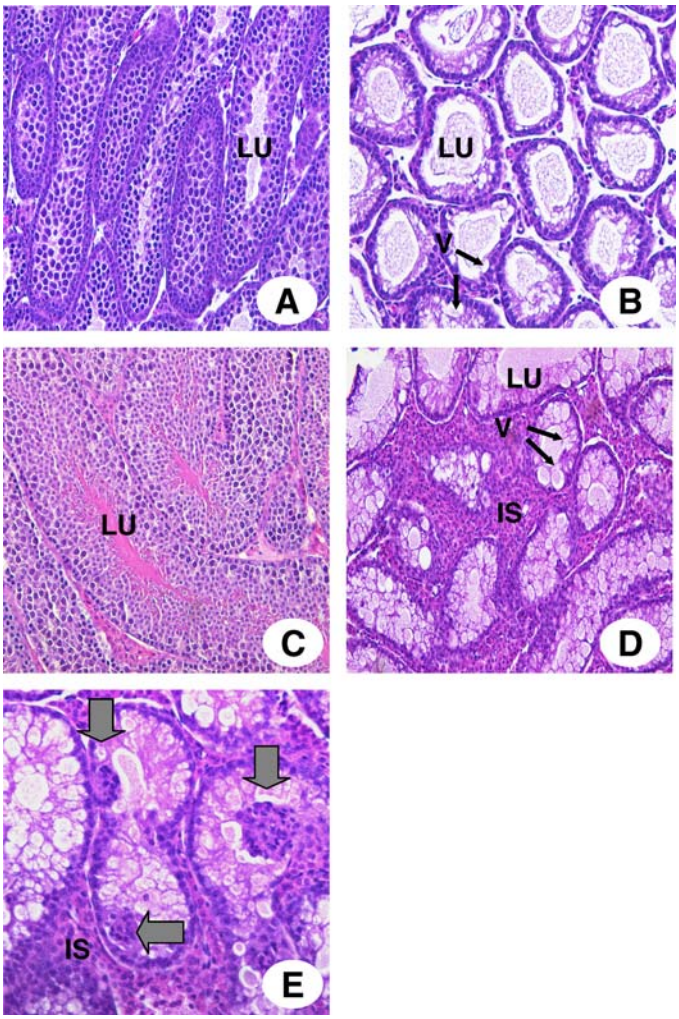

FIG. 4. Testicular histology of 20-day-old wt (**A**), 60-day-old wt (**C**), 20-day-old SC-*Cx43* KO (**B**), and 60-day-old SC-*Cx43* KO (**D** and **E**) mice. Spermatogenesis was absent in SC-*Cx43* KO mice at both ages (**B**, **D**, and **E**). The SC-*Cx43* KO testis had a Sertoli cell-only phenotype with a few germ cells in the seminiferous tubules at both 20 days (**B**) and 60 days (**D** and **E**). Vacuoles (V) were present in tubules of SC-*Cx43* KO mice (black arrows) at both 20 and 60 days. In SC-*Cx43* KO testis there was a relative increase in interstitial cells (IS) due to reduced overall testicular size (**D** and **E**). In addition, the presence of clusters of sloughed Sertoli cells (arrows) inside the lumen (LU) was pronounced in 60-day-old SC-*Cx43* KO testis **(E)**. Original magnification ×100 (**A**–**D**) and ×200 (**E**).

clusters inside tubule lumens, and this was more prominent at 60 days (Fig. 4E).

MKI67 staining indicated that some germ cells were proliferating at 20 and 60 days of age in SC-*Cx43* KO mice (not shown). Thus, lack of Sertoli cell CX43 does not preclude proliferation of remaining germ cells.

Loss of CX43 in Sertoli Cells Extends Their Proliferation

We observed a lack of Sertoli cell proliferation in wt testes at 20 and 60 days of age in the present study (Fig. 5, A and B), consistent with previous reports that Sertoli cells normally cease proliferation by approximately 2 wk of age in mice [27]. In contrast, in SC-*Cx43* KO mice, Sertoli cells were still proliferating at 20 and even 60 days of age (Fig. 5, C and D). Sertoli cell proliferation, as determined by labeling index (n = 4 per genotype), was 2.2% ± 0.3% ($P < 0.00001$) and 1.3% ± 0.1% ($P < 0.000001$) in 20- and 60-day-old SC-*Cx43* KO mice, whereas in wt mice Sertoli cell proliferation was 0.02% ± 0.02% at 20 days of age and was absent at 60 days (Fig. 6A).

Effect of Loss of Sertoli Cell CX43 on Adult Sertoli Cell Number

Sertoli cell number was increased in SC-*Cx43* KO mice compared with wt mice at 20 and 60 days of age (Fig. 6B). There was no significant difference in Sertoli cell numbers between 20-day-old and 60-day-old wt mice, which is consistent with earlier findings that Sertoli cell mitogenesis ceases before weaning [27]. Sertoli cell numbers in 20-day-old ($6.66 \pm 0.98 \times 10^6$, $P < 0.05$) and 60-day-old ($6.06 \pm 0.99 \times 10^6$, $P < 0.05$) SC-*Cx43* KO mice were significantly increased compared with wt mice ($3.85 \pm 0.14 \times 10^6$ and $3.50 \pm 0.37 \times 10^6$, respectively). Sertoli cell number was increased by 73% in both 20-day-old and 60-day-old SC-*Cx43* KO mice, despite 41% and 76% reductions in testis weight, respectively, compared with wt mice.

*Effect of Loss of Sertoli Cell-*Cx43 *on* Thra *Expression*

THRA expression in Sertoli cells is indicative of maturational state, with high neonatal expression followed by steady decreases to minimal levels in adults [28–30]. There was a significant 166% increase ($P < 0.05$) in *Thra* expression in SC-*Cx43* KO mice compared with wt mice at 20 days (Fig. 6C). At 60 days, *Thra* expression in SC-*Cx43* KO mice was increased 44% and showed a trend toward an increase compared with age-matched wt mice, but this did not reach significance ($P = 0.16$).

DISCUSSION

CX43 is the predominant gap junctional protein in the testis of both the rat [3, 31] and the human [7, 32], and it appears to have critical effects on spermatogenesis [4, 5]. Work in other cell types suggests that CX43 may also regulate cell proliferation [8, 9] and thus could have effects on Sertoli cell mitogenesis and the establishment of the ultimate Sertoli cell number. However, this question was problematic to address due to the neonatal lethality of the global knockout [33]. In this study we have used a Sertoli cell-specific *Cx43* knockout to determine the role of CX43 in Sertoli cell development.

The lack of CX43 expression in Sertoli cells of the SC-*Cx43* KO mice in this study indicates that the Cre/lox methodology used has resulted in a conditional knockout of Sertoli cell *Cx43* expression. The lack of Sertoli cell *Cx43* contrasts with the continued robust expression in other testicular cell types, such as peritubular, myoid, Leydig, and germ cells. Similarly, heart cells normally express CX43, and mice with a global knockout of *Cx43* die from heart abnormalities [33]. Our results show that heart, which is dependent on CX43 for normal development, expresses CX43 normally and is functional in SC-*Cx43* KO mice. These results further indicate that we have successfully produced mice lacking Sertoli cell CX43, but that body weight was not significantly different. This contrasted with the sharp decreases in testis weight seen at 20 and especially at 60 days of age in SC-*Cx43* KO mice. The lack of Sertoli cell CX43 resulted in the absence of spermatogenesis and concomitant decreases in overall testis weight compared with the wt control, where spermatogenesis was normal. The lack of effect on body weight is consistent with immunohistochemical results that indicate a loss of CX43 exclusively in Sertoli cells of the testis.

Mice in which *Amh*-Cre and/or floxed *Cx43* is present—but do not have the combination of two floxed alleles and *Amh*-Cre necessary to produce a deletion of *Cx43*—function as critical

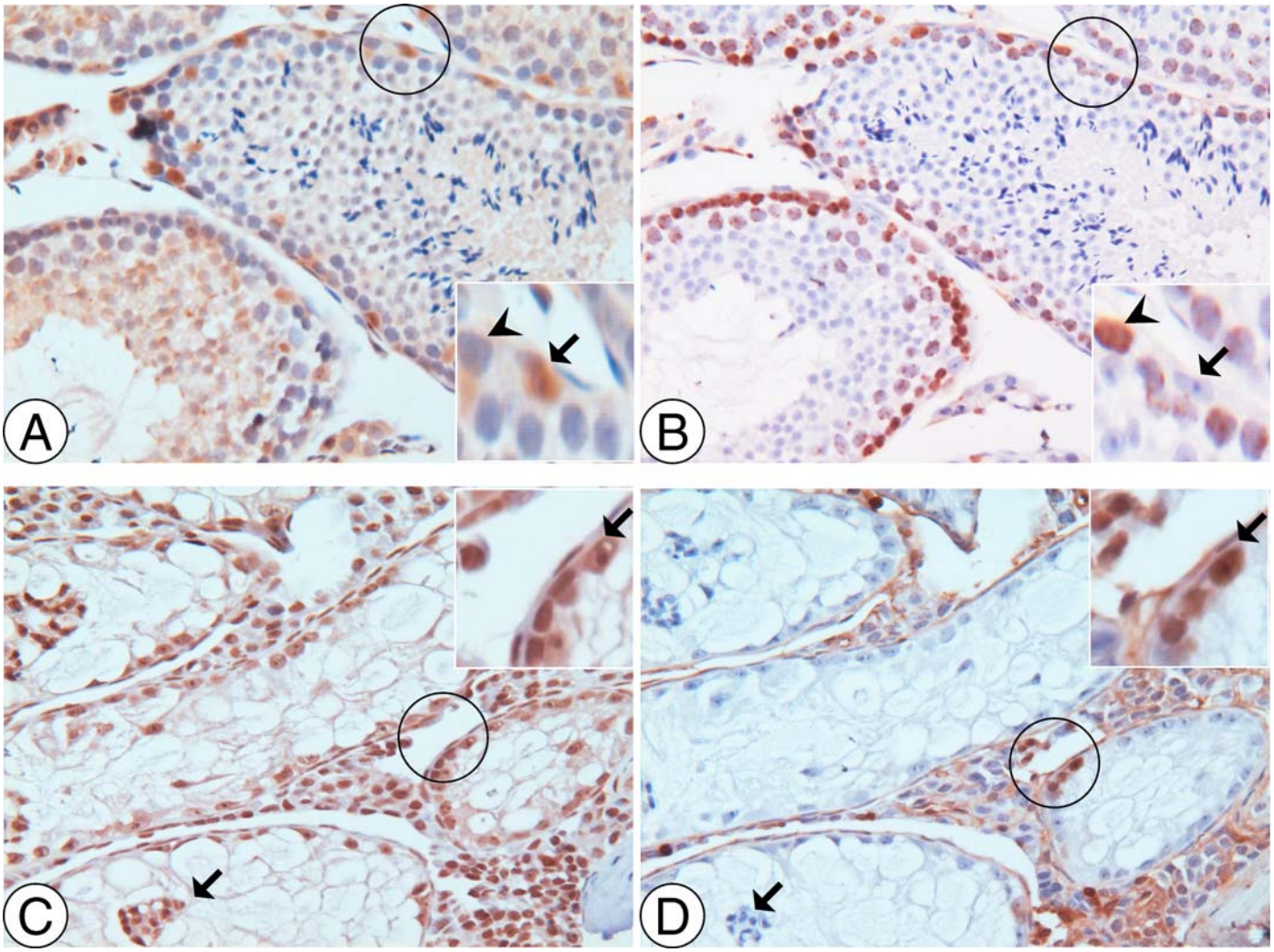

FIG. 5. Immunohistochemistry of serial testicular sections for WT1 (**A** and **C**) and MKI67 (**B** and **D**). Insets in **A**, **B**, **C**, and **D** are magnifications (2-fold) of corresponding circled areas. Cells stained for WT1 are Sertoli cells, and those immunopositive for MKI67 are proliferating cells. **A**) In 60-day wt testis, cells immunopositive for WT1 (inset, black arrow) are Sertoli cells, and all germ cells (inset, arrowhead) were immunonegative for WT1. **B**) In the adjacent section stained for MKI67, cells immunopositive for WT1 (**A**) were immunonegative for MKI67 (inset, black arrow), whereas some of the germ cells (inset, arrowhead) were immunopositive for MKI67. **C**) In 60-day SC-*Cx43* KO testis, cells immunopositive for WT1 are Sertoli cells (inset, black arrow). Sloughed cells (black arrow) forming clusters in the lumen stained for WT1, indicating that they were Sertoli cells. **D**) In the corresponding serial section stained for MKI67, some cells immunopositive for WT1 (**C**) were immunopositive for MKI67 (inset, black arrow), indicating that in SC-*Cx43* KO mice Sertoli cells were proliferating even at 60 days of age. Original magnification ×400.

controls to ensure that observed effects on various testicular parameters are indeed due to specific deletion of Sertoli cell *Cx43*, rather than to potential nonspecific deleterious effects caused by independent effects of either floxing the *Cx43* gene or the expression of *Amh*-Cre. The lack of any statistically significant changes in either body or testicular weights in mice expressing *Amh*-Cre and/or floxed *Cx43* that did not have the genotype that results in a conditional knockout indicates that neither the floxed *Cx43* nor *Amh*-Cre alone had significant deleterious effects on either of these parameters.

In mice, Sertoli cells are proliferating actively at birth and then show declining rates of proliferation during the neonatal period up to approximately 2 wk of age. Sertoli cell mitogenesis then ceases, and the cells undergo terminal differentiation [34], which involves the expression of a variety of factors implicated in supporting spermatogenesis and the development of the actin cytoskeleton [35]. In addition, morphologic changes such as the formation of the Sertoli cell barrier are accompanied by adluminal secretion of Sertoli cell fluid, which leads to canalization of the seminiferous cord. Once Sertoli cells are terminally differentiated, they remain nonmitogenic under normal conditions in mice. The mechanisms involved in Sertoli cell proliferation and differentiation, as well as the transition between these two states, are not fully understood.

Our present results indicate that the lack of CX43 expression in Sertoli cells allows them to remain proliferative well into adulthood. The oldest age examined in our present study was Day 60, but it is likely that Sertoli cells in SC-*Cx43* KO mice continue proliferating throughout life. The continued Sertoli cell proliferation in SC-*Cx43* KO mice suggests that maturation of these cells must be inhibited, as full maturational changes in Sertoli cells cannot occur until proliferation has ceased. THRA expression is a sensitive indicator of maturational state in Sertoli cells, being high during the neonatal proliferative period and declining to minimal levels as the Sertoli cells mature and become postmitotic. The increased *Thra* expression in Sertoli cells of SC-*Cx43* KO mice

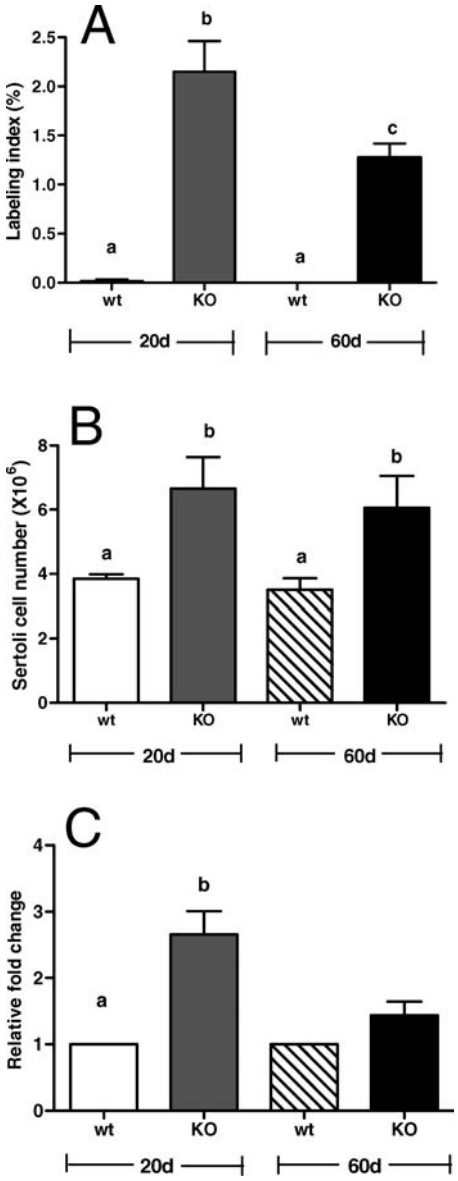

FIG. 6. Effects of loss of Sertoli cell CX43 on proliferation of Sertoli cells (**A**), Sertoli cell number (**B**), and *Thra* mRNA expression (**C**), in testes of 20-day-old and 60-day-old wt and SC-*Cx43* KO mice. **A**) Significant Sertoli cell proliferation was seen in SC-*Cx43* KO mice compared with wt mice at both 20 days and 60 days, where Sertoli cell proliferation was essentially absent. **B**) Sertoli cell number was increased in SC-*Cx43* KO compared with wt mice at both 20 and 60 days. **C**) Relative *Thra* mRNA expression for SC-*Cx43* KO mice at 20 days was significantly higher ($P < 0.05$) than age-matched wt mice. At 60 days, mRNA expression was 40% more than that of age-matched wt mice, although this did not reach statistical significance. Data are expressed as mean ± SEM (n = 4 per genotype except in **C**, where n = 3 per genotype). Values that do not share a common superscript are significantly different.

compared with wt mice strongly supports the idea that Sertoli cell differentiation is inhibited by lack of CX43, and these results are consistent with the observed continued proliferation of these cells. Thus, absence of CX43 blocks the maturational sequence that Sertoli cells normally undergo during neonatal life that culminates in them eventually becoming postmitotic and showing morphologic and functional differentiation.

CX43 expression in Sertoli cells first occurs at puberty and serves as a pubertal differentiation marker in species such as pig, guinea pig, and human [36]. In the rat, CX43 was initially localized to both the plasma membrane and the cytoplasm of Sertoli cells during postnatal development. By Day 30, CX43 expression was entirely along the basement membrane [3, 31]. In contrast, hypothyroidism resulted in impairment of this normal maturational sequence and continued localization of CX43 in the cytoplasm at Day 30, where it is presumed to be nonfunctional. Hypothyroidism is associated with an increased period of neonatal proliferation [37] and an ultimately large increase in adult Sertoli cell numbers [26]. The present results showing a critical regulatory role for CX43 in Sertoli cell proliferation, in concert with previous data showing that thyroid hormone affects developmental expression of CX43 [16, 38] as well as Sertoli cell proliferation in vivo [37] and in vitro [39], indicate that thyroid hormone effects on CX43 may be critical to understanding how thyroid hormones induce maturational changes that normally render Sertoli cells postmitotic during juvenile development in rodents in vivo. This conclusion is strongly supported by recent in vitro data using a Sertoli cell line, in which thyroid hormone increased CX43 and inhibited proliferation of these cells [16].

The continued Sertoli cell proliferation in SC-*Cx43* KO mice would be expected to lead to increased adult Sertoli cell populations in these mice, and indeed such increases in Sertoli cell populations were observed. However, increases in Sertoli cell populations in SC-*Cx43* KO mice were modest based on the continued robust proliferation of Sertoli cells in SC-*Cx43* KO mice into adulthood. This apparent discrepancy is explained by our observations that SC-*Cx43* KO mice have aggregates of cells in the lumen of the seminiferous tubules. Immunostaining identified these as Sertoli cells, and these cells appeared to have been sloughed from the seminiferous epithelium. The continued proliferation of Sertoli cells in SC-*Cx43* KO mice into adulthood may lead to an excessive buildup of Sertoli cells along the basement membrane of the tubule, and this may result in sloughing and loss of some Sertoli cells as their proliferation continues.

In SC-*Cx43* KO mice, the seminiferous tubules had Sertoli cells and a few early-stage spermatogonia; some of those spermatogonia were actively proliferating. Our results with the conditional *Cx43* KO confirm the earlier study of Roscoe et al. [5], who also observed proliferation of early-stage germ cells when they grafted *Cx43* KO testes under the renal capsules of adult male mice for up to 3 wk. The lack of continued development of germ cells past an early stage likely results from the immaturity of the Sertoli cells lacking CX43 as well as the lack of gap junctional communication with the Sertoli cells. However, our results and the previous work of Roscoe et al. [5] indicate that even in the absence of CX43, Sertoli cells can support development of fetal germ cells (primordial germ cells and gonocytes) and the differentiation of spermatogonia from these cells, although the Sertoli cells are not capable of supporting more advanced stages of spermatogenesis.

FSH is the major mitogen for Sertoli cells, but Sertoli cell proliferation decreases and then stops neonatally despite continued presence of FSH [40] and increasing expression of FSH receptor [41]. Thyroid hormones stimulate maturational events that result in the cessation of Sertoli cell proliferation and the concomitant stimulation of functions characteristic of the mature Sertoli cell, as described above. These thyroid hormone effects on Sertoli cell proliferation may involve effects on cell cycle proteins such as CDKN1B, CDKN1A (also known as $p21^{Cip1}$), and SKP2 [12–14, 42]. In addition, elegant work by Chaudhary et al. [43] demonstrated that overexpression of the inhibitor of differentiation proteins in postmitotic, terminally differentiated Sertoli cells caused them to reenter the cell cycle and proliferate indefinitely. The

potential interrelationships between CX43, cell cycle proteins such as CDKN1B, CDKN1A, and SKP2, and the inhibitor of differentiation proteins in the events that normally accompany the transition of a Sertoli cell from a proliferative to a mature, postmitotic state during development still remain to be established, but work in this area should yield additional insights into the complex and important process of Sertoli cell differentiation.

In conclusion, we have successfully generated a Sertoli cell-specific *Cx43* knockout and shown that CX43 in Sertoli cells is essential for spermatogenesis but not for maintenance and proliferation of spermatogonia. Critically, SC-*Cx43* KO mice showed continued Sertoli cell proliferation long after Sertoli cell proliferation had ceased in wt mice, but they had inhibited Sertoli cell differentiation. Our results suggest that CX43 plays a key inhibitory role in controlling Sertoli cell proliferation and is essential for normal maturation of Sertoli cells in mice.

ACKNOWLEDGMENTS

The authors thank Sara Edwards and Evemie Dube (INRS-Institut Armand Frappier, Université du Québec, QC, Canada) for help with CX43 immunostaining.

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