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ORIGINAL ARTICLE

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Homeobox transcription factor Meis1 is crucial to Sertoli cell mediated regulation of male fertility

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Abstract

Background: Infertility has become a global phenomenon and constantly declining sperm count in males in modern world pose a major threat to procreation of humans. Male fertility is critically dependent on proper functioning of testicular Sertoli cells. Defective Sertoli cell proliferation and/or impaired functional maturation may be one of the underlying causes of idiopathic male infertility. Using high-throughput "omics" approach, we found binding sites for homeobox transcription factor MEIS1 on the promoters of several genes up-regulated in pubertal (mature) Sertoli cells, indicating that MEIS1 may be crucial for Sertoli cell-mediated regulation of spermatogenesis at and after puberty.

Objective: To decipher the role of transcription factor MEIS1 in Sertoli cell maturation and spermatogenesis.

Materials and methods: Sc-specific *Meis1* knockdown (KD) transgenic mice were generated using pronuclear microinjection. Morphometric and histological analysis of the testes from transgenic mice was performed to identify defects in spermatogenesis. Epididymal sperm count and litter size were analyzed to determine the effect of Meis1 knockdown on fertility.

Results: Sertoli cell (Sc)-specific *Meis1* KD led to massive germ cell loss due to apoptosis and impaired spermatogenesis. Unlike normal pubertal Sc, the levels of SOX9 in pubertal Sc of *Meis1* KD were significantly high, like immature Sc. A significant reduction in epididymal sperm count was observed in these mice. The mice were found to be infertile or sub-fertile (with reduced litter size), depending on the extent of *Meis1* inhibition.

Discussion: The results of this study demonstrated for the first time, a role of *Meis1* in Sc maturation and normal spermatogenic progression. Inhibition of *Meis1* in Sc was associated with deregulated spermatogenesis and a consequent decline in fertility of the transgenic mice.

Conclusions: Our results provided substantial evidence that suboptimal *Meis1* expression in Sc may be one of the underlying causes of idiopathic infertility.

Rajesh Kumar Sarkar and Souvik Sen Sharma are contributed equally to this work.

KEYWORDS
sertoli cell, Meis1, knockdown, germ cell apoptosis, male fertility

1 | INTRODUCTION

Declining sperm count and their inability to fertilize egg leading to live birth is a matter of concern as it brings threat to human population.[1] A decline in the quantitative and qualitative parameters of spermatozoa of men is detrimental to reproductive ability of humans. Nearly half of all reported cases of male infertility are idiopathic in nature wherein exogenous hormone supplementation fails to ameliorate infertility symptoms.[2,3] Genetic anomalies and defective methylation in Gc have been shown to cause developmental arrest of germ cells (Gc) at various levels.[4,5] Similarly, impaired maturation of Sc and/or non-responsiveness of Sc to hormones are likely to contribute to infertility. Unless we divulge the underlying causes of idiopathic male infertility and correct them in men, use of assisted reproductive technologies (ART) involving unnecessary invasive procedures in females cannot be curtailed.

The process of spermatogenesis involves extensive cross talk between the developing germ cells and the somatic cellular components of the testis. The development of germ cells occurs in the milieu provided by the Sc. Sc plays an indispensible role in germ cell division and differentiation.[6,7] During neonatal/infantile period, Sc proliferate in response to FSH and other factors.[8-10] The postnatal development of Sc involves two distinct phases—a proliferative phase which is observed during the neonatal period, when the animal is sexually immature, and a functionally mature phase which is attained at puberty.[11] Sc proliferation during the neonatal phase is crucial for fertility as the total Sc population established during this stage determines the final spermatogenic output during adult life.[12] Functional maturation of Sc occurs at the onset of puberty.[11] Pubertal maturation of Sc involves cessation of Sc proliferation and establishment of a well defined apico-basal polarity.[11] Functionally mature Sc exhibits enhanced responsiveness to hormones (FSH and T) which in turn are involved in up-regulating the expression of Sc genes such as *Abp* and *Scf* essential for germ cell differentiation.[10,13,14] Functionally mature Sc also forms the blood-testis barrier (BTB), an impermeable immunological barrier that plays a major role in safeguarding advanced germ cells.[15] It is reasonable to believe that even if hormone levels are sufficient, defects in Sc maturation can adversely affect germ cell development leading to impaired sperm production and consequently, sub-fertility or infertility.

In a previously reported study from our laboratory, the binding sites of a number of TFs in genes up-regulated in mature Sc and infant (immature) Sc were identified using TRANSFAC analysis.[16] Analysis of the TRANSFAC data revealed that the binding site for the transcription factor MEIS1 was significantly enriched on the promoters of the genes up-regulated in functionally mature pubertal Sc. This led us to hypothesize that MEIS1 might be involved in the Sc maturation. MEIS1 knockout mice exhibit embryonic lethality due to failure of lymphatic-venous separation and absence of megakaryocytes.[17] Members of Meis1 family are also expressed in the brain and sensory organs.[18] Haploinsufficiency of MEIS1 has been reported to adversely affect differentiation of the retina leading to microphthalmia in humans.[19] While studies indicate that MEIS1 is crucial for normal embryonic development and maintenance of homeostasis, there are studies showing crucial role of Meis1 in regulation of hematopoietic stem cell (HSC) differentiation.[20] Similar to the HSCs, the Sc in the testes undergoes proliferation and differentiation post-birth. However, the role of MEIS1 in Sc is not known. In the present study, we have deciphered a role of MEIS1 in Sc maturation and regulation of spermatogenesis using a transgenic mouse model.

2 | MATERIALS AND METHODS

2.1 | Reagents and animals

Mice (strain FVB/J) were obtained from the small animal facility of National Institute of Immunology, New Delhi, India. Mice were housed and used as per the guidelines laid down by the CPCSEA (Committee for the Purpose of Control and Supervision of the Experiments on Animals). Protocols for experiments were approved by the Institutional Animal Ethics Committee (IAEC), constituted by CPCSEA. All reagents were purchased from Sigma unless mentioned otherwise.

2.2 | Generation of transgene cassette of Meis1 shRNA

The seed sequence of *Meis1*shRNA was obtained from web database of Origene (USA). The oligos for both strands were procured from Sigma Aldrich. Restriction sites for EcoRI (5' end) and SalI (3'end) were introduced at the seed sequence for subsequent cloning in dual PEM vector. The *Meis1*shRNA was cloned under the promoter of *Pem* (*Rhox5*) gene which ensured the expression of the shRNA specifically in Sertoli cells at the onset of puberty.[21] Sequence of Meis1 shRNA used in this study is provided in Table 1.

2.3 | Pronuclear microinjection of Meis1 shRNA transgene cassette and oviductal embryo transfer

For pronuclear microinjection, female mice were super-ovulated using PMSG and hCG followed by cohabitation with male mice. Oviducts were collected from donor mice (euthanized by cervical dislocation) and placed into Brinster's Modified Oocyte Culture

TABLE 1 List of primers and shRNAs used in the study

Primer	Sequence
*Meis1*shRNA Forward	AATTCGATGACACGGCATCCACTCGTTC AGGAGGTCAAGAGCCTCCTGAACG AGTGGATGCCGTGTCATCTTTTTTAC CGGTG
*Meis1*shRNA Reverse	TCGACACCGGTAAAAAAGATGACACGGC ATCCACTCGTTCAGGAGGCTCTTGA CCTCCTGAACGAGTGGATGCCGTG TCATCG
Sox9 Forward	AAGAACGGACAAGCGGAGG
Sox9 Reverse	AGATTGCCCAGAGTGCTCG
Gja1 Forward	TAAGTGAAAGAGAGGTGCCCAG
Gja1 Reverse	CCCAGGAGCAGGATTCTGAAA
Scf Forward	GGTCCCGAGAAAGATTCCAGAG
Scf Reverse	CTGTCCATTGTAGGCCCGAG
Cldn11 Forward	GCCATCTTGCTGCTGTTGAC
Cldn11 Reverse	CGCAGAGAGCCAGCAGAATAA
Hif1α Forward	CACAGAAATGGCCCAGTGAGA
Hif1α Reverse	AATATGGCCCGTGCAGTGAA

Media (BMOC) containing hyaluronidase (1 mg/mL). Embryos were collected and transferred to the 60 mm dish contain 100 µL drops of BMOC overlaid with mineral oil. Linearized *Meis1*shRNA transgene cassette was prepared at a final concentration of 4ng/µl for microinjection. DNA was microinjected into the male pronucleus of fertilized eggs using Narishighe micromanipulator. Each manipulated embryo was then transferred into the pre-incubated BMOC containing dish and maintained at 37°C in a 5% CO_2 incubator. Embryos were incubated till 2-cell stage. Pseudo-pregnant female mice were generated by mating with vasectomized male mice. 2-cell stage embryos were transferred into the ampulla of the oviduct of pseudo-pregnant recipient mice. Approximately 20 microinjected embryos were transferred in the oviductal ampulla. After the gestation period (21 ± 2 days), recipient pups were born. Pups born were analyzed for transgene integration by slot blot.

2.4 | Genomic DNA isolation and Slot blot analysis

Screening of the transgenic mice was done by radioactive slot blot method as described previously.[22] A radiolabeled probe against the GFP DNA fragment was prepared using radiolabeled α^{32}p dCTP, procured from BRIT. Probe preparation was done with DNA High prime labeling kit (Roche) as per the manufacturer's protocol. The genomic DNA (gDNA) was isolated from the tail snips of mice. Briefly, the tail sample was lysed in tail lysis buffer containing Proteinase K (10 µL of 20 mg/mL). Salting out method was used for DNA isolation. 5M NaCl (300 µL) added to the lysed samples. Excess protein was removed by centrifuging the lysate at 13 000 RPM for 10 minutes. The clear supernatant containing gDNA was transferred to a fresh microfuge tube. Genomic DNA was precipitated by adding an equal volume of absolute alcohol followed by centrifugation at 13 000 RPM for 20 minutes. The gDNA pellet was washed in 70% ethanol and air dried for 5-10 minutes. The pellet was dissolved in 100 µL nuclease-free water. The Genomic DNA concentration in the samples was estimated using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

For slot blot analysis, 1µg of gDNA was transferred on to Nitrocellulose membrane (MDI, India) using Slot blot apparatus (Cleaver Scientific Ltd.). Hybridization buffer (Millipore) was used for pre-hybridization and hybridization. A radiolabeled DNA fragment complementary to the EGFP sequence in the vector was used as a probe for detecting transgene integration in the genome of the mice. The membrane was incubated with the radiolabeled probe (in hybridization buffer) overnight at 60°C. Excessive radioactive probe was removed by washing the membrane a low stringency buffer (2X SSC and 0.1% SDS- 2 washes, 10 minutes each at RT) followed by 2 washes in a high stringency buffer (0.5X SSC, 0.1%SDS, 5 minutes each at 65°C). The membrane was incubated for overnight exposure in a phosphor screen (GE Healthcare), and images were scanned using typhoon phosphor imager. Dual PEM plasmid DNA was used as a positive control and genomic DNA from wild-type mice was used as negative control in the slot blot analysis. Upon exposure in phosphor screen, genomic integration of the construct was confirmed by analyzing the presence of bands on the phosphor screen (Figure 1B).

2.5 | Immunoblot analysis

Total protein was extracted from whole testis and quantified using the bicinchoninic acid assay kit (Pierce) as per the manufacturer's protocol. A total of 70 µg of total testicular protein was denatured in 5X Lamelli buffer (with Beta-mercaptoethanol) at 99°Cfor 10 minutes. Denatured protein samples were resolved in a 10% sodium dodecyl sulfate-polyacrylamide (SDS) gel using a vertical gel electrophoresis apparatus (Bio-Rad). The resolved protein was electro-transferred to a polyvinylidene fluoride membrane (MDI). The blot was blocked using 5% bovine serum albumin (2 hours at RT) and incubated overnight with Anti-MEIS1 antibody (Abcam) at 4°C. Excess primary antibody was removed by washing the blot with PBST (0.1% Tween-20), thrice for 5 minutes each. This was followed by incubation with HRP-labeled secondary antibody for 45 minutes at RT. Protein bands corresponding to MEIS1 protein were detected using ECL kit (Thermo Fischer Scientific) using a Chemidoc Apparatus. Beta-actin was used as a loading control for the immunoblot experiments. Details of the antibodies used in the study are provided in Table 2.

2.6 | RNA isolation and c-DNA preparation

Testicular tissue from Meis1 KD and wild-type mice were homogenized in TRI reagent (Sigma Aldrich). Total RNA was isolated from the samples as previously described.[23] The quantity and quality of the RNA were determined using usingNanodrop2000

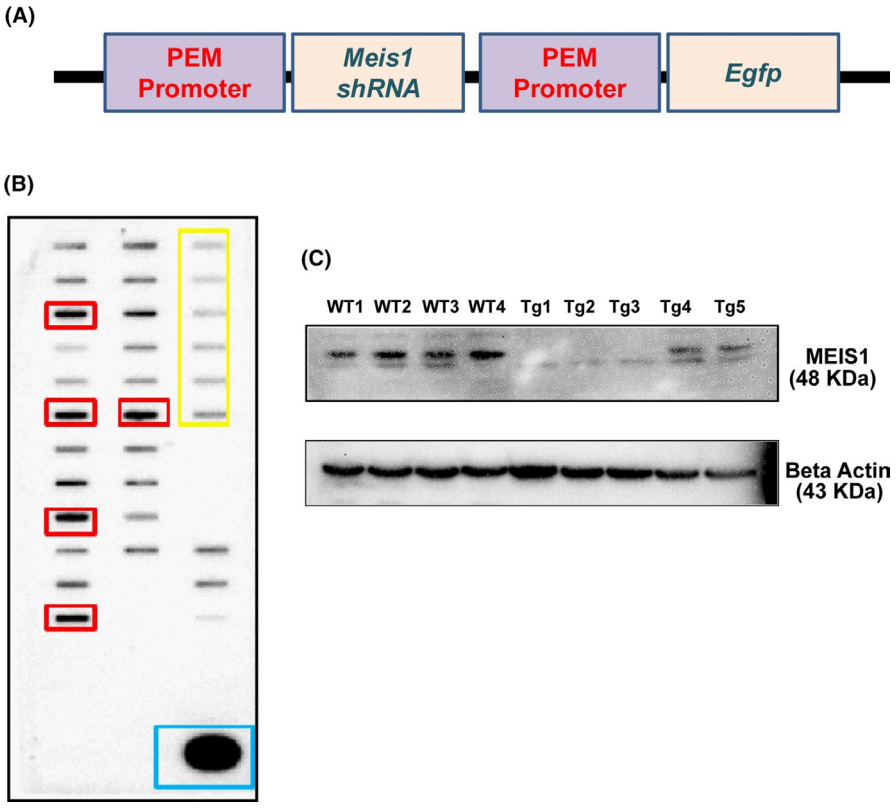

FIGURE 1 Generation of Meis1 KD transgenic mice. (A) Schematic representation of the transgene construct used in the study. (B) Representative slot blot used for detection of transgene-positive mice. Bands enclosed in red boxes indicate transgene-positive mice; bands enclosed in yellow box are wild type. pCX-EGFP plasmid (band enclosed in blue box) was used as a positive control for slot blot analysis. (C) Detection of MEIS1 knockdown using immunoblot analysis. Representative immunoblot showing MEIS expression in wild-type and MEIS1 KD mice. Beta-actin was used as a loading control

TABLE 2 Details of antibodies used in the study

Antibody	Dilution	Manufacturer	Catalogue No.
Anti-MEIS1	1:1500 (IB)	Abcam	Ab19867
Anti-SOX9	1:100 (IHC)	Abcam	ab185230
Anti-PCNA	1:250 (IHC)	Thermo Scientific	MA5-11358
Anti-Beta actin	1:3000 (IB)	Cell Signaling Technologies	4967L
Alexa Fluor 488 goat anti-rabbit	1:500 (IHC)	Life technologies	A1108
Alexa Fluor 488 goat anti-mouse	1:500 (IHC)	Life Technologies	A11001
Alexa Fluor 546 goat anti-rabbit	1:500 (IHC)	Life Technologies	A11010
HRP-conjugated Goat anti-rabbit	1:5000 (IB)	Epitomics	30 531

spectrophotometer (Thermo Fisher Scientific). A total of 1µg of total RNA was subjected to DNaseI treatment to remove any contaminating gDNA in the samples. This was followed by c-DNA preparation using M-MLV Reverse transcriptase (cat.no.-M170BPromega) as per the manufacturer's protocol. The c-DNA was used for assessing gene expression using quantitative real-time PCR.

2.7 | Quantitative real-time PCR

Q-RT-PCR was performed in Realplex^4 master cycler (Eppendorf) using Kapa Sybr green master mix (Sigma Aldrich). A total of 1µl of c-DNA was used per reaction mixture containing 5 µL Kapa Sybr, 0.5 µM primers, and 3 µl nuclease-free water. Reaction conditions involved initial denaturation of c-DNA at 95°C for 3 minutes, followed by 40 cycles of amplification (95°C for 10 seconds, 60°C for 1 minute). Single amplification peak was detected using melt curve analysis. Fold change in gene expression was calculated using $2^{(-\Delta\Delta Ct)}$ method as described previously.[24] *Sox9* was used as a reference for normalization of gene expression data. Details of the primers used in the study are provided in Table 1.

2.8 | TUNEL assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was used for detecting apoptotic cells in Meis1 KD and wild-type mice testicular sections. TUNEL assay

was performed using DeadEnd™ Colorimetric TUNEL System (Promega) as described previously.[25] The total number of apoptotic germ cells was recorded from ten random fields (under 20× magnification) of the testicular sections of wild-type and transgenic mice. The average number of apoptotic germ cells per field was calculated. This was repeated for three wild-type and three transgenic mice samples.

2.9 | Immunohistochemistry

Testis samples were fixed in OCT media and stored in −80°C. 5 µm cryosections were prepared using Shandon Cryotome®E (Thermo USA). The sections were permeabilized using 0.1% Triton X-100 (in 1X PBS) followed by blocking in 3% BSA for 2 hrs at RT. The sections were then incubated with Anti-SOX9 antibody overnight at 4°C. Unbound primary antibody was removed by washing the sections with 1X PBS, thrice for 5 minutes each. This was followed by incubation with flurophore-labeled secondary antibody for 3 hrs at RT. Unbound secondary antibody was removed by washing the sections with 1XPBS, thrice for 5 minutes each. Nuclei were stained using Hoechst-33342 dye. Imaging was done using Ri-2 epifluorescence microscope (Nikon). For quantification of SOX9-positive cells, three testicular sections, each with approximately 10 seminiferous tubules, were analyzed from each wild-type and transgenic animal. Details of the antibodies used in this study are provided in Table 2.

2.10 | Hematoxylin and eosin staining

The testicular cryosections were stained in hematoxylin for three minutes, then washed in tap water for 5 minutes followed by destaining with acid ethanol. Sections were then washed in tap water twice. The sections were immediately dipped in eosin for 1 minute. Sections were rinsed in tap water and mounted in DPX.

2.11 | Epididymal sperm count and litter size analysis

Wild-type and Meis1 KD transgenic mice were euthanized at the age of 80 days. The testes and epididymis were dissected out separately. Testis was weighed and stored as per experimental design. Epididymis was dissected out and kept in 1 mL of 1X PBS. The epididymis was punctured at several sites and shaken in microfuge tube containing PBS in order to release the spermatozoa. Punctured epididymis was kept at 37°C for 10-20 min. This facilitated the release of spermatozoa. Total numbers of spermatozoa were then counted using a Hemocytometer (PolyopticsGmBH). For fertility assessment, transgenic and wild-type mice were bred with at least three wild-type females separately. Copulatory plugs were checked post-mating. Pups obtained from at least three different females were recorded for each of the transgenic mice.

2.12 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. $P < .05$ was considered to be statistically significant. Details of the statistical tests are provided in the figure legends.

3 | RESULTS

3.1 | Generation of Meis1 KD transgenic mice

In order to knock down *Meis1* expression specifically in pubertal Sc, the shRNA targeting *Meis1* was cloned under PEM (Rhox5) promoter (Figure 1A). Transgenic mice were generated using pronuclear microinjection, and transgene-positive mice were identified using radiolabeled slot blot analysis of the genomic DNA isolated from the tail snips (Figure 1B). The presence of prominent radioactive signal from the DNA confirmed transgene integration in the genome of the mice. No signals were detected from the genomic DNA of the wild-type mice (Figure 1B).

Meis1 knockdown in the transgenic mice was confirmed using immunoblot analysis. Transgenic mice from 2 independent lines were studied to understand the effect of *Meis1* knockdown on Sc maturation and spermatogenesis. A remarkable decline in MEIS1 expression was observed in Meis1KD mice as compared to age matched wild-type control animals (Figure 1C). This confirmed efficient knockdown of *Meis1* in the transgenic mice.

3.2 | Testicular phenotype of Meis1 KD transgenic mice

Meis1 KD transgenic mice had significantly ($P < .05$) smaller testes than the age-matched wild-type mice (Figure 2A, Table 3). Histological analysis of testicular cross-sections revealed a significant reduction in seminiferous tubule diameter in Meis1KD mice as compared to age-matched wild-type controls (Figure 2B, Figure S1). Mesi1 KD mice had defects in seminiferous tubule architecture with many tubules displaying germ cell loss and compromised spermatogenesis (Figure 2B).

3.3 | Germ cell apoptosis in Meis1 KD transgenic mice

In order to check whether the observed testicular defect was due to germ cell apoptosis, TUNEL assay was performed on testicular sections of Meis1 KD transgenic mice and age-matched wild-type mice. A significant ($P < .05$) increase in the number of apoptotic germ cells was observed in the Meis1 KD transgenic mice as compared to age-matched wild-type control mice (Figure 3A,B).

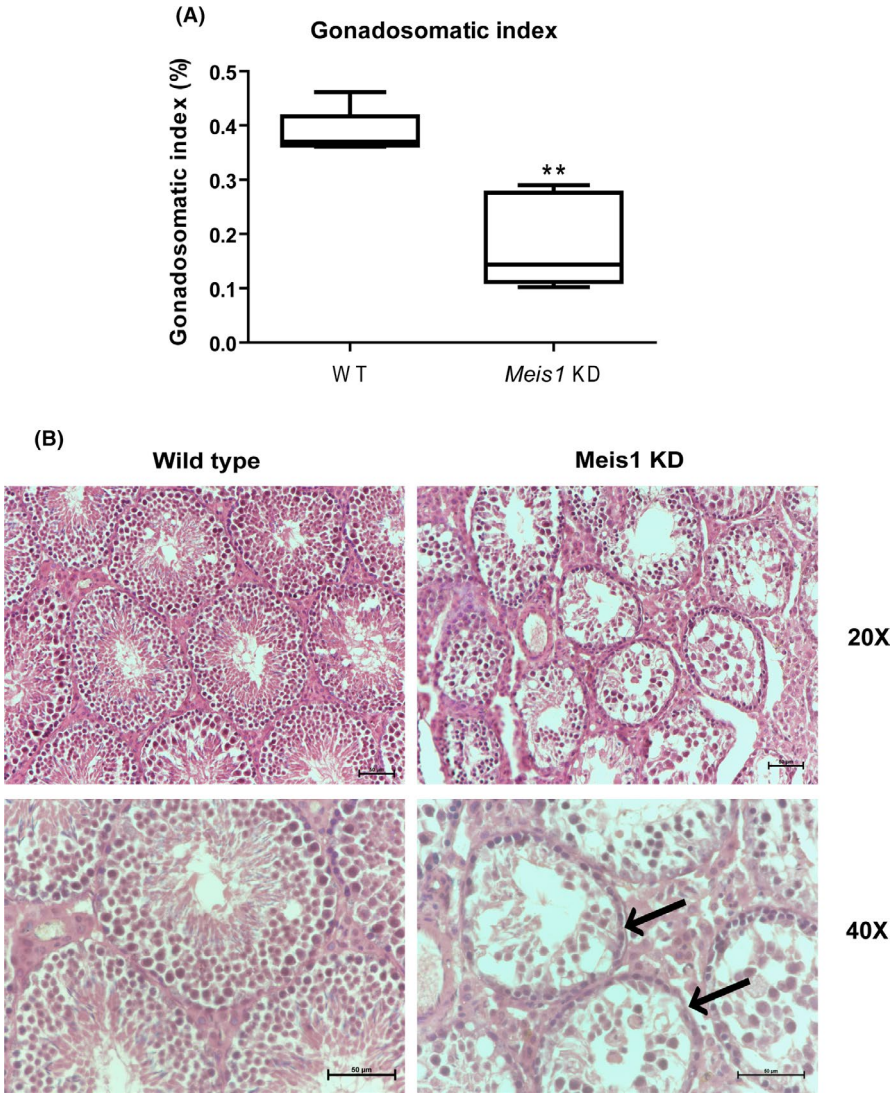

FIGURE 2 Testicular phenotype of Meis1 knockdown transgenic mice. (A) Gonado-somatic index (testes weight/body weight) of MEIS1 KD and wild-type mice. The GSI was significantly reduced in MEIS1 KD mice as compared to age-matched wild-type control mice. Unpaired Student's t test was used for determining statistical significance. $P < .05$ was considered to be statistically significant; n = 5 (5 transgenic and 5 wild-type mice). (B) Hematoxylin and eosin staining of testicular sections from MEIS1 KD and wild-type mice. A decline in tubule diameter was evident in MEIS1 KD mice (ii and iv). Black arrows indicate tubules with disrupted spermatogenesis

3.4 | SOX9 expression in Meis1 KD transgenic mice

The expression of Sc-specific marker, SOX9, was found to be significantly ($P < .05$) up-regulated in Meis1 KD transgenic mice as compared to wild-type controls (Figure 4C). Immunostaining of testicular sections revealed a significant increase in the number of SOX9-positive Sertoli cells in *Meis1* KD mice as compared to wild-type mice (Figure 4A,B). We also checked if the increase in the number of Sc was due to continued proliferation of these cells in adulthood. To this end, we checked the expression of proliferating cell nuclear antigen (PCNA) in testicular sections from transgenic and wild-type mice. However, no PCNA was detected in the nuclei of Sc in the transgenic and age-matched wild-type mice (Figure S2).

3.5 | Expression of Sc maturation markers in Meis1 KD transgenic mice

It was observed that there was a significant ($P < .05$) decline in the expression of Sc maturation markers such as *Scf*, *Gja1*, and *Cldn11* in Meis1 Knockdown transgenic mice as compared to age-matched wild-type mice (Figure 5A-C). A significant ($P < .05$) decline in the expression of Hif1α, an established target of MEIS1 was also observed in the Meis1 KD mice (Figure 5D).

TABLE 3 Absolute testis weight and body weight of Meis1 KD and wild-type mice

Animal	Testis weight (g)	Body weight (g)
Tg1	0.032	22.3
Tg2	0.029	24.04
Tg3	0.03	29.32
Tg4	0.077	29.4
Tg5	0.089	30.7
WT1	0.097	26.2
WT2	0.108	23.4
WT3	0.094	25.8
WT4	0.089	23.9
WT5	0.091	25.2

Abbreviations:: Tg, transgenic (Meis1 Knockdown); WT, wild type.

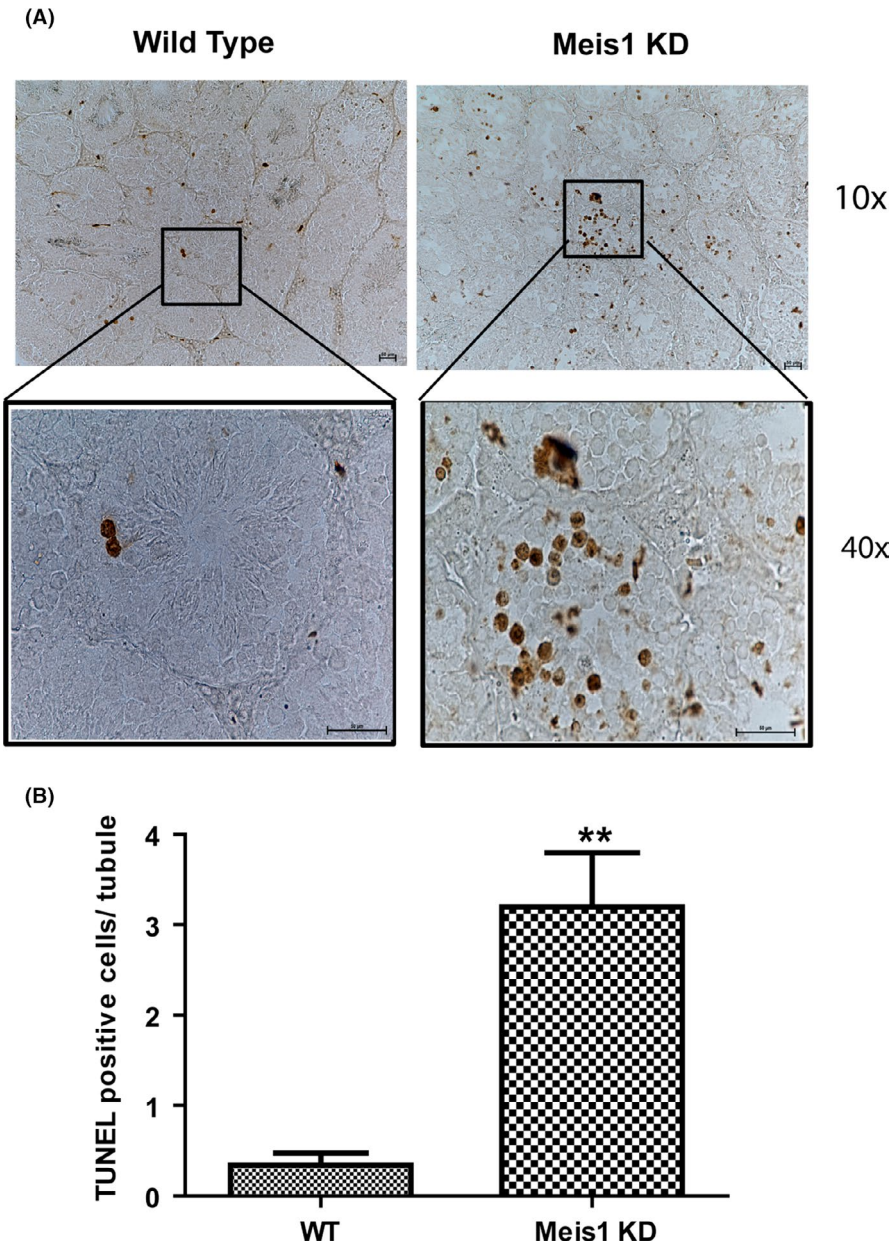

FIGURE 3 Germ cell apoptosis in Meis1 knockdown transgenic mice. (A) Representative images of testicular sections of wild-type and Meis1 knockdown transgenic mice showing apoptotic germ cells as detected by TUNEL assay. (B) Quantification of TUNEL-positive cells per tubule in wild-type and Meis1 KD mice. A statistically significant increase in the number of apoptotic germ cells was observed in Meis1 KD mice. Unpaired Student's t test was used for determining statistical significance. $P < .05$ was considered to be statistically significant; n = 3 (3 transgenic and 3 wild-type mice). At least 3 different sections from each animal (each with at least 10 seminiferous tubules) were analyzed to determine the extent of germ cell apoptosis

3.6 | Fertility assessment of Meis1 KD transgenic mice

Analysis of epididymal sperm count revealed a significant ($P < .05$) decline in sperm count in *Meis1* KD mice as compared to age-matched wild-type mice (Figure 6). The decline in sperm count was associated with a significant ($P < .05$) decline in the fertility of the Meis1 transgenic mice (Figure 6). The decline in fertility of Meis1 KD mice correlated with the extent of MEIS1 knockdown in these transgenic animals (Figure 7 and Figure 1C). Presence of copulatory plugs confirmed that transgenic mice mated successfully.

4 | DISCUSSION

Differentiation of Gc in males is initiated at the onset of puberty when Sc maturation occurs after birth.[11] Testicular Sc plays an indispensible role in regulating the complex process of spermatogenesis. The robust initiation of spermatogenesis at the onset of puberty occurs due to functional maturation of Sc.[6,11,26] FSH and testosterone, having their receptors in Sc, play a crucial role in maturation and regulation of Sc function and spermatogenesis.[10,26]Impaired maturation of Sc due to defective hormone signaling and/or deregulated gene expression may adversely affect spermatogenic progression.

We have shown in the past that besides hormones, a number of Sc gene products play an important role in maintenance of Sc function, crucial for spermatogenesis.[16,25,27] A number of transcription factors such as SOX9, WT1, and GATA4 are known to regulate Sc development and maturation.[28] In the present study, we demonstrate a role of homeobox transcription factor MEIS1 in maturation of Sc and, hence, spermatogenesis. We selected Meis1 from the list of transcription factors whose binding sites were found by us to

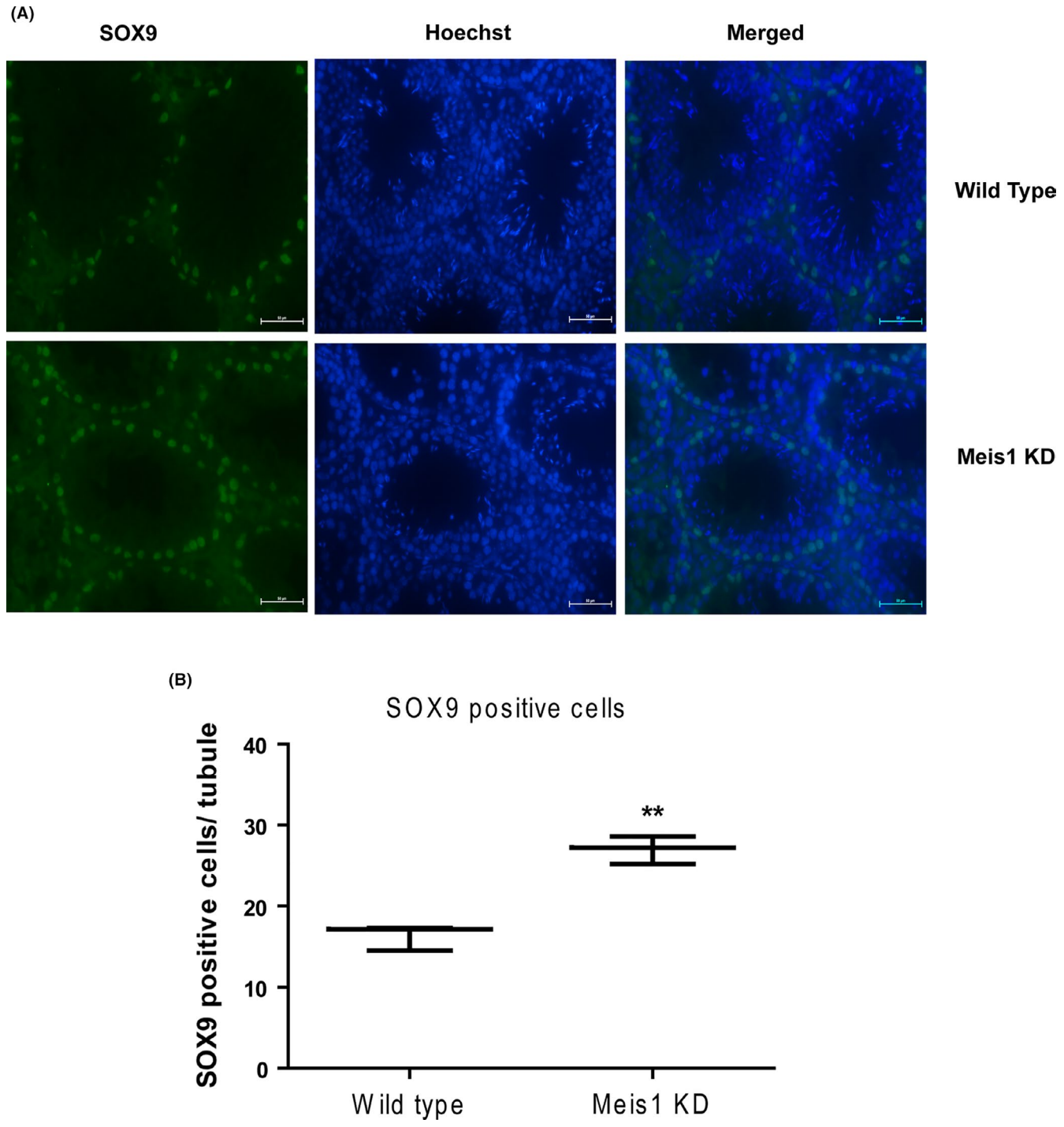

FIGURE 4 SOX9 expression in Meis1 knockdown transgenic mice. (A) Testicular sections from wild-type and Meis1 KD mice immunostained for detection of SOX9 expression. Nuclei were stained using Hoescht-33342 dye. Magnification 40×. (B) Quantification of SOX9-positive cells per tubule in wild-type and Meis1 KD mice. SOX9 positive cells were significantly higher in number in Meis1 KD mice as compared to wild-type control mice. Unpaired Student's t test was used for determining statistical significance. $P < .05$ was considered to be statistically significant

be enriched in the genes which gets up-regulated in Sc during puberty.[16] As the binding sites of MEIS1 are significantly enriched on the promoter of genes up-regulated in pubertal Sc, we hypothesized that MEIS1 could be playing an important role in regulating pubertal Sc function which is also associated with robust Gc differentiation, first time after birth.

In order to understand the role of Meis1 in Sc, transgenic mice with Sc-specific knockdown of Meis1 were generated. Since Pem (Rhox5) promoter used by us becomes active at the onset of puberty, the expression of shRNA was restricted to Sc in an age-specific manner, that is puberty onwards. MEIS1 expression in the transgenic mice was found to be significantly down-regulated as compared to age-matched control

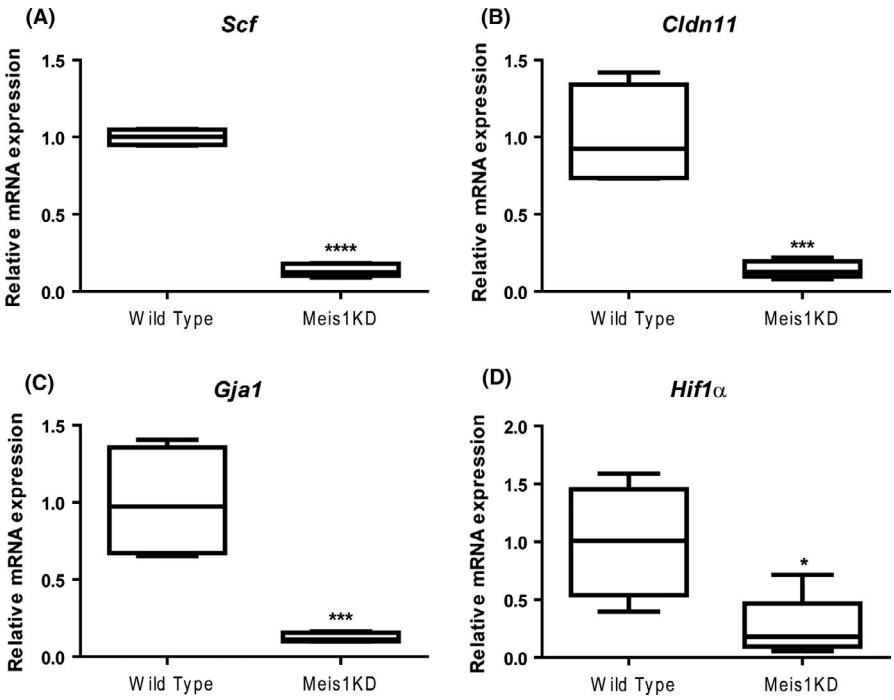

FIGURE 5 Expression of Sc maturation markers in Meis1 Knockdown transgenic mice. (A-C) q-RT PCR data showing the expression of Scf, Gja1, and Cldn11 in the testes of Meis1 knockdown transgenic mice and age-matched wild-type controls. A significant ($P < .05$) decline in the expression of Scf, Gja1, and Cldn11 was observed in the transgenic mice as compared to the controls. The expression of Hif1α, a target of Meis1, was also down-regulated in the transgenic mice (D). *Sox9* was used for normalizing gene expression. Unpaired Student's t test was used for determining statistical significance. $P < .05$ was considered to be statistically significant

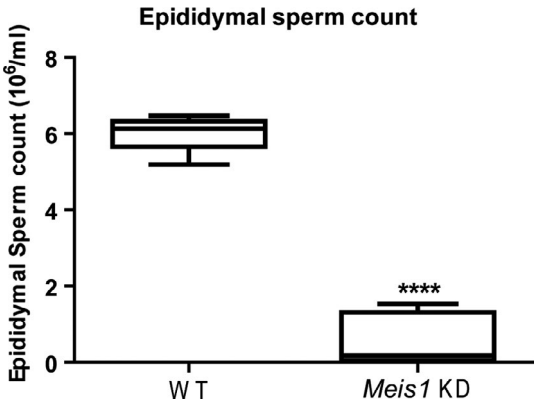

FIGURE 6 Epidiymal sperm count analysis of Meis1 knockdown transgenic mice. The epididymal sperm count of Meis1 KD mice was significantly ($P < .05$) reduced as compared to age-matched wild-type mice. Unpaired Student's t test was used for determining statistical significance. $P < .05$ was considered to be statistically significant, n = 5 (5 transgenic and 5 wild-type animals)

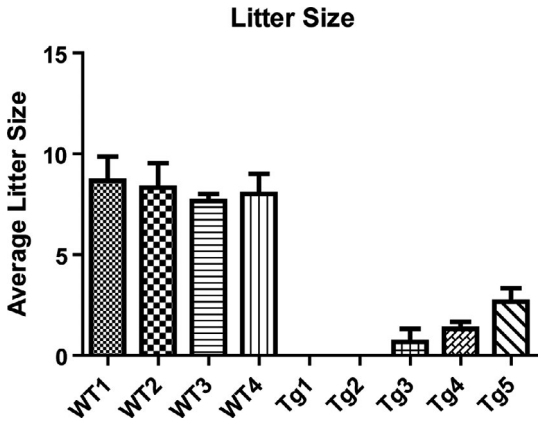

FIGURE 7 Fertility analysis of Meis1 knockdown transgenic mice. Graph showing average litter size (as a consequence of 3 mating cycles) of wild-type and Meis1 KD mice

animals, indicating efficient knockdown of MEIS1 by shRNA in the transgenic mice. Wild-type mice were used as a control in our experiments as we have previously demonstrated that LacZ knockdown transgenic mice are phenotypically similar to wild-type mice.[25]

A significant decline in testicular size and seminiferous tubule diameter, in addition to disturbed tubular architecture in the mice expressing *Meis1*shRNA, indicated a crucial role of Meis1 in spermatogenesis. Increased Gc apoptosis and a decline in advanced Gc population in the Meis1 KD transgenic mice testes suggested that the observed effects could probably be due to restricted Sc maturation in the testes of the transgenic mice. Sertoli cells in the transgenic mice were identified using SOX9 immunostaining. SOX9 is a transcription factor that is expressed specifically in Sc within the testes.[6] Interestingly, it was observed that there was a significant increase in the number of SOX9-positive cells in the testis of Meis1 KD transgenic mice as compared to age-matched wild-type

control mice indicating Sc is engaged more in proliferation than maturation in these transgenic pubertal mice. However, we could not detect the expression of the proliferative marker PCNA in the Sc of the transgenic mice. It is possible that the observed increase in the number of Sc took place early during puberty in the transgenic mice. This observation is supported by a previous report in men where the expression of SOX9 has been reported to be significantly elevated in sub-fertile or infertile testiculopathic patients.[29] The Sc of Meis1 KD mice had impaired functional maturation as was evident by a significant decline in the expression of genes like stem cell factor, gap junction protein connexin-43 (Gja1) and tight junction protein Claudin 11 (*Cldn11*). Stem cell factor produced by Sc is essential for the spermatogonial differentiation; *Gja1* and *Cldn11* are essential components of the blood-testis barrier.[30-32] It is likely that a decline in the expression of these genes in Meis1 KD transgenic mice contributed to the testicular defects observed in these mice.

Expectedly, Meis1 KD transgenic mice exhibited a significant decline in epididymal sperm count as well as fertility. The transgenic mice mated successfully as confirmed by the presence of copulatory plugs. It is important to note here that the severity of infertility or reduced sperm count in the transgenic mice directly correlated with the extent of MEIS1 knockdown in these animals and varied from animal to animal. This suggested that defective Meis 1 expression due to any reason may be one of the reasons for falling sperm count.

These results clearly suggested that Sc-specific expression of MEIS1 is crucial for proper spermatogenic progression. Interference in expression of MEIS1 within Sc led to an increased Gc apoptosis and reduced testicular weight. A concomitant decline in the epididymal sperm count and a subsequent decline in the fertility of the transgenic mice were also discerned. It is important to note that nearly 50% of male infertility cases are idiopathic in nature wherein hormone supplementation fails to ameliorate the infertility symptoms (Pei et al 2005). Defects in Sc proliferation or impaired maturation are some of the likely causes that may contribute to idiopathic infertility. The present study demonstrated that a knockdown of Meis1 in Sc was associated with a significant increase in the number of SOX9-positive Sertoli cells in the transgenic mice and a significant decline in the expression of functional maturation markers like *Scf*, *Gja1*, and *Cldn11* suggesting that Sc maturation was compromised due to shRNA-mediated suppression of Meis1 expression. Unless we generate such important information about molecular basis of idiopathic male infertility, participation of males toward correcting their own reproductive health cannot be encouraged. Such observations are crucial for reducing the burden on females for resolving infertility of couples by avoiding various steps of assisted reproductive technologies (ART). Identification of such novel male factors associated with spermatogenesis would provide useful insights into the complex regulation of spermatogenesis by various factors of Sc origin and would also open new avenues for diagnosis and treatment of male infertility.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

RKS, UR, and SSM designed the experiments. NW and NK generated the Meis1 KD transgenic mice. RKS, SSS, KM, and AG performed the experiments. SSM, RP, UR, RKS, and SSS analyzed the data and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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