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Declining levels of miR-382-3p at puberty trigger the onset of spermatogenesis

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A major change in the transcriptome of testicular Sertoli cells (Scs) at the onset of puberty enables them to induce robust spermatogenesis. Through comprehensive literature mining, we generated a list of genes crucial for Sc functioning and computationally predicted the microRNAs regulating them. Differential expression analysis of microRNAs in infant and pubertal rat Scs showed that miR-382-3p levels decline significantly in pubertal Scs. Interestingly, miR-382-3p was found to regulate genes like *Ar* and *Wt1*, which are crucial for functional competence of Scs. We generated a transgenic (Tg) mouse model in which pubertal decline of miR-382-3p was prevented by its overexpression in pubertal Scs. Elevated miR-382-3p restricted the functional maturation of Scs at puberty, leading to infertility. Prevention of decline in miR-382-3p expression in pubertal Scs was responsible for defective blood-testis barrier (BTB) formation, severe testicular defects, low epididymal sperm counts and loss of fertility in these mice. This provided substantial evidence that decline in levels of miR-382-3p at puberty is the essential trigger for onset of robust spermatogenesis at puberty. Hence, sustained high levels of miR-382-3p in pubertal Scs could be one of the underlying causes of idiopathic male infertility and should be considered for diagnosis and treatment of infertility.

INTRODUCTION

The continuously evolving human lifestyle has led to various changes in the bodily functions of humans, and fertility is no exception.[1–3] Almost 15% of healthy couples globally are affected by infertility, of which male factors contribute a share equal to female factors.[4,5] A substantial percentage of male infertility is idiopathic in nature, which is currently untreatable by conventional hormonal therapy, as the etiologies are not well established yet.[6,7] Spermatogenesis is a multi-step process of germ cell (Gc) division and differentiation that occurs in the seminiferous tubules of the testes under the regulation of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH).[8–10] It is a highly coordinated process regulated by the surrounding somatic testicular cells, such as the Sertoli cells (Scs), Leydig cells (Lcs), and peritubular myoid cells (PTcs).[11,12] Scs are intimately associated with the developing germ cells, providing them support and nourishment, thereby regulating proper spermatogenic progression.

Scs undergo proliferation during the neonatal/infantile period. During this phase, immature Scs remain incapable of supporting adequate hormonal signaling necessary for the robust initiation of spermatogenesis.[13,14] The functional maturation of Scs occurs at the onset of puberty, when these cells gain the ability to respond toward hormones optimally to promote robust germ cell division and differentiation.[15–18] An increase in the expression of androgen receptor (AR) and Wilms tumor 1 (WT1) play a crucial role in the maturation of Scs. The maturation of Scs is associated with the establishment of tight junction proteins to form an immunological barrier called the blood-testis barrier (BTB), which spatially separates the neoantigens expressed during the development of new germ cells from the reach of immune surveillance.[19,20] Mature Scs show an increased responsiveness to hormonal cues and upregulated expression of genes such as Kit-Ligand (*Kitlg*) or Stem Cell factor (*Scf*), Glial-cell Derived Neurotropic Factor (*Gdnf*), Androgen binding protein (*Abp*), Transferrin *(Tf),* etc., which are essential for spermatogenesis. The transition of Scs from a proliferative to a differentiated state is associated with a remarkable change in the transcriptome/proteome profile of the cell.[21–26] Any defect in the Sc signaling or impaired maturation in response to various environmental factors or lifestyle has a negative impact on the sperm production and can lead to subfertility or infertility.

One of the major factors regulating the transcript levels of any cell are the microRNAs (miRNAs). MicroRNAs are ~22 bp small non-coding RNAs that play a critical role in regulating cellular transcriptomic fate determination.[27] The miRNAs are mostly transcribed by RNA polymerase II, which mostly binds to the 3′ UTR of the target gene, leading to its degradation or translational repression.[28,29] Indeed, the Sc- or Gc- specific ablation of the DICER and/or DROSHA, the crucial components of miRNA processing machinery, results in total loss of spermatozoa, leading to testicular degeneration and eventually infertility.[30–34] MicroRNAs are also known to be regulated by testosterone (T) and FSH.[35–37] Furthermore, specific association of

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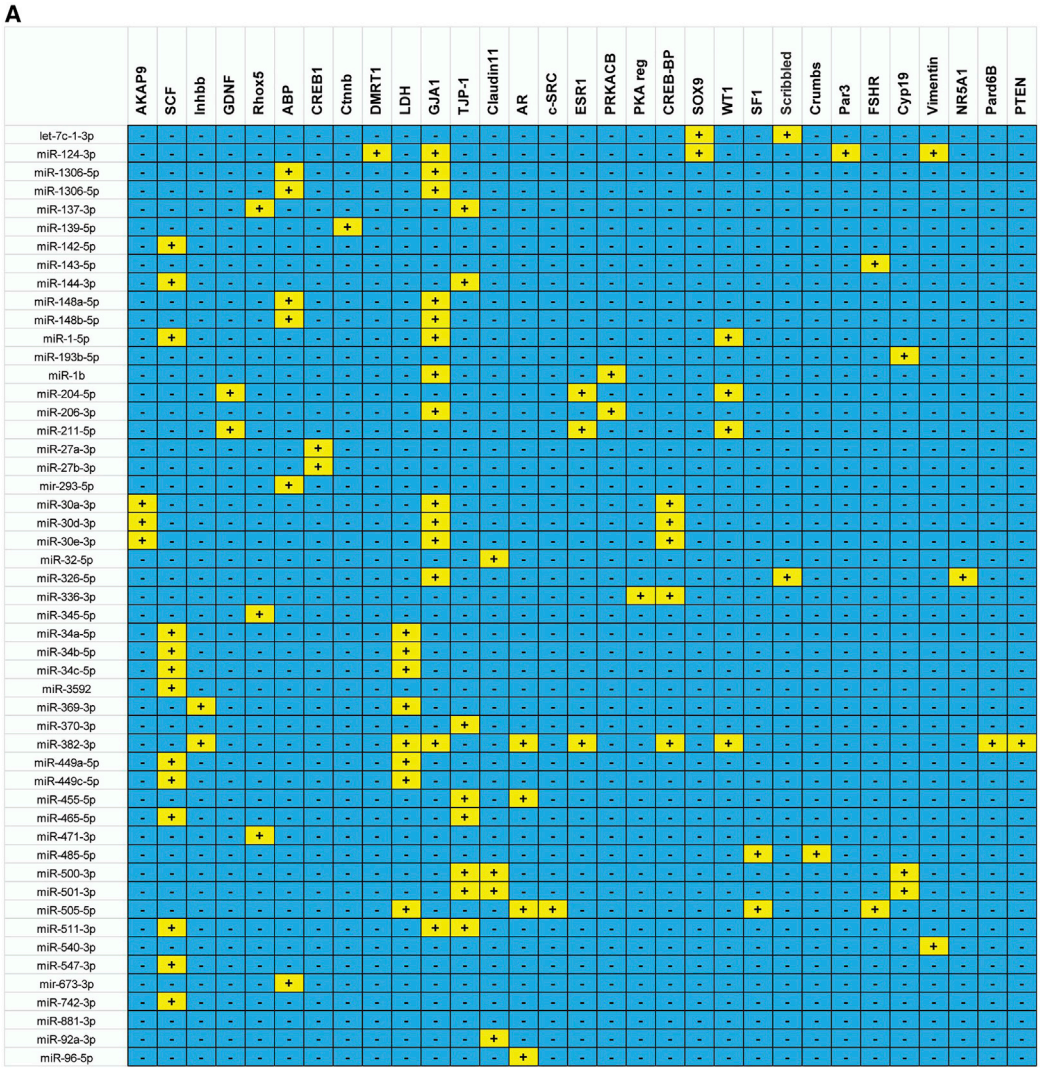

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multiple miRNAs have also been identified in endocrine-disrupting chemical (EDC) induced dysfunction in Scs.[38,39] The microRNA profiling of sexually immature (infant) versus mature (pubertal) testis of porcine and rhesus monkeys displayed substantial alterations between the two age groups.[40–42] It has also been shown that Sertoli cell only syndrome (SCOS) patients have an altered miRNA profile as compared to healthy fertile individuals.[43]

The rise in cases of male infertility has become a global concern. Failure of testis to produce sperm in spite of hormonal replenishment still remains unexplained. Hence, it is necessary to find causes underlying such infertility. Since Scs regulate onset of spermatogenesis, this study was initiated with the primary objective to identify microRNAs playing crucial roles in Sc maturation, which is necessary for onset of robust spermatogenesis at puberty. We hypothesized that the microRNAs that are predicted to target genes known to be crucial for Sc functioning would also be important for Sc development and maturation. To address this notion, we predicted the microRNAs that may potentially bind to the 3′ UTR of the genes already known to be crucial in Sc functioning and determined expression levels of such microRNAs in infant (5 days old, devoid of spermatogenesis) and pubertal (19 days old, with visible onset of spermatogenesis) rat Scs. In the current study, we intended to identify a crucial microRNA that regulates the expression of several genes that are known to be essential for Sc functioning. We found that miR-382-3p expression is naturally diminished in functionally mature pubertal Scs as compared to immature infant Scs. We hypothesized that decline in the levels of mir-382-3p at the onset of puberty is critical to Sc maturation. Indeed, overexpression of miR-382-3p in pubertal Scs was found to downregulate the expression of crucial genes like *Ar* and *Wt1* and consequently their target genes as well. Our studies in a transgenic mouse model with pubertal Sc-specific overexpression of miR-382-3p showed that the augmentation of miR-382-3p in pubertal Scs leads to low sperm counts that lead to infertility in the transgenic male mice. This study for the first time demonstrates a crucial role of miR-382-3p in Sc maturation, maintenance of BTB integrity, and spermatogenic progression.

RESULTS

Differentially expressed microRNAs in infant (5 days old) and pubertal (19 days old) rat Scs

Our comprehensive literature survey showed 39 genes that are known to have crucial role(s) in Sc functioning (Table S1). Standard databases like miRDB and TargetScan revealed a putative list of 289 miRNAs predicted to target these genes. 51 miRNAs (Table S2) were found to be putatively targeting 31 genes out of the initial list of 39 genes (Figure 1A) and were therefore selected for further studies. The expression levels of these microRNAs were then determined in cultured infant (5 days old) and pubertal (19 days old) Scs that were subjected to pulsatile hormone treatment with FSH and T. Forty miRNAs were detected in Scs from both ages (Figure S1), and 11 miRNAs were found to be significantly differentially expressed between these two age groups (Figure 1B). Four miRNAs were significantly ($p < 0.05$) upregulated in infant Scs, and 7 miRNAs were significantly high in pubertal Scs (Figure 1B). miR-1b, miR-92a-3p, miR-369-3p, and miR-382-3p were abundant in infant Scs, while mir-204-5p, miR-211-5p, 471-3p, miR-449-5p, miR-547-3p, miR-742-3p, and miR-881-3p were significantly ($p < 0.05$) upregulated in pubertal Scs. MiR-382-3p levels showed the maximum decline (89%) in pubertal Scs as compared to infant Scs and was predicted to target various genes that are indispensable for Sc functioning. Thus, we decided to overexpress miR-382-3p in pubertal Scs for *in vitro* and *in vivo* studies to decipher its role in Sc functioning and spermatogenesis.

In vitro validation of genes predicted to be targeted by miR-382-3p

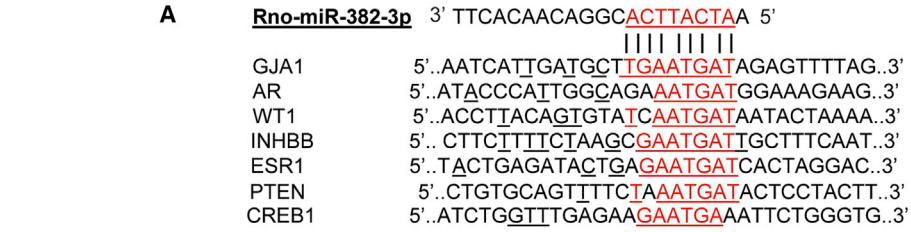
We performed luciferase assay in HEK293T (human kidney cells), which were used just as a host cell for conducting luciferase assay, as they are easy to transfect. For validating the predicted targets of miR-382-3p, we co-transfected miR-382-3p mimic with 3′ UTR (of target genes) bound luciferase in HEK293T cells. Genes like *Ar*, *Wt1*, *Gja1*, *InhbB*, *Esr1*, *Pten*, and *Creb1* were found to be directly targeted by mir-382-3p (Figures 2A and 2B). Then we sought to validate these direct targets by quantitative real-time PCR in pubertal (19 days old) Scs that were transfected with miR-382-3p mimics (miRNA overexpression) or scrambled controls. miR-382-3p overexpression significantly ($p < 0.05$) downregulated the transcript levels of *Ar*, *Wt1*, *Gja1*, *Inhbb*, *Esr1*, *Creb1*, and *Pten* in cultured 19-day rat Scs (Figure 2C). Immunoblot analysis revealed a significant ($p < 0.05$) decline in the protein levels of WT1 and Cx43 (coded by *Gja1*) in pubertal Scs overexpressing miR-382-3p as compared to scrambled miRNA-transfected Scs (Figure 2D).

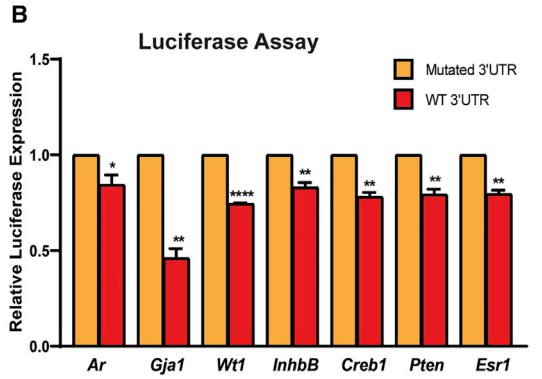
MiR-382 overexpression in cultured Scs leads to disruption of AR signaling and impaired Sc maturation

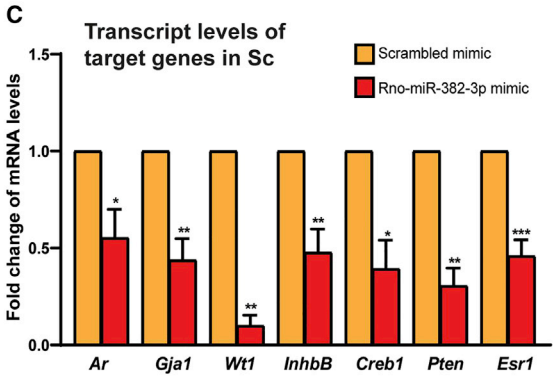
MiR-382-3p-overexpression-mediated downregulation of AR in cultured pubertal (19 days old) Scs led to a significant ($p < 0.05$) decline in the transcript levels of AR-responsive genes *Cldn11*, *c-Myc*, and *Rhox5* (Figure 2E). The mRNA levels of *Wt1*-responsive genes like *Pard6b* and *E-Cadherin* had significantly ($p < 0.05$) declined in response to miR-382-3p overexpression in Scs as compared to scrambled transfected Scs (Figure 2F). Moreover, the expressions of genes like *Abp*, *Kitlg*, *Gdnf*, *Transferrin*, and *Ldhb*, which are already established to be essential for spermatogenesis, were significantly downregulated in mir-382-3p-overexpressing Scs as compared to scrambled miRNA-transfected Scs (Figure 2G).

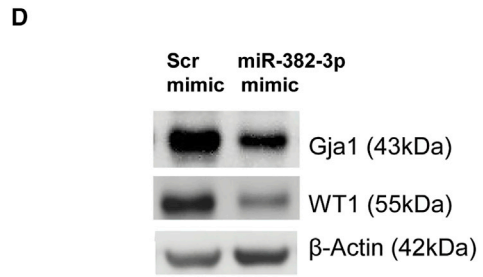
Figure 1. Differentially expressed microRNAs in infant (5 days old) and pubertal (19 days old) rat Scs

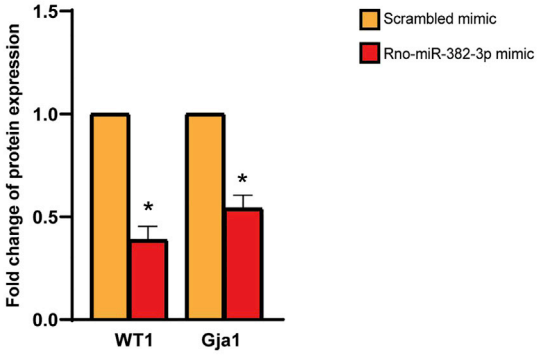
(A) Matrix showing 51 microRNAs and their predicted target genes as identified from online databases such as miRDB and TargetScan. +, predicted target gene; −, non-target gene. (B) Quantitative real-time PCR data for microRNA levels showing the differential expression of 11 microRNAs in infant (5 days old) and pubertal (19 days old) cultured rat Scs. Values are mean ± SEM of 3 independent biological replicates. Unpaired Student's t test was used for determining statistical significance. $p < 0.05$ was considered to be statistically significant.

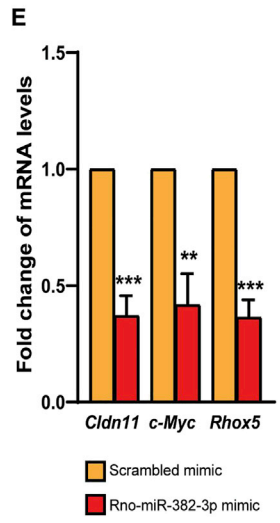


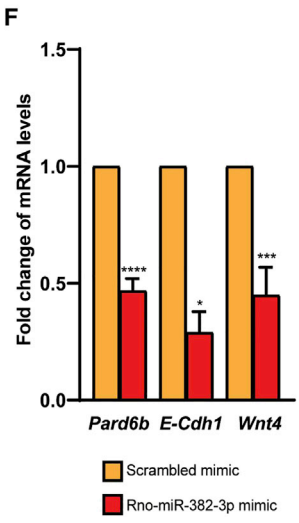


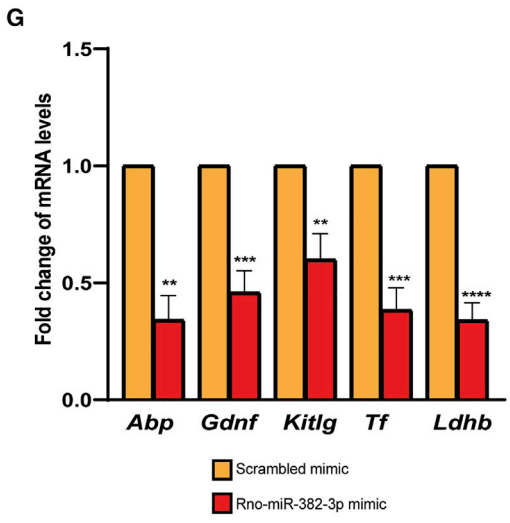












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Generation of transgenic mice with Sc-specific overexpression of miR-382-3p

As miR-382-3p overexpression in cultured pubertal Scs had a major impact on the functional maturation markers of the cell, we generated a transgenic mouse model overexpressing miR-382-3p in Scs. The expression of miRNA was driven under the Rhox5 promoter, which ensured the overexpression of miR-382-3p specifically in Scs at the onset of puberty (Figure S2A). Slot blot analysis of the F1 generation from the electroporated fore-founder mice identified the transgene-positive animals (Figure S2B). miR-382-3p levels were significantly ($p < 0.05$) upregulated in the transgenic testis as compared to age-matched wild-type (WT) controls (Figure 3A). Despite a similar body weight and serum testosterone levels in miR-382-3p-overexpressing transgenic mice and WT controls, there was a significant ($p < 0.05$) decline in the testis weight of the transgenic mice (Figures S2C–S2E). The testis size (Figure 3B) was evidently reduced in the miR-382-3p transgenic mice from the F1 generation and the F2 generation (Figure S2F). The percentage ratio of testis weight to body weight, gonadosomatic index (GSI %), also showed a sharp decline in the transgenic mice (Figure 3C).

MiR-382-3p-overexpressing transgenic mice were infertile and had testicular atrophy

The total epididymal sperm count was lower than 1 million/mL in the transgenic mice as compared to WT, having average sperm count of more than 5 million/mL (Figure 3D). The transgenic animals were completely infertile and produced no litter when mated with age-matched WT females (Figure 3E). In contrast to the WT testis, the seminiferous tubules in the testis of miR-382-3p-overexpressing transgenic mice from both F1 and F2 generations had significantly ($p < 0.05$) reduced tubule size and discernible histological differences with disrupted seminiferous tubule architecture as observed with hematoxylin and eosin staining on tissue paraffin sections (Figures 3F and 3G; Figure S2G). The tubules also had a vacuolated phenotype toward the basal lamina in the transgenic mice testes (Figure 3F).

MiR-382 overexpression impaired Sc maturation and increased germ cell apoptosis

The testicular mRNA levels of Sc functional maturity markers like *Abp*, *Gdnf*, *Kitlg*, *Ldhb*, and *InhbB* were significantly ($p < 0.05$) downregulated in miR-382-3p-overexpressing transgenic mice testes as compared to those of control animals (Figure 4A). These results were in concordance with the results obtained in our *in vitro* experiments with cultured pubertal (19 days old) Scs from rats. The decline in the Sc maturation markers was also associated with an increase in the number of apoptotic germ cells. The number of apoptotic cells per tubule, as detected by TUNEL assay, were significantly ($p < 0.05$) higher in the transgenic mice testis as compared to WT testis (Figures 4B and 4C).

MiR-382-3p-overexpressing transgenic mice had compromised AR signaling and deregulated expression of cell polarity genes

miR-382-3p overexpression in transgenic mice led to direct decline in the testicular mRNA levels of *Ar*. AR-responsive genes like *Rhox5*, *Creb1*, and *c-Myc* were also significantly ($p < 0.05$) downregulated due to miR-382-3p-mediated decline in levels of *Ar* (Figure 5A). The transgenic mice showed significantly reduced transcript levels of *Wt1* and its target genes like *Pard6b* and *E-Cdh1*, which are crucial for maintaining Sc polarity (Figure 5B). The decline in mRNA levels of miR-382-3p target genes and their downstream genes followed a similar trend as observed in microRNA overexpression experiments with cultured pubertal (19 days old) rat Scs. miR-382-overexpressing transgenic mice had significantly ($p < 0.05$) reduced testicular protein levels of AR and WT1 (Figure 5C).

MiR-382-3p overexpression in transgenic mice led to disruption of the BTB

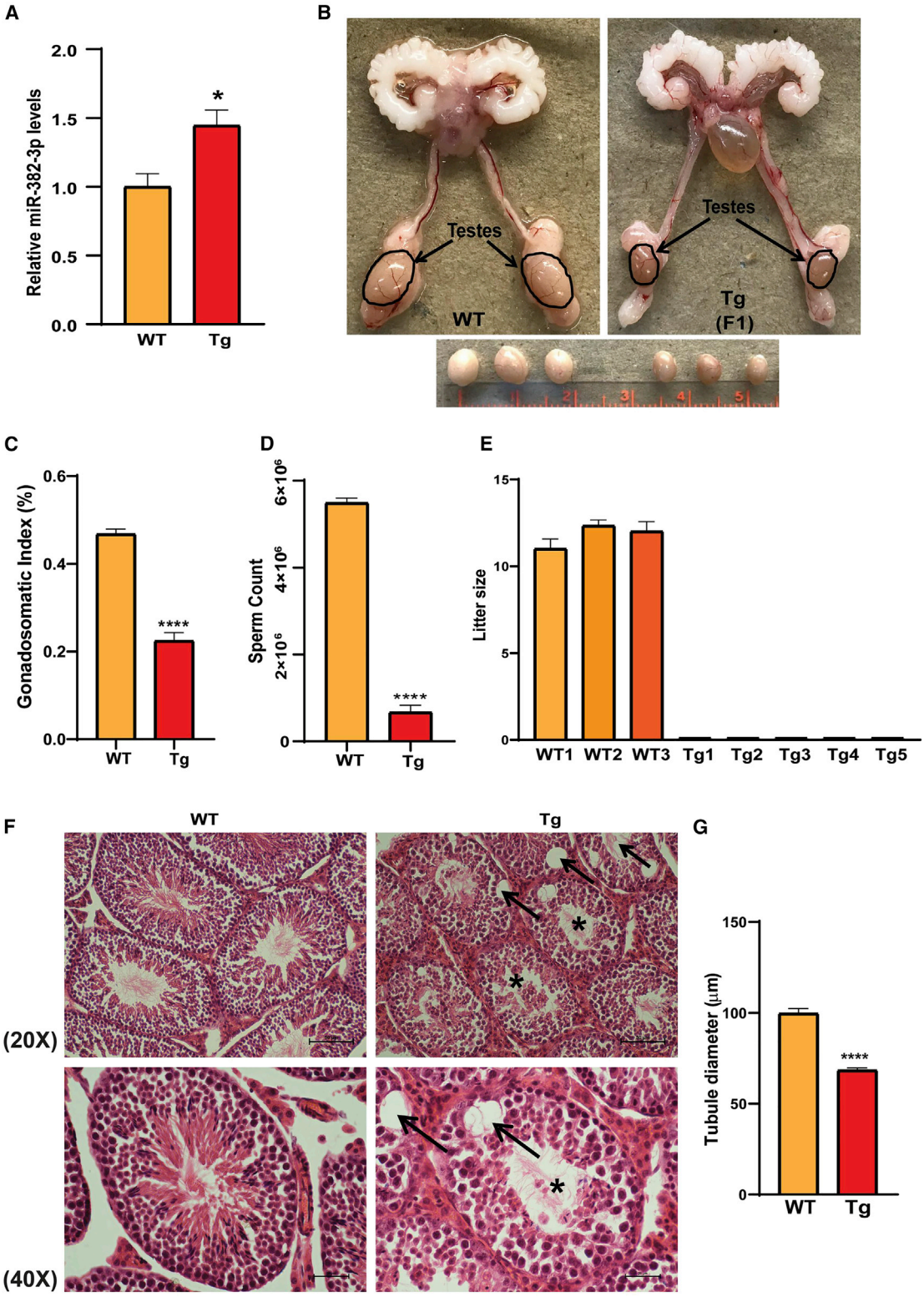
The testicular mRNA levels of BTB components such as *Cldn11* and *Gja1* were significantly ($p < 0.05$) reduced in miR-382-3p-overexpressing transgenic mice as compared to age-matched WT mice (Figure 6A). Protein level of CX43and CLDN11 in the transgenic testis was also significantly reduced as compared to WT control mice (Figure 6B). Immunohistochemistry for Claudin11 and Connexin43 on testicular frozen sections (7 μm) showed that the transgenic mice testis expressed very low and almost undetectable levels of these proteins, which are the major constituents of the BTB, as compared to age-matched WT testis (Figures 6C and 6D). Transmission electron microscopy (TEM) revealed a discontinuous and relatively narrow BTB in the transgenic mice as compared to the intact BTB in age-matched WT control testis (Figure 6E).

MiR-382-overexpressing transgenic mice had an increased number of Scs

We hypothesized that the deregulated Sc maturation in the miR-382-3p transgenic mice was due to delayed maturation of these cells, which in turn meant that these cells might have undergone a

Figure 2. Validation of miR-382-3p predicted target genes and effect of miR-382-3p overexpression in pubertal (19 days old) rat Scs

(A) miR-382-3p sequence (3′ → 5′) and its binding site on the 3′ UTR of the predicted target genes. (B) Luciferase analysis of 3′ UTRs of predicted target genes of miR-382-3p in HEK293T cells. (C) mRNA expression for direct targets of miR-382-3p in cultured pubertal (19 days old) rat Scs as compared to scramble controls. (D) Representative immunoblot and its densitometric quantification for change in protein levels of WT1 and CX43 in cultured pubertal (19 days old) rat Scs after miR-382-3p overexpression using microRNA mimics as compared to scramble-mimic-transfected Sc. β-actin was used as a loading control. (E) Quantitative real-time PCR data showing the levels of AR-responsive genes in miR-382-3p-overexpressed cultured pubertal (19 days old) rat Scs. (F) Quantitative real-time PCR data showing the transcript levels of WT1-responsive genes in cultured pubertal (19 days old) rat Scs that were transfected with miR-382-3p mimics as compared to scrambled controls. (G) Quantitative real-time PCR data showing change in levels of Sc functional maturation markers in miR-382-3p-mimic-transfected cultured rat pubertal (19 days old) Scs as compared to scramble-transfected Scs. All values are mean ± SEM of at least 3 independent biological replicates. Paired Student's t test was used for determining statistical significance. $p < 0.05$ was considered to be statistically significant.



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prolonged proliferative phase. To this end, we performed immunostaining for Sc-specific SOX9 on tissue sections from transgenic and WT mice (Figure 7A). The total number of SOX9-positive cells per tubule was significantly ($p < 0.05$) higher in the transgenic mice as compared to age-matched WT controls (Figure 7B). In addition to this, there was a significant ($p < 0.05$) increase in the testicular transcript levels of *Sox9* in the transgenic mice as compared to age-matched WT control mice (Figure 7C).

DISCUSSION

Spermatogenesis is an elaborate multi-step event that involves the rapid proliferation and differentiation of spermatogonial stem cells into highly differentiated spermatozoa. Scs are a major somatic component of the testis and play a critical role in the regulated differentiation of germ cells.[12] The functional maturation of Scs during puberty directs the robust initiation of first spermatogenic wave, whereas the final number of Scs in the pubertal testes determines the maximal spermatogenic output.[17,44,45] MicroRNAs are well-established *trans*-acting factors for optimizing developmental gene expression in various cellular processes, including gonadal differentiation. Several studies have shown microRNAs like miR-34/449, miR-10a, miR-100-3p, miR-383, etc., to be crucial for mammalian spermatogenesis.[46–49] In the current study, we for the first time have deciphered a microRNA miR-382-3p in the regulation of Sc maturation and spermatogenesis. Based on the available literature on Sc maturation, we listed out 39 genes crucial for Sc functioning, having varied roles such as hormone receptors, transcription factors, differentiation markers, secretory molecules, etc. Online available databases (miRDB and TargetScan) provided a list of microRNAs that are predicted to target these genes. We selected a differentially expressed microRNA - miR-382-3p, that is almost 10 times more abundantly transcribed in immature proliferating Scs and is naturally downregulated in the mature Scs at the onset of puberty. Our results suggested that the decline in levels of miR-382-3p in testicular Scs at puberty is crucial for the functional maturity of Scs so as to attain the ability to support germ cell division and robust sperm production at that stage of development.

We overexpressed miR-382-3p in pubertal Scs *in vitro*, using commercial microRNA mimics. The validated direct targets of miR-382-3p included genes like *Ar*, *WT1*, *Gja1*, etc., which are known to be essential for Sc maturation and its ability to support spermatogenesis. Our *in vitro* data showed that overexpression of miR-382-3p in pubertal Scs led to significant decline in important genes like *Kitlg*, *Gdnf*, *Ldhb*, etc., which are produced by Scs to nurture the germ cells and support spermatogenesis. Low levels of Kit-Ligand or SCF and GDNF have been previously reported to increase germ cell apoptosis and are associated with azoospermia or oligozoospermia.[50,51] Sc-Sc tight junction proteins like Claudin11 and Connexin 43, which are essential components of the BTB, were also significantly reduced in miR-382-3p-overexpressing cultured pubertal Scs, indicating the importance of this microRNA.

To confirm our *in vitro* finding, we generated a Sc-specific transgenic mouse model overexpressing miR-382-3p specifically in mature Scs. The overexpression of miR-382-3p in the transgenic mice was driven by proximal *Rhox5* promoter, which gets activated in Scs at around 14 days of post-natal age and continues to express during adulthood in all stages of seminiferous cycles, in particular stages VI to VIII.[52–54] The Sc-specific overexpression of miR-382-3p in the transgenic mice led to severe testicular impairment with non-obstructive oligozoospermia and complete infertility. A significantly reduced testis size and low epididymal sperm counts in transgenic mice as compared to age-matched WT control mice provided substantial evidence to support our *in vitro* finding, confirming the critical role of miR-382-3p in spermatogenesis. The Lc physiology in adult transgenic testes was not compromised, as shown by the comparable serum testosterone levels and the size of seminal vesicles between transgenic and WT mice. The testicular architecture of the miR-382-3p transgenic animals was considerably compromised. Adult Scs from the transgenic mice, being functionally incapable of supporting massive germ cell division and differentiation, seemed to have failed to undergo maturation and may have remained in the proliferative phase for a prolonged duration. This was evident from the massive increase in germ cell apoptosis and an increase in the number of Scs per tubule in the transgenic testis.

MiR-382-3p directly targeted AR by binding to its 3′ UTR, and overexpression of miR-382-3p in transgenic mice resulted in decreased levels of AR in testis. AR is a ligand-activated transcription factor that shuttles between the cytoplasm and nucleus and is activated by binding to androgens like testosterone and dihydrotestosterone. The classical AR signaling in testis regulates the expression of genes involved in Sc maturation, formation of the immunological BTB, and meiotic progression of germ cells.[55,56] The non-classical arm works in sync with other molecules of the ERK/MAPK pathway.[57] The BTB, which is composed of tight/gap junctions, ectoplasmic

Figure 3. MiR-382-3p in Sc-overexpressing transgenic mice had reduced testis size and low epididymal sperm count

(A) Quantitative real-time PCR data showing the levels of miR-382-3p in transgenic mouse testis as compared to age-matched WT control mouse testis. (B) Representative image of testis size and seminal vesicles of mir-382-3p-overexpressing transgenic mice from the F1 generation as compared to age-matched WT controls. (C) Gonado-somatic index (testis weight/body weight × 100) of WT and transgenic mice overexpressing miR-382-3p. (D) Epididymal sperm count (million per milliliter) in control and miR-382-3-overexpressing transgenic mice. (E) Average litter produced by miR-382-3p transgenic mice as compared to age-matched WT controls. (F) Representative images of hematoxylin and eosin staining of testicular paraffin sections from miR-382-3p transgenic mice from the F1 generation and age-matched WT controls. Images captured at 20× (scale bar, 50 μm) and 40× (scale bar, 20 μm) objective magnifications. Black arrows point at vacuoles observed toward basal lamina. Asterisks represent tubules with very low numbers of mature sperm. (G) Tubule diameter as measured in the testis from miR-382 transgenic and WT mice. At least 3 different sections from each animal (each with at least 5 seminiferous tubules at 20× magnification) were analyzed to measure the tubule diameter. All values are mean ± SEM of at least 5 WT and 5 transgenic mice. Unpaired Student's t test was used for determining statistical significance. $p < 0.05$ was considered to be statistically significant.

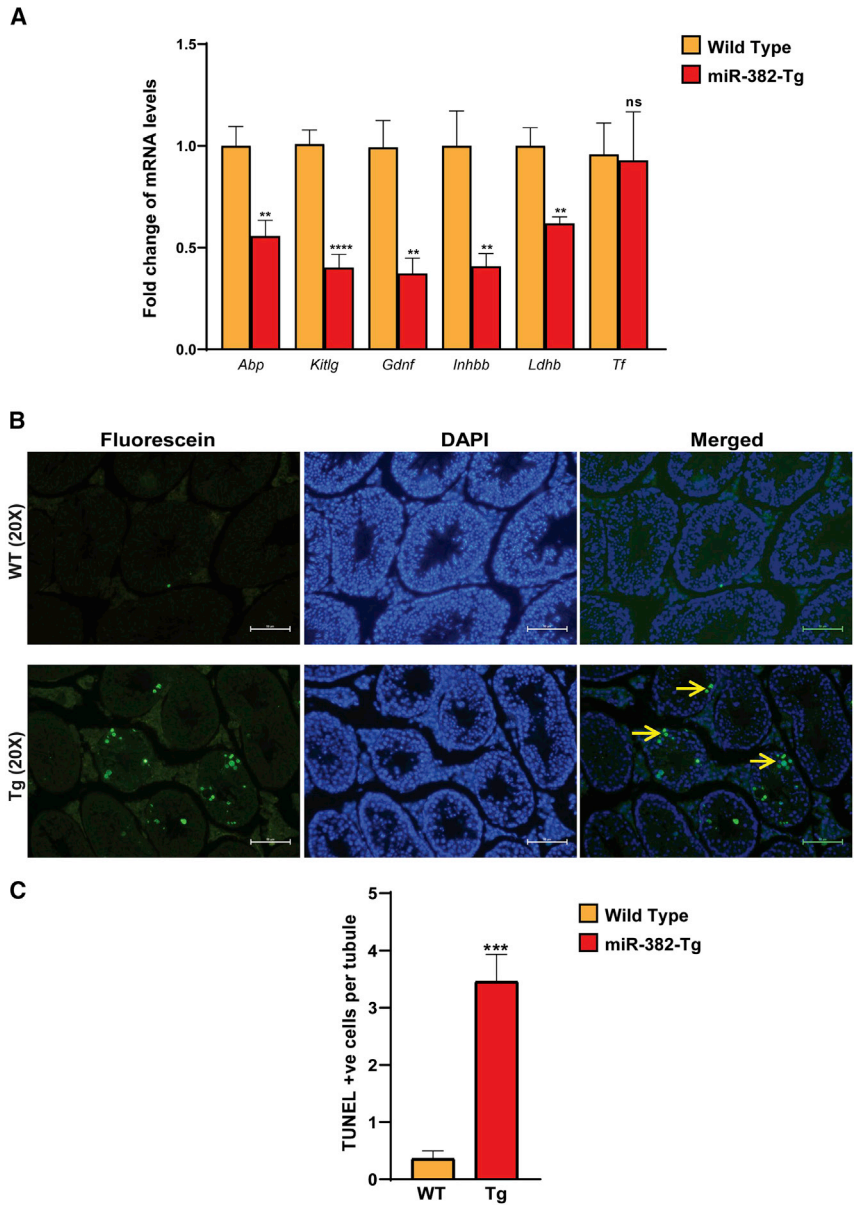


Figure 4. MiR-382-3p overexpression in transgenic mice impaired Sc maturity and was associated with germ cell apoptosis

(A) mRNA expression data showing levels of Sc functional maturation marker genes in miR-382-3p-overexpressing transgenic mouse testis as compared to age-matched WT mouse testis. (B) Representative image of testicular sections (7 μm) of miR-382-3p transgenic and WT mice showing apoptotic germ cells (yellow arrows) as detected by TUNEL assay. Images captured at 20× (scale bar, 50 μm) objective magnification. (C) Quantification of TUNEL-positive cells per tubule in transgenic mouse testis as compared to WT testis. At least 3 different sections from each animal (each with at least 10 seminiferous tubules at 10× magnification) were analyzed to determine the extent of germ cell apoptosis. Nuclei were stained with DAPI. All values are mean ± SEM of at least 5 WT and 5 transgenic mice. Unpaired Student's t test was used for determining statistical significance. $p < 0.05$ was considered to be statistically significant.

specializations, etc., undergoes remodeling during the seminiferous epithelial cycle to aid in the movement of the pre-leptotene spermatocytes from the basal to the adluminal compartment, which is indispensable for basal-to-adluminal movement of the germ cells.[58,59] Our results show that the testicular mRNA and protein levels of tight junction component Claudin11 (coded by *Cldn11*) or gap junction Connexin43 (coded by *Gja1*) were significantly downregulated in adult miR-382-3p-overexpressing transgenic mice as compared to age-matched WT controls. Our *in vivo* results obtained from miR-382-Tg mice validated our *in vitro* findings from cultured pubertal (19 days old) rat Scs, where we overexpressed miR-382-3p using synthetic mircoRNA mimics. Furthermore, TEM imaging showed a discontinuous and non-uniform architecture of BTB in the transgenic testis, which in turn may explain the severe oligozoospermia observed in the transgenic mice. The disruption of BTB is a well-established cause for male infertility, as reported in Sc-specific ablation of *Cldn11* or *Gja1*, where the maturational status and the polarity of Sc get impaired.[60,61]

Our study also demonstrated that miR-382-3p directly downregulated the mRNA and protein expression of WT1 with compromised expression of its target genes *Pard6B* and *E-Cadherin1*. WT1 is known to be crucial for spermatogenesis, as it plays an important role in regulating the Sc polarity through its direct targets like Pard6B and E-Cadherin1.[62–64] WT1 deficiency in Scs has been reported to cause declined levels of Claudin11, and WT1-deficient testes show accumulation of undifferentiated spermatogonia that exhibit meiotic arrest.[65]

In summary, this study for the first time has highlighted the potential of a single microRNA, miR-382-3p, to directly regulate Sc function and male fertility. Our results suggested that persistent presence of miR-382-3p in adult Scs results in compromised AR and WT1 signaling, severe impairment in Sc maturation, Sc polarity, dysfunctional BTB, and massive germ cell apoptosis, leading to acute oligozoospermia and finally complete male infertility. We therefore propose that the pubertal decline in the levels of miR-382-3p is a pivotal step for the adequate AR signaling critical for Sc maturation and subsequent spermatogenic onset. To the best of our knowledge and belief, this is the first direct *in vivo* demonstration of an Sc-specific microRNA-mediated direct regulation of male fertility.

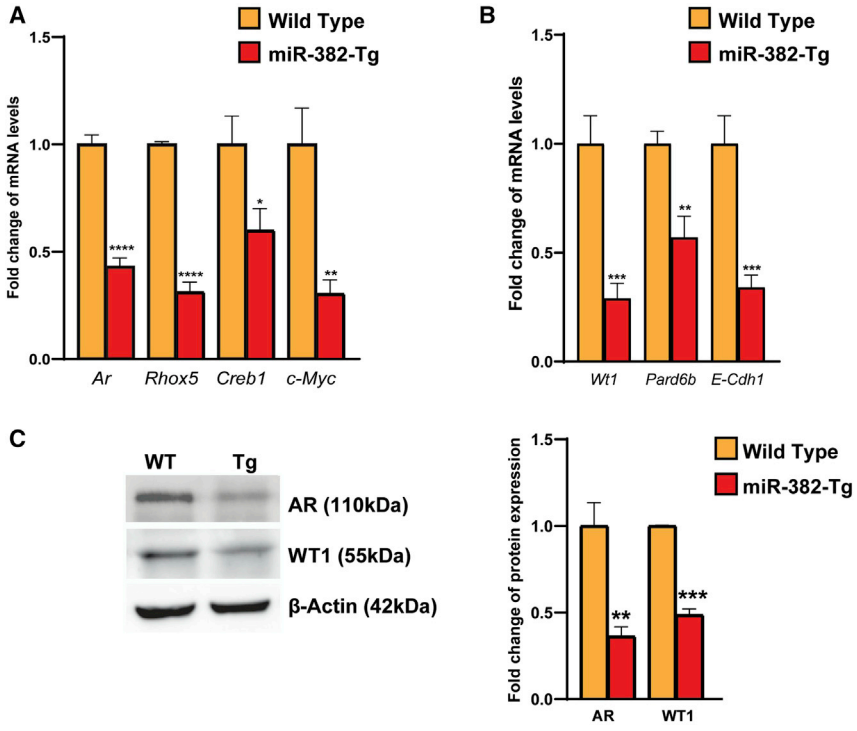


Figure 5. MiR-382-3p overexpression in transgenic mice led to decline in the testicular levels of its direct targets, AR and WT1, and their downstream responsive genes

(A) mRNA expression data showing transcript levels of *Ar* and its downstream target genes in miR-382-3p-overexpressing transgenic mouse testis as compared to age-matched WT controls. (B) Quantitative real-time PCR data showing levels of *Wt1* and its responsive genes in miR-382-3p-overexpressing transgenic mouse testis as compared to WT controls. (C) Representative immunoblot and its densitometric quantification to show testicular protein levels of AR and WT1 in transgenic and WT mice. β-actin was used as a loading control. All values are mean ± SEM of at least 5 WT and 5 transgenic mice. Unpaired Student's t test was used for determining statistical significance. $p < 0.05$ was considered to be statistically significant.

The causes of about 33% of male infertility are yet unknown. This study showed that deregulated expression of miR-382-3p due to genetic or lifestyle factors may be one of the important etiologies for some forms of idiopathic male infertility, and if diagnosed appropriately such individuals may be treated by manipulating expression of miR-382-3p in the testis. We propose that downregulating the levels of miR-382-3p in the testes of infertile men can help restore spermatogenesis in such individuals. *In vivo* knockdown of microRNAs using anti-sense oligonucleotides (antagomirs) or miRNA sponge is a promising strategy for small RNA therapy. Cell-specific delivery of the antagomirs can be achieved using viral or non-viral delivery systems in testis. Alternatively, small testicular tissue biopsy samples from infertile men can be manipulated *in vitro* to achieve such knockdown, potentially culminating into sperm production, which can then be used for *in vitro* fertilization (IVF) and embryo transfer. The use of such robust gene manipulation and delivery methods can help manage infertility/subfertility in humans. However, further studies are required to dissect the regulation of miR-382-3p expression to generate useful insights on microRNA-mediated changes in the testicular transcriptome. Given the vital functions of the genes that are directly targeted by miR-382-3p, this microRNA candidate has the potential to be studied in other cells to understand regulation of cellular fate, cellular differentiation, and cell-cell adhesion.

Conclusion

Our study showed, for the first time, a role of miR-382-3p in regulating Sc maturation and spermatogenesis. Our data suggested that the natural downregulation of miR-382-3p in Scs at the onset of puberty is essential for robust spermatogenesis, and overexpression of this microRNA in pubertal Scs resulted in impaired spermatogenic progression leading to infertility in mice. Overexpression of miR-382-3p in transgenic mice interrupted functional maturation of Scs, as evidenced by impairment of the BTB, which is made by Sc-Sc junctions and compromised expression of genes associated with Sc polarity, leading to complete infertility in the transgenic male mice. Thus, the sustained expression of miR-382-3p in post-pubertal adults may be an underlying cause of idiopathic male infertility, and overcoming this by modulating microRNA expression, *in vivo* or *in vitro*, can serve as potential therapy for male infertility.

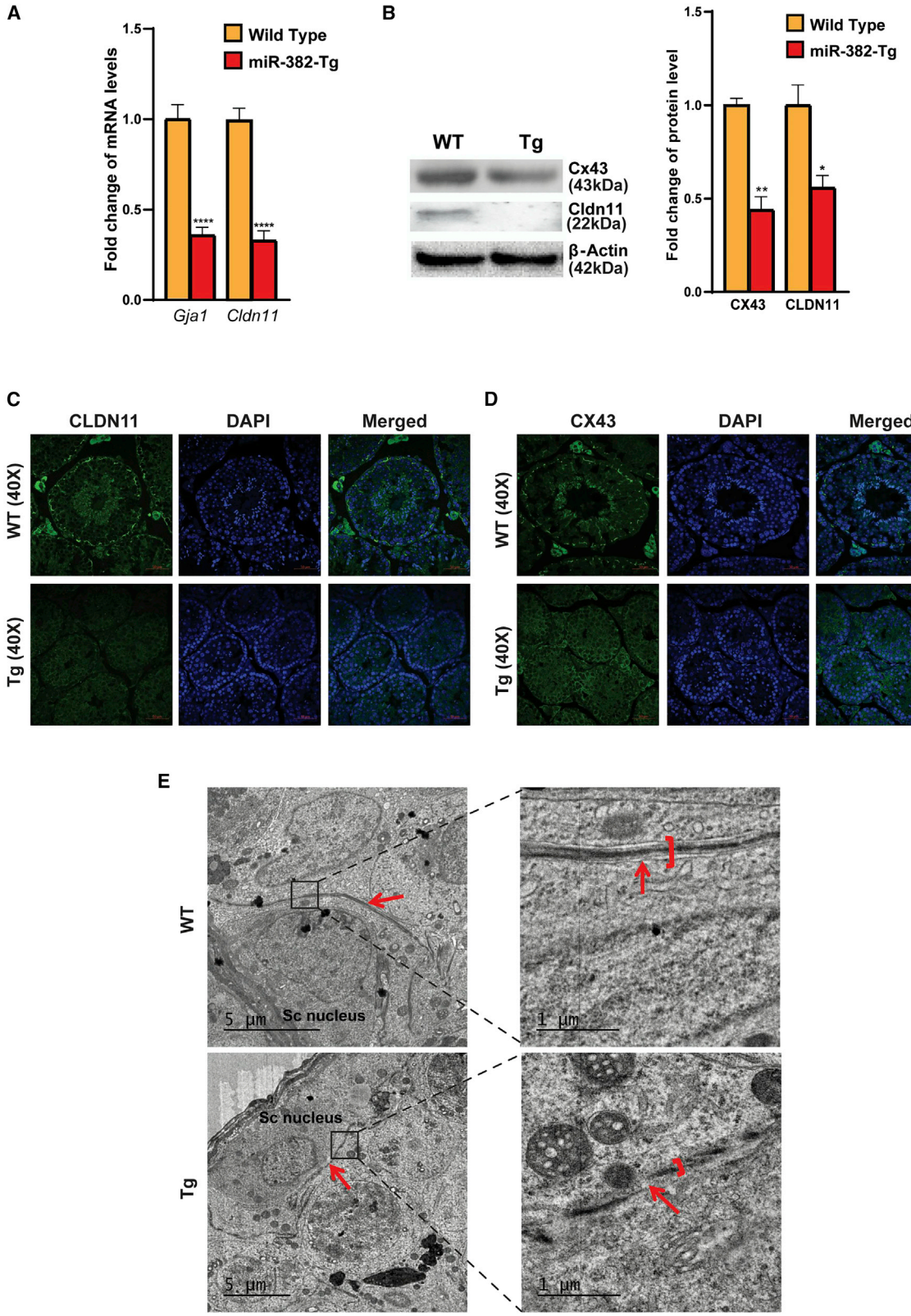
MATERIALS AND METHODS

Animals and reagents used

Wistar rats and FVB/J mice used in this study were procured from the Small Animal Facility of the National Institute of Immunology, New Delhi, India. All animals were housed and used as per the guidelines laid down by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA). All animal-related experimental protocols followed were approved by the Institutional Animal Ethics Committee (IAEC). All reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

Listing of microRNAs for differential expression analysis

A comprehensive literature study was done to select genes that are known to be important for Sc functioning. The microRNAs predicted to target these genes were listed out from online databases like miRDB, TargetScan etc. 51 microRNAs that were predicted to target 31 genes important for Sc functioning were shortlisted and taken forward for evaluation of their expression in cultured infant (5 days old) and pubertal (19 days old) rat Scs.



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Cell culture

Sc culture

Testes were obtained from 20–22 infant (5 days old) and 5–6 pubertal (19 days old) Wistar rats for Sc isolation and culture as described previously.[66,67] Briefly, the animals were euthanized by CO_2 asphyxiation, and the testes were dissected out and decapsulated using fine forceps to unpack the seminiferous tubules, which were then chopped with a sterile scalpel. These finely chopped tubules were subjected to sequential enzymatic digestion with collagenase (3 mg/20 mL, for 30–35 min), and pancreatin (3 mg/20 mL, for ~2 min). This ensured the removal of other testicular cells, namely interstitial cells and peritubular myoid cells. The cells so obtained were plated in 12-well cell culture plates at a density of 8–9 $\times$ 10^5 cells/clusters per well in DMEM/HAM's F-12 media with 1% fetal bovine serum (FBS) and were kept in a humidified cell culture incubator maintained at 34°C with 5% CO_2. After 24 h, cells were replenished with fresh serum-free media supplemented with 1% growth factor (5 μg/mL sodium selenite, 10 μg/mL insulin, 5 μg/mL transferrin, and 2.5 ng/mL epidermal growth factor). On the third day, cells were subjected to a brief hypotonic shock using 20 mM Tris-HCl dissolved in culture media to remove any contaminating germ cells. The cells were subjected to various treatments on day 4 of culture. Sc culture purity was accessed by SOX9 immunostaining along with Hoechst stain (Figure S4).

HEK293T cell culture

HEK cells were cultured in DMEM high-glucose media supplemented with 10% FBS and were maintained in humidified cell culture incubator at 37°C with 5% CO_2. All experiments were performed at 3rd or 4th passage of culture, at a seeding density of 0.05 $\times$ 10^6 in 24-well cell culture plates and transfected at ~70% cell confluency.

Plasmid cloning

Cloning of UTRs in pmirGLO

The 3′ UTRs of the predicted targets of miRNA-382-3p were obtained from the miRDB database (http://mirdb.org/). The miRNA binding site on the 3′ UTR, along with its flanking sequences (25 bp each), were taken from the miRDB database. This sequence was then synthesized as a pair of complementary single-stranded DNA oligos (Sigma-Aldrich). The 3′ UTRs were mutated for use as control plasmids, where the seed sequence binding region was replaced by T's. All oligos were designed such that upon annealing, they generated overhangs compatible with those generated by NheI (3′) and SalI (5′) digestion. The oligos were annealed and the product was checked on 4% agarose gel to ensure the annealing efficiency. The annealed insert was then ligated to the pmirGLO dual-luciferase plasmid digested with the same set of restrictions enzymes (NheI and SalI). To assist the screening of the bacterial colony harboring the desired clone, a unique restriction enzyme site (EcoRI) was introduced at the 3′ end of the UTR sequences during its design. The ligated product was transformed into *E. coli* DH5α-competent cells, and positive clones were identified by double digesting the isolated plasmid samples with EcoRI and HindIII. The plasmid was then used for transfecting HEK cells. The list of UTRs cloned is provided in Table S3.

Cloning of miR-382 overexpression plasmid in dual PEM (Rhox5) vector

The primary microRNA (pri-miRNA) sequence for Mmu-miR-382 was obtained from the miRBase database (http://www.mirbase.org/). The pri-miRNA sequence and the upstream/downstream 200 bp flanking sequences were PCR amplified from mouse genomic DNA. The forward and reverse primers were designed to have EcoRI and SalI restriction sites, respectively, to facilitate cloning in dual PEM plasmid vector double digested with the same set of enzymes. The vector and insert were ligated and transformed into *E. coli*. The colonies obtained were screened by colony PCR with specific primers on the vector backbone and the insert.

In vitro treatments

Pulsatile hormone treatment of Scs

The cultured Scs were subjected to hormone treatment on day 4 of the culture in a pulsatile manner.[68] The cells were treated together with ovine FSH (50 ng/mL o-FSH) and testosterone (10^{-7} M) for 30 min followed by 2.5 h incubation with hormone-free media for 4 cycles. The cells were harvested in TRI reagent at the end of fourth cycle for RNA isolation.

Mimic transfection in Scs

On day 4, cultured Scs were transfected in 12-well plates with Rno-miR-382-3p mimic (200 pmol) procured from Dharmacon, using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol. Scrambled mimic was used as a negative control. Briefly, the miRNA mimic and Lipofectamine were separately diluted (quick vortex) in 50 μL of Opti-MEM media and mixed together upon 5 min of incubation at room temperature (RT) to make the transfection mixture. This mixture was incubated further for 20–30 min at RT. Meanwhile, the Scs were drained of existing media and gently washed once with 1× phosphate-buffered saline (PBS) and kept in 150 μL Opti-MEM (Invitrogen) media, and the transfection mixture was added

Figure 6. MiR-382-3p overexpression in transgenic mice led to a defective BTB

(A) mRNA expression data showing testicular transcript levels of *Gja1* and *Cldn11* in miR-382-3p-overexpressing transgenic mice as compared to age-matched WT mice. (B) Representative immunoblot and its densitometric quantification to show testicular protein levels of CX43 and CLDN11 in transgenic and WT mice. β-actin was used as a loading control. (C) Representative image of immunostaining for Claudin11 on testis frozen sections (7 μm) from miR-382-3p-overexpressing transgenic mice and WT mice. Nuclei were stained with Hoechst. Images captured at 40× (scale bar, 20 μm) objective magnifications. (D) Representative image of immunostaining for Connexin43 on testis frozen sections (7 μm) from transgenic mice and age-matched WT mice. Nuclei were stained with Hoechst. Images captured at 40× (scale bar, 20 μm) objective magnifications. (E) TEM images from transgenic mouse testis and WT testis showing the BTB near the Sc nucleus (marked with red arrows and braces; scale bar, 5μm). Higher magnification (scale bar, 1μm) of marked area to show discontinuous BTB in transgenic testis as compared to an intact continuous BTB in WT controls. All values are mean ± SEM of at least 5 WT and 5 transgenic mice. Unpaired Student's t test was used for determining statistical significance. $p < 0.05$ was considered to be statistically significant.

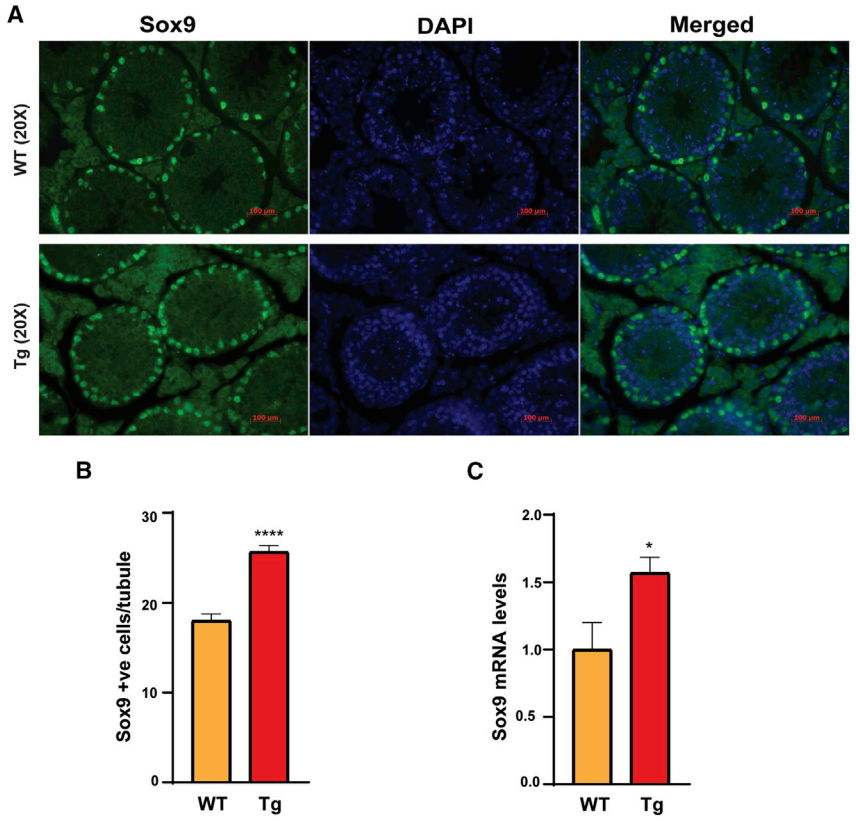

Figure 7. MiR-382-3p transgenic mice had an increased number of Scs

(A) Representative image of immunostaining for Sc-specific SOX9 on testis paraffin section (7 μm) from transgenic and WT mice. Nuclei were stained with Hoechst. Images captured at 20× (scale bar, 50 μm) objective magnifications. (B) Quantification of SOX9-positive cells per tubule in transgenic mouse testis as compared to WT testis. At least 3 different sections from each animal (each with at least 10 seminiferous tubules at 10× magnification) were analyzed to count the number of SOX9-positive cells. (C) Testicular mRNA levels of *Sox9* in transgenic and WT testis. All values are mean ± SEM of at least 5 WT and 5 transgenic mice. Unpaired Student's t test was used for determining statistical significance. $p < 0.05$ was considered to be statistically significant.

drop-wise to the Scs. The cells were then incubated at 34°C for 9 h, after which the media was changed back to DMEM/Ham's F-12 (with 1% growth factors). The cells were finally harvested in TRI reagent or as cell pellets 24 h post media change for RNA and protein isolation, respectively.

Plasmid transfection in HEK293T

HEK293T cells were cultured, and at ~70% confluency the cells were co-transfected with Rnu-miR-382-3p mimic (20 pmol) and pmirGLO dual-luciferase plasmid containing WT or mutated 3′ UTR of the target gene (500 ng), using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol. Briefly, the mimic and the plasmid were mixed in Opti-MEM and incubated to make the transfection mixture. The existing media of the cells was replaced with the transfection mixture and incubated for 6 h, after which the media was changed back to complete media (DMEM high glucose + 10% FBS). The cells were harvested at 24 h post media change, and the cell pellet was stored at −20°C for Luciferase assay.

RNA isolation and cDNA preparation

Total RNA was isolated from Scs frozen in TRI reagent (Sigma-Aldrich) using chloroform as described previously.[69] Additionally, sodium acetate (0.3 M) and glycogen (0.2 mg/mL) were added for enhanced precipitation of small RNAs. The quantity and quality (260/280) of RNA was determined using NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). 1 μg of RNA was treated with 0.5 U DNaseI (Thermo Scientific, Waltham, MA, USA) to remove any contaminating genomic DNA fragments. This was followed by single-strand cDNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, USA) as per the manufacturer's protocol.

For the cDNA synthesis of microRNAs, 1 μg RNA was treated with *E. coli* PolyA Polymerase (NEB) to generate a poly(A)-tail, followed by reverse transcription using 0.5 mg/mL oligo-dT and M-MLV reverse transcriptase. A 32 bp unique sequence was added to the 5′ end of the oligo-dT (GCGAGCACAGAATTAATACGACTCACTATAGGTTT TTTTTTTTTT), which served as a template for a universal reverse primer for the downstream qPCR analysis of all the miRNA. The entire microRNA sequence was used as the forward primer. Universal reverse primer sequence is shown in Table S2.

Quantitative real-time PCR

Quantitative real-time PCR was performed in Realplex[4] master cycler (Eppendorf, Germany) using Kapa Sybr mix as per the manufacturer's protocol. 1 μL of the cDNA preparation was taken for each reaction, along with 5 μL Kapa Sybr, 0.5 μM each primer (forward and reverse), and 3 μL nuclease-free water (NFW). Melting curve was analyzed to detect single-amplification peak, and the differential expression of genes or miRNA in terms of fold change was calculated using the $2^{(-\Delta\Delta Ct)}$ method as described previously.[70] Each reaction was set in 3 technical replicates and at least 4 biological replicates. The expression level of 18S rRNA and Let-7a were used for normalizing genes and miRNA expression level, respectively.[71–74] The list of primers used in the study has been provided in Tables S2 and S4.

Luciferase assay for target validation

The harvested HEK cells (co-transfected with miRNA mimic and pmirGLO dual-luciferase plasmid) were re-suspended in 150 μL

PBS, and 10 μL of the suspension was taken to perform luciferase assay using Dual-Glo Luciferase Assay Kit (Promega, E2920) following the manufacturer's protocol. Briefly, the luciferase assay reagent (LAR) was added to the cell suspension. The LAR comprises the lysis solution and the substrate for firefly luciferase protein. After measuring the firefly luciferase activity, a Stop and Glo solution was added, which stalled the firefly activity and induced the Renilla luciferase activity. The ratio of firefly/Renilla luciferase activity was calculated and compared between mutated and WT UTR samples for estimating the decline in firefly activity, which reflects the translational repression of the transcripts and thus the miRNA-mediated suppression. Each reaction was set in technical duplicates and at least 4 biological replicates.

Protein extraction and immunoblot analysis

Protein was extracted from Scs by resuspending the cells in RIPA Lysis buffer (G Biosciences) with 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail followed by freeze-thaw in liquid nitrogen. For the testis, the tissue was homogenized in RIPA lysis buffer (with 1× protease and phosphatase inhibitor cocktail). Protein was quantified using Bradford solution,[75] and 20–30 μg total protein was run on 10% or 12% resolving SDS-PAGE gel at 100 mV. The protein was transferred onto PVDF membranes and incubated with blocking solution (5% skimmed non-fat milk in 1× Tris buffered saline with 0.1% Tween 20 detergent or 1XTBST) for 1–2 h at room temperature. The membrane was briefly washed with 1× TBST and incubated overnight with the primary antibody dissolved in 1× TBST with or without 1% BSA. The next day the membrane was washed thrice with 1× TBST (5 min each at RT under rapid shaking condition) and incubated for 1 h with the horseradish peroxidase (HRP)-labeled secondary antibody in 1× TBST. The membrane was then developed using BioRad Clarity Western ECL substrate and imaged in a chemi-doc (ImageQuant Las 500, GE Healthcare). The protein bands obtained were quantified using ImageJ. β-actin was used as loading control. Primary antibody dilutions are listed in Table S5.

Generation of transgenic mice

Transgenic mice were generated using non-surgical testicular electroporation as described previously by our lab.[76] Briefly, the miR-382 overexpression plasmid DNA was prepared in high quantity using GenElute HP Plasmid Maxi prep (Sigma-Aldrich) and was linearized using StuI restriction enzyme that cuts the plasmid in the backbone. The linearized plasmid was purified by ethanol precipitation and quantified using NanoDrop. The integrity of the linearized DNA was verified by running it on 1% agarose gel. 15 μg of the linearized plasmid DNA at a concentration of 500 ng/μL was injected into each testis of anesthetized 30-day-old FVBJ male mice followed by electroporation (60 V for 50 ms, 4 forward + 4 reverse pulses) using tweezer electrodes (Electro Square Porator, ECM 830, BTX, Holliston, MA, USA). The fore-founder animals were housed for 30 days and then mated with age-matched WT females. The pups born (F1 generation) were screened for transgene integration using slot blot analysis (as described below). Several individuals from the F1 generation were transgenic. Infertility was confirmed in F1 generation transgenic males using experiments described below. To generate more transgenic animals, WT males and transgenic females from the F1 generation were mated to generate a F2 generation, and all experiments were performed on transgenic males from the F2 generation at around 80–90 days of age (Figure S3). A control transgenic animal was made by expressing a stem loop structured short hairpin RNA (shRNA) against bacterial LacZ to mimic the pri-microRNA structure, in the same vector backbone. LacZ mice were compared to WT mice to check for any probable effects of transgene overexpression in the animals. As none were found, WT was thereafter used as control for all experiments (Figure S4).

Genomic DNA isolation and slot blot analysis

Genomic DNA was isolated from 3–4 mm of tail snippets using salt precipitation method.[77] The isolated DNA was quantified using NanoDrop, and 1 μg of DNA of each sample was used for slot blot analysis as described previously by our lab (Usmani et al.[76]). Briefly, each genomic DNA sample was denatured at 95°C for 10 min and immobilized and cross-linked on a positively charged nylon-66 transfer membrane (MDI Membrane Technologies, Ambala Cantt, India, CL-1000 UV crosslinker, UVP). A radioactive DNA probe complementary to the reporter GFP sequence in the plasmid was synthesized by PCR amplifying the GFP coding region. The clean PCR product was denatured and incubated with αP32-CTP to produce the radioactive probe. The membrane was hybridized with a αP^{32}-CTP radiolabeled probe in a rotating chamber overnight at 60°C. The membrane was washed gently with low-stringency and high-stringency 2× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0] for 20× SSC) buffer twice for 10 min each and kept in exposure cassettes for 12–24 h, which were then scanned using phosphorimager Typhoon 9400 (GE Healthcare). Genomic DNA from WT mice was used as negative control, and 10 ng of the plasmid (which contained the probe region) was used as a positive control.

Immunohistochemistry

The freshly collected testes from WT and transgenic mice were fixed in 4% paraformaldehyde for 48 h, washed well to remove the fixative, and then used for paraffin embedding.[78,79] 7 μm sections were cut from paraffin-embedded blocks and used for immunostaining. The sections were de-paraffinized using xylene-alcohol, subjected to antigen unmasking by boiling the sections for 10 min in antigen unmasking solution (Vector Laboratories, H3300), and cooled to room temperature under tap water. The sections were permeabilized by treating them with 0.1% Triton X-100 for 5 min at room temperature followed by washing with 1× PBS thrice, for 5 min each, and then blocked with 3% BSA solution for 1 h. The sections were then incubated overnight with the primary antibody in 1% BSA at 4°C in a moist chamber. The primary antibody was removed, and sections were washed with 1× PBS to remove any residual antibody and then kept in secondary antibody solution for 3–4 h, washed again, and finally stained with 20 μg/mL Hoechst-3342 to stain the nuclei. The sections were mounted on glass slides using ProLong Gold Antifade mounting media (Life Technologies, Carlsbad, CA, USA) and viewed under a

fluorescence microscope (Nikon Eclipse TE2000-E). Primary antibody dilutions are listed in Table S5.

Fertility assessment

The fertility assessment was done by measuring parameters like gonadosomatic index, sperm count, and litter size in the transgenic and age-matched wild-type mice.[53,80–82] The body weight and testis weight for each mouse (WT or transgenic) was noted, and the gonadosomatic index (body weight/testis weight × 100) was calculated. The testis size was also observed for any visible morphological changes. Transgene-positive animals were euthanized at around 80–90 days of age along with their age-matched WT counterparts. The entire intact epididymis was surgically removed and transferred into a 1.5 mL micro-centrifuge tube containing 1 mL 1× PBS and ruptured thereafter for sperm count analysis using a hemocytometer. The litter size for each transgene-positive animal was also noted for at least 5 mating cycles with at least 3 age-matched females.

TUNEL assay

Paraffin sections of WT and transgenic testis were taken, deparaffinized using xylene, and subjected to antigen unmasking using 20 μg/mL Proteinase K solution. Apoptosis was detected by performing the TUNEL assay using Promega DeadEnd Fluorometric TUNEL System (G3250) as per the manufacturer's protocol.[83–86] Briefly, the sections were fixed in 4% paraformaldehyde for 20–30 min and then permeabilized using 0.2% Triton X-100 in 1× PBS for 5 min. The sections were then equilibrated and incubated with the enzyme mix containing the fluorophore. The enzyme reaction was stopped using 2× SSC buffer, and the sections were stained with 20 μg/mL Hoechst for 5 min, washed, and finally preserved using antifade (ProLong Gold Antifade, Invitrogen). Slides were viewed under a fluorescence microscope (Nikon Eclipse TE2000-E) to detect fluorescent TUNEL-positive cells.

Electron microscopy

Whole testes from WT and transgene-positive animals were fixed in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M phosphate buffer solution for 6–8 h and cut into small pieces of approximately 2 mm × 2 mm dimension. These pieces were washed once with 1× PBS and incubated for 1 h in osmium tetroxide for secondary fixation. The tissue was dehydrated using ethanol and infiltrated using epoxy resin, which was allowed to settle and polymerize at 60°C overnight. Ultrathin sections of approximately 70 nm were cut at Sophisticated Analytical Instrumentation Facility, All India Institute of Medical Science, New Delhi, India. TEM imaging was done using Tecnai G2 20 Twin at National Institute of Immunology, New Delhi, India.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.2 software. Data (mean ± SEM) from at least three independent experiments were used for calculating statistical significance. Details of the statistical tests are provided in the figure legends. p value < 0.05 was considered to be statistically significant, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.07.001.

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AUTHOR CONTRIBUTIONS

A.G., K.M., and S.S.M. designed the study. A.G. performed most experiments with help from P.S. R.S. A.G., K.M., and S.S.M. analyzed the experimental data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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