A Sertoli Cell-Specific Knockout of Connexin43 Prevents Initiation of Spermatogenesis

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The predominant testicular gap junctional protein connexin43 (cx43) is located between neighboring Sertoli cells (SCs) and between SCs and germ cells. It is assumed to be involved in testicular development, cell differentiation, initiation, and maintenance of spermatogenesis with alterations of its expression being correlated with various testicular disorders. Because total disruption of the cx43 gene leads to perinatal death, we generated a conditional cx43 knockout (KO) mouse using the Cre/loxP recombination system, which lacks the cx43 gene solely in SCs (SCCx43KO), to evaluate the SC-specific functions of cx43 on spermatogenesis *in vivo*. Adult SCCx43KO^{-/-} mice showed normal testis descent and development of the urogenital tract, but testis size and weight were drastically lower compared with heterozygous and wild-type littermates. Histological analysis and quantitation of mRNA expression of germ cell-specific marker genes revealed a significant reduction in the number of spermatogonia but increased SC numbers/tubule with only a few tubules left showing normal spermatogenesis. Thus, SC-specific deletion of cx43

mostly resulted in an arrest of spermatogenesis at the level of spermatogonia or SC-only syndrome and in intratubular SC clusters. Our data demonstrate for the first time that cx43 expression in SCs is an absolute requirement for normal testicular development and spermatogenesis. (Am J Pathol 2007, 171:19–31; DOI: 10.2353/ajpath.2007.061171)

Somatic Sertoli cells (SCs) play a central role in testicular development. Within the undifferentiated gonad, the first histological sign of male sex differentiation is the appearance of pre-SCs forming primitive testicular cords.¹ Pre-SCs 1) integrate primordial germ cells migrating from the yolk sac into the primitive seminiferous cords and prevent these cells from entering meiosis²; 2) are supposed to predominantly express the gene for the sex-determining region of the Y chromosome (SRY),^{3,4} initiating the genetic sex determination of the embryo; and 3) secrete the anti-Muellerian hormone (AMH), which causes regression of the Muellerian ducts initiating the hormonal sex determination of the embryo.^{5,6} At puberty, SCs develop complex morphological interactions with each other and with adjacent germ cells and undergo terminal differentiation resulting in their adult, mature phenotype. This process involves important functional and structural changes with SCs now supporting spermatogenesis through complex cell-cell interactions.^{7–10} SCs form a complex network of specific intercellular junctions with individual adjacent germ cell populations. In addition, SCs are in contact with each other via the so-called SC-junctional complex that consists of tight, adherens, and gap junctions constituting the anatomical basis of the blood-testis barrier.^{11–13}

Gap junctions are formed between adjacent homologous and heterologous cell types in nearly all epithelia. A gap junction channel consists of two hemichannels (connexons)

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contributed separately by each of the two participating cells. Each connexon is again formed by the hexameric assembly of protein subunits known as connexins (cx). The cx family consists at least of 20 members in human and 19 members in rodents.^{14,15} In testis, the occurrence of gap junctions has been firmly established by various morphological, immunocytochemical, and functional assays.^{16–21} Within the interstitium, Leydig cells are solely immunopositive for cx43,^{22–26} as are peritubular cells.^{22,27} Within the seminiferous epithelium, gap junctions containing cx43 are an integral component of the junctional complex between SCs, and they do also occur between SCs and spermatogonia and between SCs and primary spermatocytes.^{22,25,26,28–32} Furthermore, cx43 in SCs is believed to play a role in the regulated formation of the blood-testis barrier at puberty.^{13,33,34} Thus, SCs guarantee metabolic and signaling coupling to germ cells and allow synchronization of male germ cell proliferation and differentiation.^{18,35} Among testicular cx, cx43 represents the predominant gap junction protein.^{13,22,24–26}

For the elucidation of the contribution of cx43 to the function of gap junctions in molecular physiology during embryonic development and/or in the adult animal *in vivo*, constitutive cx43 knockout (KO), knockin (KI), and transgenic mouse models have been generated.¹⁵ Unfortunately, total disruption of the cx43 gene was found to lead to altered cardiac morphology and perinatal death.^{15,36} However, the importance of cx43 to gametogenesis is indicated by severe depletion of germ cells in prenatal male mice lacking the cx43 gene.²⁸ Postnatal proliferation of spermatogonia is also impaired in cx43-null mutants.³¹ Insertion of cx32 or cx40 coding regions into cx43 coding region of constitutive cx43-KO mice restored other deficiencies caused by cx43 deletion,³⁶ but spermatogonial amplification and spermatogenesis remained defective.³⁷ Thus, expression of cx43 is considered to be an essential component of the communication pathways starting in early embryogenesis in rodents. Furthermore, cx43 plays an integral part with the onset of spermatogenesis at puberty concomitant with functional maturation and terminal differentiation of SCs.^{13,17,19,21}

In the present study, a conditional cx43-KO mouse line (SCCx43KO) was generated using the Cre/loxP recombination system by crossing two transgenic mouse lines, AMH-Cre mice and cx43-floxed LacZ mice. Our data indicate that cx43 expression in SCs is an absolute requirement for normal testicular development and spermatogenesis.

Materials and Methods

Generation of Transgenic AMH-Cre Mice

An established transgenic mouse line expressing Cre recombinase under the control of the AMH gene promoter selectively in SCs was used. Generation of AMH-Cre mice is described in detail elsewhere.⁶

Generation of Transgenic Floxed cx43-LacZ Mice

We used an established floxed cx43-LacZ transgenic mouse line carrying a cx43 coding region flanked by loxP recognition sites for the Cre recombinase in all cells. In addition, a silent LacZ reporter gene was integrated into the floxed cx43 allele.^{38,39}

Fertility and Expression of the cx43 Gene in Floxed cx43 Mice and AMH-Cre Mice Is Unaltered

Mice of both transgenic lines and sexes were viable, fertile, and reproduced normally. We also used immunohistochemistry (IHC) and Western blot analysis to assess the levels of cx43 protein synthesis. All male mice revealed histologically normal spermatogenesis, and results showed that the expression level and cellular distribution of the cx43 gene in testis of heterozygous, homozygous floxed cx43 mice and Cre mice were unchanged, as compared with those of wild-type (WT) mice (data not shown).

Breeding Strategy, Generation of SCCx43KO Mice, and Polymerase Chain Reaction (PCR) Genotyping

SC-specific deletion of the cx43 coding region was achieved by crossing floxed mice with mice harboring the Cre transgene under control of SC-specific AMH transcriptional elements. Briefly, homozygous male (female) cx43-floxed LacZ mice were crossed with corresponding female (male) mice expressing Cre recombinase (Cre) exclusively in SCs [(P)arental generation]. The transgenic mice from the F1 generation (SCCx43KO^{+/-}) were then backcrossed to homozygous Cx43-floxed LacZ mice to generate SCCx43KO^{+/-} and SCCx43KO^{-/-} mice. Mice of this F2 generation were born with the expected Mendelian frequency, were grossly indistinguishable from their non-KO littermates, and were genotyped using appropriate primer pairs (Table 1). DNA for all genotyping experiments was prepared from mouse tails using DirectPCR lysis reagent (Viagen Biotech, Los Angeles, CA) according to the manufacturer's protocol and subjected to Cre-PCR. Briefly, 1 μ l of DNA was added to 5 μ l of 5 $\times$ Colorless GoTaq Flexi PCR buffer (Promega, Mannheim, Germany), 2 μ l of MgCl₂ (25 mmol/L; Promega), 0.5 μ l of dNTPs (Promega), 0.15 μ l of GoTaq DNA polymerase (Promega), 0.5 μ l of each primer (10 μ mol/L; MWG, Ebersberg, Germany), and diethylpyrocarbonate (DEPC)-H₂O to a final volume of 25 μ l. PCR conditions were as follows: 1 $\times$ 95°C for 2 minutes; 39 $\times$ [95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds], 72°C for 7 minutes. PCR products were separated in a 1.5% agarose gel and visualized with SYBR Green (Sigma-Aldrich, Munich, Germany). For simultaneous detection of the cx43-floxed allele and the cx43 WT allele, appropriate primers UMP and UMPR (Table 1) were applied, generating a 1.1-kb floxed amplicon and a 987-bp WT amplicon, spanning the junction between the intron of

Table 1. Primers Used for PCR (Genotyping) and RT-PCR

Gene/mRNA	Forward primer	Reverse primer	Size of amplicon
Cre	5'-CCTGGAAATGCTTCTGTCCG-3' (Cre26)	5'-GCAGGCGCAGGAGCTGGTGC-3' (MT1)	1.914 bp
Cx43 WT and floxed	5'-TCATGCCCGCACAAAGTGAGAC-3' (UMP)	5'-TCACCCCAAGCTGACTCAACCG-3' (UMPR)	1.100 bp (flox), 988 bp (WT)
Cx43 del	5'-GGCATACAGACCCCTTGGACTCC-3' (delforw)	5'-TGCGGGCTCTTCGCTATTACG-3' (delrev)	670 bp (del)
Cx43	5'-CCATCTCTAACTCCCATGCACAGC-3' (F)	5'-TGGCAGCACTGCTGGCTCTGCTT-3' (R)	138 bp
Prm1	5'-AAGATGTTCGAGACGGAGGAG-3' (F)	5'-TATGGTGTATGAGCGGCGG-3' (R)	79 bp
Prm2	5'-GCTGTCTCTCGTAAGAGGC-3' (F)	5'-ACATTTCTGACCTGCATCT-3' (R)	146 bp
Tnp1	5'-CGACCAGCCGCAAACTAAAG-3' (F)	5'-GCAATTACCGATCCCACTTGT-3' (R)	162 bp

Cx43, connexin43; Prm1, protamine 1; Prm2, protamine 2; Tnp1, transition protein 1.

cx43 and the cx43 coding region. For cx43flox PCR, 1 μ l of DNA was added to 2 μ l of 10 $\times$ Gold PCR buffer (Applied Biosystems, Darmstadt, Germany), 1 μ l of MgCl₂ (25 mmol/L; Applied Biosystems), 0.25 μ l of dNTPs (Applied Biosystems), 0.15 μ l of Gold AmpliTaq DNA polymerase (Applied Biosystems), 0.25 μ l of each primer (10 μ mol/L⁻¹; MWG), and DEPC-H₂O to a final volume of 25 μ l. PCR conditions were as follows: 1 $\times$ 94°C for 10 minutes; 34 $\times$ [92°C for 1 minute, 65°C for 1 minute, and 72°C for 2 minutes], 72°C for 10 minutes. PCR products were separated in a 2% agarose gel and visualized with SYBR Green (Sigma-Aldrich).

Fertility Assessment of SCCx43KO^{+/-} and SCCx43KO^{-/-} Mice

We investigated the reproductive capacities of male SCCx43KO^{+/-} and SCCx43KO^{-/-} mice by mating these males with two or three WT females each for up to 6 weeks. Female mice were checked for vaginal plugs each morning.

Tissue and Histochemical Techniques

Animal experiments were approved by the animal rights committee at the regional commission of Giessen, Germany (decision V54-19c 20/15 c GI 18/1) and the ethics commission at the University of Giessen, Giessen, Germany (decision 56/05). Genotyped SCCx43KO^{+/-} and SCCx43KO^{-/-} mice as well as WT mice [normal mice, ie, no transgenic mice and mice from P generation (floxed mice not expressing Cre and Cre mice that are not floxed)] at different ages (Table 2) were anesthetized with an intraperitoneal injection of a high-dose cocktail of ketamine hydrochloride (Medistar, Holzwickede, Germany) and xylazine (Serumwerk Bernburg, Bernburg, Germany). The body weight was defined. Then, the urogenital or genital system, including both testes, and heart were immediately removed. The relative testis weight was defined as the ratio between the weights of both testes and the body weight (Table 2). From each mouse, the right testis and heart were fixed in liquid nitrogen, and the left testis was fixed by perfusion in Bouin's fixative for 24 hours and then transferred to 70% ethanol. Testes were dissected and embedded in paraffin wax using standard techniques. Five- μ m sections were stained with hematoxylin and eosin (H&E) and evaluated according to the methods described by Russell and colleagues.⁴⁰ For

comparative analysis, age- and sex-matched animals were chosen, and littermates were used whenever possible. Sections were always processed simultaneously.

Histomorphometric and Statistical Analysis

Quantitative microscopic analysis was performed using a Leica DM LB microscope (Leica, Wetzlar, Germany) at a magnification of $\times$ 400. The number of germ cells and SCs per seminiferous tubule was determined by counting spermatogonia and SCs in 20 tubules from each genotype at days 30, 60, 90, and 120 postpartum. The effects of genotype and age were tested by a two-way analysis of variance without interaction because, for each combination between genotype and age group, one animal was analyzed. To get approximate homogeneity of variances, data were transformed by square root transformation before analysis was performed. A level of $P < 0.05$ was considered statistically significant, and analysis was performed using the statistical program package BMDP/Dynamic, release 7.0 (BMDP Statistical Software, Los Angeles, CA). For testis weights, data were analyzed by one-way analysis of variance followed by nonparametric Kruskal-Wallis test. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using GraphPad InStat 3 (GraphPad Software, Inc., San Diego, CA).

DNA and RNA Extraction, DNase Treatment, cDNA Synthesis, and Reverse Transcriptase (RT)-PCR from Testis Homogenates

Total DNA (for genotyping PCR) and RNA (for RT-PCR) were extracted with TRIzol reagent (Life Technologies, Karlsruhe, Germany), according to the manufacturer's protocol. Isolated RNA was then incubated with RNase-free DNase I (1 to 3 U/ μ g RNA; Roche, Mannheim, Germany) for 40 minutes at 37°C. First-strand cDNA synthesis was performed using Superscript II Reverse Transcriptase, according to the manufacturer's protocol (Gibco BRL, Eggenstein, Germany).

Confirmation of Loss of the Floxed cx43 Gene Using the Cre/loxP-Recombination System by cx43 del PCR

For detection of the deleted cx43-floxed (cx43 del) allele, primers cx43delforw and cx43delrev (Table 1) were

Table 2. Mouse Data (Genotype, Age, Body Weight, Total and Relative Testis Weight), Histological Diagnosis after H&E Staining, and Total and Relative Number of Seminiferous Tubules per Cross Section with Normal Spermatogenesis

Mouse (genotype)	Days	BW (g)	TW (g)	RTW (%)	Phenotype (histology)		
					TNoT per cross section with		
					Nsp	SCO/sga	RNoT with nsp (%)
SCCx43KO ^{-/-}	30	24.2	0.03	0.12	3	87	3.33
SCCx43KO ^{-/-}	30	25.3	0.03	0.12	5	96	4.95
SCCx43KO ^{-/-}	30	25.8	0.03	0.11	2	57	3.39
SCCx43KO ^{+/-}	30	26.1	0.06	0.22	96	0	100
SCCx43KO ^{+/-}	30	25.4	0.07	0.27	68	0	100
SCCx43KO ^{+/-}	30	26.4	0.06	0.23	83	0	100
WT	30	26.0	0.07	0.27	57	0	100
WT	30	25.8	0.06	0.23	89	0	100
WT	30	26.7	0.07	0.26	48	0	100
SCCx43KO ^{-/-}	60	36.8	0.04	0.10	3	135	2.17
SCCx43KO ^{-/-}	60	36.1	0.04	0.11	1	98	1.01
SCCx43KO ^{-/-}	60	37.0	0.05	0.13	0	85	0
SCCx43KO ^{+/-}	60	35.6	0.13	0.37	98	0	100
SCCx43KO ^{+/-}	60	36.2	0.14	0.39	87	0	100
SCCx43KO ^{+/-}	60	35.9	0.13	0.36	74	0	100
WT	60	36.1	0.14	0.38	122	0	100
WT	60	37.1	0.15	0.40	103	0	100
WT	60	36.6	0.14	0.38	94	0	100
SCCx43KO ^{-/-}	90	35.3	0.03	0.08	0	87	0
SCCx43KO ^{-/-}	90	36.2	0.02	0.05	0	78	0
SCCx43KO ^{-/-}	90	35.8	0.03	0.08	1	87	1.14
SCCx43KO ^{+/-}	90	37.9	0.14	0.36	117	0	100
SCCx43KO ^{+/-}	90	35.8	0.15	0.41	126	0	100
SCCx43KO ^{+/-}	90	36.4	0.15	0.41	88	0	100
WT	90	36.7	0.14	0.38	113	0	100
WT	90	35.9	0.14	0.39	102	0	100
WT	90	36.0	0.15	0.42	74	0	100
SCCx43KO ^{-/-}	110	35.9	0.02	0.05	2	47	4.25
SCCx43KO ^{+/-}	110	35.0	0.14	0.40	132	0	100
WT	110	36.8	0.15	0.40	109	0	100
SCCx43KO ^{-/-}	120	32.9	0.03	0.09	5	97	5.15
SCCx43KO ^{-/-}	120	33.5	0.03	0.09	3	85	3.4
SCCx43KO ^{-/-}	120	33.2	0.03	0.09	0	94	0
SCCx43KO ^{+/-}	120	29.8	0.12	0.40	96	0	100
SCCx43KO ^{+/-}	120	31.3	0.13	0.40	78	0	100
SCCx43KO ^{+/-}	120	32.5	0.13	0.40	112	0	100
WT	120	31.5	0.13	0.31	112	0	100
WT	120	32.1	0.14	0.41	79	0	100
WT	120	32.4	0.13	0.40	87	0	100
SCCx43KO ^{-/-}	140	36.1	0.04	0.11	1	51	1.96
WT	150	35.7	0.15	0.42	138	0	100
SCCx43KO ^{-/-}	170	33.2	0.02	0.06	0	83	0

BW, body weight; TW, testis weight; RTW, relative testis weight; TNoT, total number of seminiferous tubules; RNoT, relative number of seminiferous tubules; nsp, qualitative normal spermatogenesis; SCO, SC-only syndrome; sga, arrest of spermatogenesis at the level of spermatogonia. WT, SCCx43KO^{-/-}, and SCCx43KO^{+/-} mice at different ages listed in triplicate represent mice of different litters. WT (*n* = 14), SCCx43KO^{+/-} (*n* = 13), and SCCx43KO^{-/-} (*n* = 15).

used, generating a 670-bp amplicon of the junction between the intron of cx43 and the β -gal coding region in mice that lost the cx43 coding region. To assess the specificity of the deletion of the cx43 gene in SCs of the testis, genomic DNA samples of testis, heart, and tail of SCCx43KO^{-/-} mice, and testis of WT mice, were isolated. DNA was prepared using DirectPCR lysis reagent (Viagen Biotech) or TRIzol reagent (Life Technologies) according to the manufacturers' protocols and subjected to PCR. Equal amounts of DNA from each sample were used. Briefly, 0.5 μ l of DNA was added to 5 μ l of 5 $\times$ Colorless GoTaq Flexi PCR buffer (Promega), 2 μ l of MgCl₂ (25 mmol/L; Promega), 0.5 μ l of dNTPs (Promega), 0.15 μ l of GoTaq DNA polymerase (Promega), 0.5 μ l of each primer (10 μ mol/L⁻¹; MWG), and DEPC-

H₂O to a final volume of 25 μ l. PCR conditions were as follows: 1 $\times$ 95°C for 2 minutes; 39 $\times$ [95°C for 45 seconds, 64°C for 45 seconds, and 72°C for 90 seconds], 72°C for 5 minutes. PCR products were separated in a 2% agarose gel and visualized with SYBR Green (Sigma-Aldrich). Experiments were repeated at least twice.

Confirmation of Loss of the Floxed cx43 Gene Using the Cre/loxP-Recombination System by β -gal IHC

IHC was performed on testicular sections of 15 SCCx43KO^{-/-} mice, of 13 SCCx43KO^{+/-}, and of 14 WT mice to confirm the efficiency of the SC-specific deletion

of the cx43 gene. Briefly, sections were treated, after deparaffinization and rehydration, with 3% H₂O₂ and blocked with bovine serum albumin (5%) for 30 minutes each and incubated with the polyclonal anti- β -gal antibody (rabbit anti- β -gal, 1:1000; Abcam, Cambridge, UK) overnight. Sections were then exposed to the biotinylated secondary antibody (goat anti-rabbit IgG, 1:200; DAKO, Hamburg, Germany) for 30 minutes and to the avidin-biotin-peroxidase complex (Vectastain Elite ABC standard kit; Vector, Grünberg, Germany) for 30 minutes. Immunoreactivity was finally visualized by diaminobenzidine. Experiments were repeated three times.

Production of Digoxigenin (DIG)-Labeled cRNA Probes for in Situ Hybridization

DIG-labeled cRNA probes were generated as described previously.²¹ The DNA sequence of the human cx43 gene (accession no. AF151980) was generated using a touch-down PCR with primers cx43F and cx43R (MWG; Table 1). PCR conditions were as follows: 1× 95°C for 3 minutes; 15× [95°C for 1 minute, 66°C for 1 minute, and 72°C for 2 minutes], 45× [95°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes], 72°C for 10 minutes. The 138-bp PCR product of the human cx43-gene was subcloned in pGEM-T (Promega). Plasmids were transformed in the XL1-Blue *Escherichia coli* strain (Stratagene, Heidelberg, Germany) and extracted by column purification, according to the manufacturer's instruction (Qiagen, Hilden, Germany). After sequencing, vectors containing the cx43 insert were digested with *Nco*I and *Not*I (New England Biolabs, Frankfurt, Germany) for the production of sense cRNA (*Nco*I) and anti-sense cRNA (*Not*I), respectively. Subsequently, *in vitro* transcription was performed using the 10× RNA-DIG labeling mix (Boehringer Mannheim, Mannheim, Germany) and RNA polymerases T7 and SP6 (Promega).

Confirmation of Loss of the Floxed cx43 Gene Using the Cre/loxP-Recombination System by cx43 in Situ Hybridization

In situ hybridization was performed on consecutive sections of the same mice used for β -gal immunostaining as described previously.²¹ In brief, deparaffinized tissue sections were incubated in active DEPC water for 2 × 12 minutes at 40°C, postfixed in 4% paraformaldehyde for 10 minutes, exposed to 20% acetic acid, and prehybridized in 20% glycerol for 30 minutes. Sections were then incubated with the DIG-labeled sense or anti-sense cRNA probes. Both cRNAs were used at a dilution of 1:25 in hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 2× standard saline citrate, 1× Denhardt's solution, 10 μ g/ml salmon sperm DNA (Sigma-Aldrich, Taufkirchen, Germany), and 10 μ g/ml yeast t-RNA (Sigma-Aldrich). Hybridization was performed overnight at 40°C in a humidified chamber containing 50%

formamide in 2× standard saline citrate after posthybridization washes. Subsequently, sections were incubated with the anti-DIG Fab antibody conjugated to alkaline phosphatase (Boehringer) overnight at 4°C. Staining was visualized by developing sections with NBT/BCIP (nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate; KPL, Gaithersburg, MD) in a humidified chamber protected from light. Finally, sections were rehydrated for 5 minutes in deionized water, dehydrated through successive baths of ethanol and xylol, and then mounted in Eukitt resin (Merck, Darmstadt, Germany). For each test, negative controls were performed using DIG-labeled cRNA sense probes. *In situ* hybridization was repeated at least twice.

Confirmation of Loss of the Floxed cx43 Gene Using the Cre/loxP-Recombination System by cx43 IHC

Immunohistochemical stainings for cx43 were performed on consecutive paraffin sections of the same mice used for *in situ* hybridization, with minor changes as described previously.²¹ In brief, sections were microwave-treated for 30 minutes at 1000 W in sodium citrate buffer (pH 6.0), blocked with 5% bovine serum albumin for 30 minutes, and incubated with the polyclonal anti-cx43 primary antibody (1:250; Zytomed, Berlin, Germany) overnight at 4°C. Sections were then exposed to the secondary antibody (1:50, mouse anti-rabbit IgG; DAKO) followed by the third antibody (1:50, rabbit-anti-mouse IgG; DAKO) and finally mouse alkaline phosphatase anti-alkaline phosphatase antibody complex (1:100; DAKO) for 30 minutes each. The immunoreaction was visualized using HistoMark Red (KPL). For each immunoreaction, control incubations were performed by substituting buffer for the primary antibody. Cx43 IHC was repeated at least twice.

RNA Analysis of Spermatogenic Cell Markers

To evaluate the gene expression pattern of spermatid-specific markers, RT-PCR was performed using primer pairs for the detection of prm1-mRNA, prm2-mRNA, and tnp1-mRNA. Table 1 shows the primer sequences. PCR conditions were as follows: 1 μ l of DNA was added to 2 μ l of 10× Gold PCR buffer (Applied Biosystems), 1 μ l of MgCl₂ (25 mmol/L; Applied Biosystems), 0.25 μ l of dNTPs (Applied Biosystems), 0.15 μ l of Gold AmpliTaq DNA polymerase (Applied Biosystems), 0.25 μ l of each primer (10 μ mol/L⁻¹; MWG), and DEPC-H₂O to a final volume of 25 μ l. PCR program: 1× 95°C for 10 minutes, 34× [92°C for 1 minute, [55°C (prm1); 53°C (prm2); 57°C (tnp1)] for 1 minute, 72°C for 2 minutes], 72°C for 10 minutes. PCR products were separated in a 2% agarose gel and visualized with SYBR Green (Sigma-Aldrich). RT-PCR was repeated at least two times per mouse.

Figure 1. PCR genotyping with primers UMP and UMPR (Cx43 flox PCR) and Cre26 and MT1 (Cre PCR) using DNA isolated from mouse tails of representative SCCx43KO^{-/-} (A), SCCx43KO^{+/-} (B), and WT males (C) of the offspring (F2 generation). The single 1.1-kb fragment (white arrow) indicates homozygosity for cx43flox (A, lane 2) and the Cre band (black arrowhead) at 1.914 bp (A, lane 4) confirms Cre expression. The double bands at 1.1 kb (white arrow) and 0.987 kb (black arrow) indicate heterozygosity for cx43flox (B, lane 2), and the Cre band (black arrowhead) at 1.914 bp (B, lane 4) confirms Cre expression. PCR genotyping of a WT male shows only a single band at 987 bp (black arrow), indicating that no floxed allele(s) (C, lane 2) are present, and no band for Cre (C, lane 4), confirming its absence. Lane 1 (A–C), 100-bp DNA ladder. Lane 3 (A–C), 0.5-kb DNA ladder.

Results

Generation of SCCx43KO^{-/-} and SCCx43KO^{+/-} Animals

To circumvent perinatal lethality and pleiotropic effects of the general cx43 deficiency and to clarify the SC-specific roles of cx43 in adult mice *in vivo*, a conditional cx43-KO mouse line has been generated. Male progeny underwent excision of one or both alleles of the cx43 exon 2 only in SCs. Newborn mice were grossly indistinguishable from their non-KO littermates and thus have been genotyped by PCR on genomic DNA prepared from tail biopsies resulting in SCCx43KO^{-/-}, SCCx43KO^{+/-}, and WT mice (Figure 1, A–C). To achieve cell-specific and/or conditional gene KO, it is critical that the insertions of loxP sites or the Cre transgenic construct (Cre recombinase) do not interfere with normal or floxed gene expression and normal protein synthesis. Survival, Mendelian breeding patterns, an apparently normal phenotype of mice homozygous for the floxed cx43 gene, and an apparently normal phenotype of mice containing the Cre gene are all indications for normal gene expression in the presence of loxP sites or Cre recombinase. The conditional allele in the nondeleted state was phenotypically identical to the WT allele and displayed the null-allele phenotype only on systemic Cre-mediated deletion (data not shown).

Fertility Assessment

Reproductive ability of male SCCx43KO^{+/-} and SCCx43KO^{-/-} mice was assessed by mating SCCx43KO^{+/-} and SCCx43KO^{-/-} males with two or three WT females each for up to 6 weeks. SCCx43KO^{+/-} males were fertile and litter sizes of their matings were unchanged compared with pure WT pairings. Although there were always vaginal plugs the morning after mating and SCCx43KO^{-/-} males showed signs of libido, WT females produced no pups, indicating that male SCCx43KO^{-/-} mice are infertile. As a control, these

same female mice (after three sets of 2-week matings with SCCx43KO^{-/-}) were always impregnated after mating with either SCCx43KO^{+/-} males or WT males.

Macroscopy

Male SCCx43KO^{+/-} and SCCx43KO^{-/-} mice showed no gross abnormalities of external genitalia. Epididymides, ductus deferens, coagulating gland, seminal vesicles, and prostate appeared to be normal. To elucidate the cause of infertility in our SCCx43KO^{-/-} males, the urogenital (Figure 2A) or genital tracts (Figure 2, B and C) of selected, genotyped mice were removed. On dissection, the testes were found to be located in exactly the same location as in WT or SCCx43KO^{+/-} males (Figure 2, B and C; insets). The size and total weight of the testes in SCCx43KO^{-/-} mice, however, was drastically lower compared with testes of SCCx43KO^{+/-} and WT littermates (Table 2). Statistical analysis confirmed that total testis weights were significantly reduced ($P < 0.001$) in adult SCCx43KO^{-/-} mice (range, 60 to 120 days) compared with WT and SCCx43KO^{+/-} controls (Figure 3). The relative testis weights were defined as the ratio between the weight of both testes to the body weight, also showing a marked reduction in our SCCx43KO^{-/-} males (Table 2).

Microscopy of Testis and Epididymidis and Phenotypic Differences between WT, SCCx43KO^{+/-}, and SCCx43KO^{-/-} Mice

H&E staining confirmed quantitative and qualitative normal spermatogenesis in WT mice and SCCx43KO^{+/-} mice with formation of a seminiferous epithelium containing all generations of germ cells up to elongated spermatids (Figure 4, A and B). In contrast, SCCx43KO^{-/-} mice revealed in 95% of the seminiferous tubules an arrest of spermatogenesis at the level of spermatogonia or SC-only syndrome and intratubular SC clusters. Most SCs exhibited immature or abnormal features such as rounded nuclei and absence of tubular lumina and cytoplasmic vacuoles (Figure 4, C and G). No mitotic figures could be observed. However, in 10 of our 15 SCCx43KO^{-/-} males, up to 5% of tubules showed only qualitative normal spermatogenesis and few elongated spermatids (Figure 4G). Furthermore, a hyperplasia of interstitial Leydig cells could be observed (Figure 4, C and G). Compared with WT and SCCx43KO^{+/-}, corresponding sections of epididymides in homozygous KO showed that no sperm could be detected, confirming azoospermia (Figure 4, D–F).

Reduced Number of Germ Cells (Spermatogonia) per Tubule in SCCx43KO^{-/-} Mice

The mean number of spermatogonia per seminiferous tubule was found to be significantly lower ($P < 0.0001$) in adult SCCx43KO^{-/-} males compared with their WT and

Figure 2. Dissection of urogenital tract of WT (A) and genital tracts of SCCx43KO^{+/-} (B) and SCCx43KO^{-/-} (C) mice at the age of 170 days. On dissection, the testes of SCCx43KO^{-/-} were found to be located in exactly the same location as in heterozygous littermates (testes are marked with a dotted line, insets B and C). Note that the size of the testes in SCCx43KO^{-/-} mice was drastically reduced compared with testes of SCCx43KO^{+/-} and WT littermates. t, testis; e, epididymis; dd, ductus deferens; sv, seminal vesicles. Scale bars = 1 cm.

heterozygous littermates. Germ cell number remained constant over investigated postpubertal ages with specific levels for the different genotypes (Figure 5).

Increased Number of SCs per Tubule in SCCx43KO^{-/-} Mice

The mean number of SCs per seminiferous tubule was found to be significantly higher ($P < 0.001$) in adult SCCx43KO^{-/-} males compared with their WT and heterozygous littermates. SC number remained constant throughout investigated postpubertal ages with specific levels for the different genotypes (Figure 6).

Figure 3. Comparison of testis weights in adult SCCx43KO^{-/-} mice. Total testis weights were significantly reduced ($P < 0.001$) in adult SCCx43KO^{-/-} mice (range, 60 to 120 days) compared with WT and SCCx43KO^{+/-} controls. Data are shown as mean $\pm$ SEM ($n = 9$ per genotype).

Confirmation of Successful Deletion of the cx43 Gene

First, for the detection of the deleted cx43-floxed allele, genomic DNA was isolated from testis of WT mice and from testis, heart, and tail from SCCx43KO^{-/-} mice. Deletion of the floxed cx43 allele was only observed in testicular DNA from SCCx43KO^{-/-} mice by gain of the 670-bp cx43del (deleted) amplicon (Figure 7). Second, to assess specificity of recombination, it was essential to monitor deletion of the cx43-floxed allele(s). Thus, a silent LacZ reporter gene has been integrated in our transgenic cx43-floxed mouse gene, which is expressed under the control of endogenous cx43 gene regulatory elements only after Cre-mediated deletion of the floxed DNA. A successful AMH-Cre-mediated deletion of cx43 thus leads to an activation of LacZ that can be detected by nuclear β -gal immunostaining in targeted SCs that had lost the cx43 gene but not in germ cells, peritubular cells, or interstitial Leydig cells. In seminiferous tubules of SCCx43KO^{+/-} and SCCx43KO^{-/-} mice, cx43 promoter activity could only be shown in SCs by nuclear β -gal immunostaining. SC nuclei of WT mice revealed no β -gal immunoreactivity (Figure 8, A–C). Control immunostaining reactions were performed by substituting buffer for the primary antibody, and the results were negative (data not shown). Third, loss of floxed cx43 gene expression was confirmed by cx43 *in situ* hybridization. *In situ* hybridization analysis of seminiferous tubules from WT animals and SCCx43KO^{+/-} revealed that cx43 mRNA was mainly

Figure 4. Comparison between testes obtained from adult WT, SCCx43KO^{+/−}, and SCCx43KO^{−/−} mice. H&E staining of testes from day 170 WT (A), SCCx43KO^{+/−} (B), and SCCx43KO^{−/−} mice (C and G) revealed clear differences in germ cell content. Complete spermatogenesis was observed in WT (A) and heterozygous males (B), but smaller tubules with SCO syndrome (not shown) or spermatogenic arrest at the level of spermatogonia (C and G) or residual spermatogenesis (G, right tubule) occurred in SCCx43KO^{−/−} animals. Corresponding sections of cauda epididymidis showed that sperm could only be detected in WT (D) and SCCx43KO^{+/−} (E), but not in SCCx43KO^{−/−} males (F). Lc, Leydig cells; **black arrowheads**, SC nuclei; **black arrows**, spermatogonia; **transparent arrows**, primary spermatocytes; **white arrowheads**, round spermatids; **transparent arrowheads**, elongated spermatids. Scale bars = 50 μ m.

detectable around the nuclei of SCs, spermatogonia, and spermatocytes. In contrast, only spermatogonia were found to express cx43 mRNA in seminiferous tubules of SCCx43KO^{−/−} mice. Knocked out SCs displayed no signal of cx43 gene expression (Figure 8,

Figure 5. Comparison of germ cell (spermatogonia) counts (from H&E staining) at days 30, 60, 90, and 120 postpartum in WT, SCCx43KO^{+/−}, and SCCx43KO^{−/−} mice. Twenty circular tubules were examined for each germ cell count, genotype, and postpubertal age. Given are means $\pm$ SD over seminiferous tubules within each animal. Significance between genotypes, $P < 0.0001$; between ages, n.s.

D–F). Controls using DIG-labeled cRNA sense probes were negative (data not shown). Successful deletion of both cx43 alleles in SCs of SCCx43KO^{−/−} mice was finally confirmed at the protein level by cx43 IHC. In the seminiferous epithelium of WT and SCCx43KO^{+/−} males, cx43 is found to be immunolocalized between SCs and between SCs and spermatogonia/primary spermatocytes. In contrast, no immunostaining at all was detected in seminiferous tubules from SCCx43KO^{−/−} mice, indicating that neither SCs nor spermatogonia are able to synthesize cx43 protein (Figure 8, G–I). Heart tissue from SCCx43KO^{−/−} mice was used as positive control because it is known that cx43 protein is intensively localized in intercalated disks (Figure 8I). Control immunostaining reactions were performed by substituting buffer for the primary antibody and the results were negative (data not shown). Taken together, all results indicate that the deletion of the cx43 gene was efficient and restricted to the SCs of SCCx43KO^{+/−} and SCCx43KO^{−/−} mice.

Figure 6. Comparison of SC counts (from H&E staining) at days 30, 60, 90, and 120 postpartum in WT, SCCx43KO^{+/-}, and SCCx43KO^{-/-} mice. Twenty circular tubules were examined for each SC count, genotype, and postpubertal age. Given are means $\pm$ SD over seminiferous tubules within each animal. Significance between genotypes, $P < 0.001$; between ages, n.s.

SCCx43KO^{-/-} Mice Display Incomplete Spermatogenesis

In testes of WT and SCCx43KO^{+/-} males, the presence of round and/or elongated spermatids was shown by RT-PCR of both protamines (prm1 and prm2) and transition protein 1 (tnp1) (Figure 9, A–C). In testes of 10 of 15 SCCx43KO^{-/-} mice, the absence or reduction of spermatids was confirmed by a failure of expression of prm1 and prm2. A weak expression of tnp1 was detected in these mice, consistent with histological findings confirming the presence of elongated spermatids (Figure 9, A–C). In testes of the five remaining SCCx43KO^{-/-} mice, neither prm1 and prm2 nor tnp1 were expressed (data not shown).

Discussion

Generation and characterization of male cx43 KO mice showed the need for cx43 signaling in male phenotype

Figure 7. Specificity and efficiency of the deletion of the Cx43 gene in SCs. PCR analysis of cx43 deletion in testis (lane 2), heart (lane 3), and tail (lane 4) of SCCx43KO^{-/-} mice and testis (lane 5) of WT mice. Only testicular samples from SCCx43KO^{-/-} males show a band at 670 bp (black arrowhead), indicating deletion of the cx43 floxed allele. Lane 1, 0.5-kb DNA ladder.

development,^{28,31,32,37} but postnatal studies have been impossible because lethality of cx43-deficient mice precluded analysis of the effects of the mutation on spermatogenesis and fertility.³⁶ In addition, because cx43 is known to be expressed from various testicular cells, observed changes could not be attributed to a specific cell type. This represents an indispensable necessity for functional studies on cx43 and cell-cell interactions during testicular development and spermatogenesis by generating conditional, cell-specific cx43 KO mice.

The present study demonstrates the generation of a conditional cx43-KO mouse line lacking a functional cx43 gene solely in SCs. Successful deletion of the cx43 gene was confirmed by β -gal IHC, by cx43 IHC, cx43 *in situ* hybridization, and cx43del PCR. Adult SCCx43KO^{-/-} mice exhibit descended testis and a normal development of the genital tract, but testes volumes and weights were drastically lower because of the absence of spermatogenesis. Thus, Cre-mediated deletion of both alleles of cx43 in SCs had profound effects on normal testis development. Similar to the results from Plum and colleagues³⁷ who worked with adult Cx43KI32 and Cx43KI40 mice and showed that heterozygous mutants were fertile, the expression level of a single deleted allele in our SCCx43KO^{+/-} males was found to be sufficient for proper development of gonads and spermatogenesis. The number of spermatogonia per tubule was significantly lower in SCCx43KO^{-/-} mice at different postpubertal stages, indicating that the germ cell population fails to expand in the absence of cx43 in SCs. These results imply an essential role for cx43 in SCs in supporting germ cell proliferation and/or survival. Loss and/or reduction of round or elongated spermatids in homozygous mutants were confirmed by RT-PCR for prm1, prm2, and tnp1. Using *in situ* hybridization and IHC, remaining spermatogonia were found to express cx43 mRNA but do not seem to synthesize cx43 protein, indicating a disturbed heterotypic communication via cx43 gap junctions between SCs and spermatogonia. In contrast, the number of SCs per tubule was significantly higher in adult homozygous mutants. These results imply an important role for cx43 also in SC proliferation and functional maturation. However, in up to 5% of seminiferous tubules of SCCx43KO^{-/-} mice, spermatocytes or even spermatids were detected for so far unknown reasons. We speculate that these rare tubules are those in which Cre-mediated cx43 inactivation did not occur or that in these single tubules loss of cx43 has been compensated for by another cx known to be expressed in SCs.

From our data, it can be postulated that loss of cx43 in SCs 1) prevents the initiation of spermatogenesis and 2) leads to a significant reduction of germ cells (spermatogonia) per seminiferous tubule resulting in infertility of adult SCCx43KO^{-/-} males. Loss of cx43 further leads to 3) the formation of intratubular SC clusters; 4) a significant increase of SCs per seminiferous tubule; 5) SCs exhibiting mostly immature or abnormal features such as rounded nuclei and absence of tubular lumina and cytoplasmic vacuoles; and 6) a hyperplasia of interstitial Leydig cells, because these cells are known to be present only in small numbers in WT mice. Because paracrine

Figure 8. Comparison between the testes obtained from adult WT (**A**, **D**, and **G**), SCCx43KO^{+/-} (**B**, **E**, and **H**), and SCCx43KO^{-/-} (**C**, **F**, and **I**) mice. Results of β -gal immunostaining (**A–C**), *cx43* *in situ* hybridization (**D–F**), and *cx43* immunostaining (**G–I**). As expected, SC nuclei of WT mice (**A**, **black arrowheads**) revealed no β -gal immunoreactivity. In seminiferous tubules of SCCx43KO^{+/-} (**B**) and SCCx43KO^{-/-} (**C**) mice, Cre-mediated deletion of one or both alleles of the *cx43* gene could only be detected in SCs by nuclear β -gal immunostaining but not in germ cells or interstitial Leydig cells. Note the few β -gal-immunonegative spermatogonia (**C**, **black arrows**). *In situ* hybridization of seminiferous tubules from WT animals (**D**) and SCCx43KO^{+/-} (**E**) showed that *cx43* mRNA was mainly localized around the nuclei of SCs, spermatogonia, and spermatocytes. Only a weak signal was detectable in the cytoplasm of round spermatids. In contrast, only few spermatogonia were found to express *cx43* mRNA in seminiferous tubules of SCCx43KO^{-/-} mice (**F**), whereas SCs displayed no signal of *cx43* gene expression. In the seminiferous epithelium of WT (**G**) and SCCx43KO^{+/-} males (**H**), *cx43* is found to be immunolocalized between SCs and between SCs and spermatogonia/primary spermatocytes. In contrast, no immunostaining at all was detected in seminiferous tubules from SCCx43KO^{-/-} mice (**I**), indicating that neither SCs nor spermatogonia are able to synthesize *cx43* protein. Note the single spermatogonium (**I**, **black arrow**) and intratubular SC cluster (**I**, **transparent arrow**). Heart tissue from SCCx43KO^{-/-} mice revealed *cx43*-immunopositive intercalated disks (**I**, **inset**). Scale bars = 50 μ m.

factors secreted by SCs are thought to be important for the development and function of Leydig cells,^{41,42} observed Leydig cell hyperplasia may be an indication for alterations in paracrine regulatory processes being in contrast to the results in conventional *cx43*-KO mice^{28,31} or *cx43* mutants³⁷ in which no morphological alterations in Leydig cells are observed.

In the present study, we showed that *cx43* in SCs is required early in development for the expansion of the germ cell population in adult mice. There are some possible reasons that adult SCCx43KO^{-/-} mice at different postpubertal ages show an arrest of spermatogenesis at the level of spermatogonia or SC-only syndrome and a significant germ cell deficiency in most seminiferous tubules. However, the molecular mechanisms by which deletion of *cx43* in SCs inhibits germ cell proliferation and

spermatogenesis remain to be elucidated. The following explanations are likely.

First, in previous dye-coupling studies it was shown that *cx43* participates in the coupling between adjacent SCs, between SCs and spermatogonia, and between SCs and early and late spermatocytes.^{18,35} Interestingly, the latter study showed that the coupling seemed unidirectional from SCs to germ cells, whereas no dye transfer was observed from germ cells to the somatic cells. Thus, observed alterations in germ cell differentiation and spermatogenesis in SCCx43KO^{-/-} males could be attributable to a lack of a possible substitute *cx* to structure functional gap junction channels containing *cx43* between SCs and germ cells, changes in specific signaling molecules that are needed for the synthesis of the *cx43* protein in spermatogonia and that pass through *cx43*

Figure 9. Representative RT-PCR for prm1 (A), prm2 (B), and tnp1 (C) using testes homogenates from SCCx43KO^{-/-} males (A–C, lane 2), SCCx43KO^{+/-} males (A–C, lane 3), and WT mice (A–C, lane 4). Specific mRNA for prm1 and prm2 (black arrows) was only detectable in SCCx43KO^{+/-} and WT mice (A and B, lanes 3 and 4), indicating the presence of spermatids but the absence in SCCx43KO^{-/-} (A and B, lane 2). Interestingly, a weak band for tnp1 (black arrow) could be detected in SCCx43KO^{-/-} animals (C, lane 2) compared with a strong expression in SCCx43KO^{+/-} mice (C, lane 3) and WT males (C, lane 4). Lane 1 (A–C), 100-bp DNA ladder.

channels, or changes in the gating properties of these cx43 channels. Our results may explain the selective ability of cx43 in SCs to support proliferation and survival of germ cells in normal mice compared with KI mice.³⁷ Diffusion of high-energy metabolites such as cAMP, ADP, or ATP from metabolically active SCs to probably less active germ cells supports their mitogenic potential. Our results show that cx43 in SCs must have unique regulatory or physiological properties that cannot be compensated in 95% of seminiferous tubules in SCCx43KO^{-/-} mice.

Second, total number of spermatogonia is established prepubertally by proliferation and apoptosis leading to a species-specific number of spermatogonia per SC.⁴³ In addition, alterations in SC-germ cell interactions, SC-germ cell contacts, and/or functional SC-germ cell coupling are known to play a role in germ cell apoptosis.^{44–47} It has been demonstrated previously that primordial germ cells are gap junction communication-competent cells and that germ cell deficiency in cx43-KO embryos may arise from increased apoptosis.⁴⁸ Furthermore, blockage of cx in seminiferous epithelium is able to induce germ cell death.⁴⁹ Based on these observations, the present results allow the hypothesis that observed germ cell deficiency in our adult SCCx43KO^{-/-} males originates from prepubertal apoptotic events. Because it is further known that migrating as well as postmigratory cells require signals from neighboring cells via cx43 gap junctions to sustain them,^{50–52} we may further say that already at early stages of germ line development migration and proliferation of germ cells is highly dependent on the functional expression of cx43 in SCs. Thus, testes from fetal, neonatal, and prepubertal SCCx43KO mice will be examined using electron microscopy and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining and compared with heterozygous and WT littermates.

Third, because it is proposed that cx43 in SCs play a role in the regulated formation of the blood-testis barrier at puberty,^{13,33,34} deletion of this cx may lead to an inhibition of blood-testis barrier assembly and disintegration of the SC junctional complexes. Further experiments

will show whether expression of some junction-related genes including occludin, claudin11, or N-cadherin are altered in SCCx43KO^{-/-} mice compared with their WT and heterozygous littermates, providing another explanation for the reported germ cell deficiency and altered spermatogenesis.

Fourth, the failure to initiate spermatogenesis and observed germ cell deficiency in our adult SCCx43KO^{-/-} mice may finally reflect an altered state of SC maturation and a sign for functional immaturity of the SC as proposed recently.⁵³ Whether the low number of spermatogonia can be related specifically to the altered number of supporting SCs per seminiferous tubule remains to be investigated. However, impaired germ cell development may represent a reflection of underlying abnormalities in SCs because there exists a well-known reciprocal regulation of SC and germ cell differentiation, and functional SCs are a prerequisite for normal spermatogenesis.^{10,54}

One advantage of our model system is that cx43 is deleted by Cre excision in SCs together with the beginning of AMH expression on fetal day 12.5, concomitant with important periods of SC and germ cell interaction and proliferation.^{10,55} In addition, compared with conventional cx43-KO and cx43-KI mice, this model allows the investigation of the functional consequences of the loss of cx43 in SCs on the SC themselves and all other cell populations in mouse testis. Finally, persistent cx43 mRNA transcription but no translation in germ cells offers the possibility to study fundamental mechanisms of gap junction formation between germ cells and somatic cells.

At least five conclusions can be drawn from the results of the present study. 1) SC-specific deletion of cx43 reveals that this cx is an absolute requirement for the initiation of spermatogenesis. 2) The lower number of spermatogonia per seminiferous tubule and the failure of these germ cells to expand after puberty reflects the loss of support from cx43-deficient SCs and demonstrate that cx43 in SCs is required to allow spermatogonia to start and/or complete mitosis. 3) The higher number of SCs per seminiferous tubule demonstrates that cx43 may also participate in the regulation of SC proliferation and maturation. 4) Selective inactivation of cx43 in SCs does not seem to interfere with testicular descent or male genital tract development. 5) Cx43 in SCs must have unique regulatory or physiological properties because its loss cannot be compensated for in most seminiferous tubules in SCCx43KO^{-/-} mice by other cx known to be expressed in SCs or spermatogonia. This new transgenic mouse model forms a unique tool for further analysis of the molecular mechanisms of cx43 action in testis and will contribute to our knowledge of its role in the etiology and pathogenesis of spermatogenic disorders.

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