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RESEARCH ARTICLE

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Sertoli cell specific decline in NOR-1 leads to germ cell apoptosis and reduced fertility

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Abstract

The somatic component of seminiferous epithelium, the Sertoli cells (Sc) respond to Follicle stimulating hormone (FSH), and Testosterone (T) to produce factors which are necessary for germ cell (Gc) survival and differentiation. Infant Sc do not support spermatogenesis in spite of sufficient hormonal milieu, a situation similar to that found in male idiopathic infertility. Sc maturation during pubertal period involves expression of some genes which may be important for initiation of spermatogenesis. Analysis of differentially expressed genes, one by one, in infant and pubertal Sc might provide useful information about the regulation of spermatogenesis. DNA microarray based analysis of mRNA from 5-days (infant) and 12-days (pubertal) old rat Sc revealed increased expression of *Nor-1* by pubertal Sc. NOR-1 is an orphan nuclear receptor involved in maintaining cellular homeostasis and disease. We generated transgenic mice using shRNA cloned under Pem (Rhox5) promoter which is activated at puberty in Sc. Such transgenic mice had reduced *Nor-1* expression and increased *Tgfβ1, Tgfβ3,* and Smad3 expression. Moreover, an increase in β-catenin expression was observed in NOR-1 knockdown testes. High β-catenin in such transgenic mice was found to be associated with disruption of Sc maturation characterized by elevated expression of Anti Mullerian hormone, Cytokeratin 18, and Sox9. This disruption of Sc maturation resulted in Gc apoptosis. Such NOR-1 knockdown mice showed reduced sperm count and litter size. We report for the first time that NOR-1 plays a crucial role in regulating sperm count and male fertility.

KEYWORDS

β-catenin, germ cell apoptosis, idiopathic male infertility, NOR-1, sertoli cell, subfertility transgenic mice

1 | INTRODUCTION

The Somatic cells of the seminiferous tubules of the testis-Sertoli cells (Sc), play a major role in regulation of spermatogenesis. These cells respond to paracrine and endocrine stimuli to regulate germ cell proliferation and differentiation.[1,2] During infancy, Sc proliferate in response to FSH, and are incapable of supporting robust germ cell (Gc) differentiation. At the onset of puberty, the Sc undergo functional maturation and gain the ability to nurse the Gc for their division and differentiation.[3] Previously, our lab and other groups have shown that in the process of gaining maturity, Sc undergo developmental switch in hormonal response, mainly to testosterone (T), and FSH.[4–8] Any aberration in the expression pattern of genes involved in the

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process of Sc maturation may hamper the functional maturation of Sc leading to abnormal division and differentiation of Gc thus affecting sperm production.

In primates and rodents, in spite of the presence of adequate concentration of reproductive hormones (FSH and T) during infancy, Sc fail to initiate robust Gc differentiation displaying lack of spermatogenesis in infants.[5] This situation bears a resemblance to idiopathic male infertility where Sc fail to initiate spermatogenesis even after adequate hormonal supplementation. It is estimated that about 40-50% of all infertile men suffer from idiopathic infertility.[9] Even after exposure to number of medical therapies, including hormone supplementation, their chances of getting fertile are doubtful.[10]

Studies to delineate functions of such differentially expressed genes would allow us to determine their role in functional maturation of Sc which is prerequisite to onset of spermatogenesis. Orphan nuclear receptors are a class of receptors which have been reported to play an important role in regulating signal transduction and cellular homeostasis.[11] NR4A subfamily of nuclear receptors are considered to be true orphan nuclear receptors as their ligand binding pocket does not have a cavity for ligand binding.[12] Members of NR4A subfamily are known regulators of apoptosis, cell proliferation, and differentiation.[13] However, the role NR4A nuclear receptor subfamily in regulation of spermatogenesis is not well defined.

Analysis of transcriptomic data previously generated in our lab revealed the differential expression NR4A subfamily of orphan nuclear receptor genes between infant and pubertal rodent Sc. Nuclear orphan receptor 1 (*Nor-1*) which belongs to the NR4A family was found to be up regulated in pubertal rat Sc as compared to infant rat Sc, indicative of a possible role of this gene in regulation of Sc maturation.

In the present study, we have assessed the role of NOR-1 in regulation of male fertility using a transgenic mouse model. Sertoli cell specific knock down of NOR-1 led to enhanced *Smad3* expression and enhanced expression of beta-catenin. NOR-1 knockdown mice showed compromised spermatogenesis as evident by germ cell apoptosis and decline in seminiferous tubule diameter. Assessment of epididymal sperm count revealed a significant decline in the number of spermatozoa in NOR-1 knockdown animals. Our results demonstrate for the first time, a critical role of NOR-1 in regulation of Sertoli cell maturation and male fertility.

2 | MATERIALS AND METHODS

2.1 | Animals

All experimental animals were kept and used as per the National guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA), Government of India. Protocols for the experiments were approved by the Institutional Animal Ethics Committee (IAEC), National Institute of Immunology constituted by CPCSEA.

2.2 | Isolation of sertoli cells

Testes were isolated from 8 to 12 mice of 5 days post partum age and 6-8 mice of 20 days postpartum age for Sc culture, as per protocol adapted from Welsh and Wiebe[14] with minor modifications as previously described by us.[15,16] After the isolation of Sc, culturable Sc clusters($1\text{-}2 \times 10^4$) were plated in 24-well plate and cultured in DMEM/nutrient mixture F-12 Ham (DMEM/F12 HAM) containing 1% FCS for 24 h in a humidified 5% CO_2 incubator at 34°C. Next day ie, day 2 of culture, cells were washed thrice with pre-warmed media (DMEM/F12 HAM) and were cultured further in media containing 5 μg/mL Sodium Selenite, 10 μg/mL Insulin, 5 μg/mL Transferrin, and 2.5 ng/mL Epidermal Growth Factor. On day 3 of culture, residual Gc, if any, were removed by hypotonic shock by incubating Sc with 20 mM Tris-HCl, pH 7.4 for 3-5 min at 34°C.[17] The cells were then washed thrice with pre-warmed media to remove dead Gc and the culture was continued in media with growth factor.

2.3 | Hormone treatment

On day 4, 24 h after hypotonic shock, cells were treated with ovine FSH (50 ng/mL) and T (10^{-7} M) in an intermittent manner to mimic in vivo hormonal pulsatility, 30 min of incubation with hormone alternating with 150 min of incubation with DMEM/HAM-F12 and serum replacement factors.[18] This pulsatile treatment was continued for 12 h. The culture (for each age group) was terminated by removing the media and treating the cells with TRI reagent (Sigma-Aldrich, St. Louis, MO). Samples were stored at −80°C and further used for mRNA extraction. The purity of Sc culture was assessed as previously described by us.[5]

2.4 | Quantitative RT PCR

All RNA extractions were done using TRI reagent according to manufacturer's recommendation (Sigma-Aldrich) and treated with DNaseI (Life Technologies, Carlsbad, CA) as per manufacturer's protocol. A total of 1 μg RNA was used to synthesize cDNA using MMLV reverse transcriptase kit (Eurogentec, Liege, Belgium) and oligo dT primer (Promega, Madison, WI). The first strand of cDNA was used for qPCR using MESA GREEN qPCR MasterMix Plus (Eurogentec) as per manufacturer's protocol. Realplex^S (Eppendorf, Hamburg, Germany) thermal cycler machine was used to run the qPCR reaction. A reaction mix of 10 μL total volume was prepared which included 1 μL of cDNA, and 5 μL of

TABLE 1 List of q-PCR primers used in this study

Gene	Accession No	Forward Primer	Reverse Primer	Tm (°C)	Amplicon length (bp)
Nor-1	NM_015743.3	CAGCAGCTGCGAACTCAA	GGAGGGAGGAGGAATGGA	60	169
Tgfβ3	NM_009368.3	GGACTTCGGCCACATCAAGAA	TAGGGGACGTGGGTCATCAC	60	111
Tgfβ1	NM_011577.2	TGTACGGCAGTGGCTGAAC	GCTGATCCCGTTGATTTCCA	60	112
Smad3	NM_016769.4	CACGCAGAACGTGAACACC	GGCAGTAGATAACGTGAGGGA	60	101
β catenin	NM_001165902.1	TGGACTGCCTGTTGTGG	GGTGTCCTGATGTGCTCG	60	175
Axin2	NM_015732.4	AGACCGGTCACAGGATG	GTCCTGGGTAAATGGGTGAG	60	205
Pitx2	NM_011098.4	GGTCGAGTTCACGGACTCTC	TGTCTGGGTAGCGGTTTCTC	60	233
Connexin43	NM_010288.3	GAGGGAAGTACCCAACAGC	TGGGCACCTCTCTTTCAC	60	151
AMH	NM_007445.2	CAGAACCTCTGCCCTACTCG	CACCTTCTCTGCTTGGTTGA	60	115
Sox9	NM_011448.4	AGTACCCGCATCTGCACAAC	TGTAATCGGGGTGGTCTTTC	60	141
Cytokeratin 18	NM_010664.2	GAGGGCTCAGATCTTTGCGA	CATGGATGTCGCTCTCCACA	60	143
Cyclophilin A	NM_008907.1	ATGGTCAACCCCACCGTGT	TCTGCTGTCTTTGGAACTTTGTCT	60	101

2× reaction buffer (dNTPs, Meteor Taq DNA polymerase, $MgCl_2$, SYBR Green I) and 0.5 μL of each 10 mM primer. PCR primer for Cyclophilin A was used as a reference gene. The qPCR analysis was initiated with the melting of cDNA at 95°C for 7 min. This was followed by 40 amplification cycles (95°C for 30 s, 60°C for 30 s, and 72°C for 30 s). Melt curve analysis was also performed to ensure that there was only one amplicon specific peak. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression.[19] Mean (± SEM) data representing at least three separate experiments were represented as histograms for graphical analyses. Primer sequences and details are given in Table 1.

2.5 | Generation of transgene cassette

Construct was designed to express shRNA to perturb the expression of NOR-1 in pubertal Sc of mice using online tools available at Whitehead Institute (http://sirna.wi.mit.edu/), Cold Spring Harbour labs (http://katahdin.mssm.edu/siRNA/RNAi.cgi?type=shRNA), and Clontech, Los Angeles, (https://www.clontech.com/US/Support/xxclt_onlineToolsLoad.jsp?citemId=http://bioinfo.clontech.com/rnaidesigner/oligoDesigner.do?overhangs=on&restrictionSite=on&section=16260&xxheight=750). shRNA sequences were verified for target specificity BLAST analysis (http://www.ebi.ac.uk/Tools/sss/ncbiblast/). A minimal PolIII terminator sequence was included at 3′ end of shRNA to facilitate transcription of exact shRNA sequence. A restriction site of AgeI enzyme was added at the end of PolII terminator sequence of the shRNA. LacZ shRNA was also designed for control experiments. Sequences of shRNA designed are given in Table 2. Designed shRNA were synthesized from Sigma-Aldrich and were cloned under Pem promoter. Pem is a PolII promoter which is active specifically in mice Sc after 9 days of age and onwards.[20–22]

2.6 | Generation of transgenic mice

The transgenic cassette was linearized with StuI enzyme and injected into the testes of male mice using in vivo electroporation technique established in our lab.[23] The electroporated animals were cohabitated with wild-type females after 35 days post-electroporation. Tail snips of the pups generated were collected and genomic DNA was isolated. Slot Blot technique was used to detect the presence of transgene. Slot positive animals were used for further analysis.

2.7 | Isolation of genomic DNA and slot blot analysis

The progeny sired by male mice electroporated with the shRNA cassette were screened for the presence of transgene

TABLE 2 Sequence of ShRNA used

Gene	shRNA sequences used for the study
Nor-1	AATTC GAAGAGGGTCGCGAGCATGG CTTCAA GAGA GCC ATGCTCGCGACCCTCTT CTTTTTTACCGGTG
LacZ	GATCTGCATCGAGCTGGATAATAATTCAAGAGATTATTA TCCAGCTCGATGCTTTTTTACCGGTG

using Slot blot analysis.[24] Genomic DNA was isolated from tail snips of pups using already published protocol.[25] Slot blot probes were labelled radioactively with α ^{32}P dCTP using High Prime DNA labelling kit from Roche, Basel, Switzerland, following the manufacturer's instructions. For slot blot analysis 2 μg of gDNA (isolated from the tail snips) was denatured at 95°C for 10 min and snap chilled on ice. It was then blotted on to (+) Nylon 66 Transfer membrane (Advanced Microdevices, Pvt. Ltd, India) using a slot blot apparatus.[26] The membrane was then cross-linked under UV light at 12×10^4 μJ/cm^2 energy in a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA). The cross-linked membrane was pre-hybridized using hybrisol (Merck, Kenilworth, NJ) at 42°C for 4 h in a hybridization oven (Amersham, UK). The labelled probe was first denatured at 95°C for 10 min, snap chilled, and then added to the reaction. Hybridization was carried out at 60°C overnight. The blots were washed twice for 5 min each at room temperature using Wash solution I (2X saline sodium citrate buffer and 0.1% SDS) followed by pre warmed Wash solution II (0.1× saline sodium citrate buffer and 0.1% SDS) for 10 min. The hybridization signals were detected by autoradiography.

2.8 | Immunoblot analysis

All protein extractions were done using RIPA Lysis and Extraction Buffer (G-Biosciences, St. Louis, MO) with 1X Protease inhibitor cocktail (Amresco, Solon, OH). Protein was estimated using BCA protein assay (G-Biosciences) following the protocol mentioned by the manufacturer. These proteins were resolved by SDS-PAGE. Molecular weight rainbow marker was also run in one of the lanes, as reference. Resolved proteins were then electro-transferred to PVDF membrane (Advanced Microdevices Pvt Ltd, India). The membranes were blocked with 5% BSA in PBST (0.5% Tween 20) overnight at 4°C. For detection of β-catenin, mouse monoclonal anti-Catenin beta antibody (Thermo Fisher Scientific, Waltham, MA) diluted with 2% blocking solution was used. For detection of AMH, goat polyclonal anti-AMH antibody (Santa Cruz Biotechnology, Dallas, TX) diluted with 2% blocking solution was used. Membranes were exposed to primary antibody for 12 h at 4°C. Anti-mouse (Pierce 31430, Thermo Fisher Scientific) and anti-goat IgG (Pierce 31402, Thermo Fisher Scientific) were used as secondary antibodies for β-catenin and AMH respectively. β actin blotting was used to check equal loading in each lane. The list of antibodies used are given in Table 3.

2.9 | TUNEL assay

Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) was performed using The DeadEnd™ Colorimetric TUNEL System (Promega). The testicular sections of wild-type and transgenic mice were deparaffinised by immersing the slides in xylene I and Xylene II (20 min each) followed by xylene-alchohol (10 min). The slides were washed by immersing the slides in series of alcohol (100%, 95%, 85%, 70%, and 50%) for 3 min in each alcohol solution. After that the slides were immersed in 0.85% NaCl for 5 min followed by PBS wash. The sections were fixed in 4% PFA for 15 min at RT. This was followed by two PBS washes of 5 min each. 10 μg/mL Proteinase K solution was prepared by diluting 20 mg/mL stock solution in PBS. The sections were incubated with 100 uL of 10 μg/mL Proteinase K solution for 5 min. The slides were washed with PBS for 5 min at RT. The sections were refixed with 4% PFA for 5 min followed by two PBS washes (5 min each). The sections were covered with 100 uL of Equlibration buffer for 5-10 min. While the sections were incubated with Equilibration buffer, the rTdT reaction mixture was prepared. Equlibration buffer (98 μL), Biotinylated nucleotide mix (1 μL), and rTdT enzyme (1 μL) were mixed together. 100 μL of this rTdT reaction mixture was added to each slide after removing the equilibration buffer. The slides were then kept at 37°C for 60 min in a humidified chamber for end labeling reaction to proceed. The reaction was terminated by immersing the slides in 2× Saline Sodium Citrate for 15 min at RT. The slides were again washed twice with PBS (5 min each) in order to remove unincorporated biotinylated nucleotides. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide for 3-5 min. The slides were washed twice with PBS (5 min each). Streptavidin HRP was diluted with PBS (1:500) and

TABLE 3 List of primary antibodies used

Gene	Catalogue No.	Type	Company
Nor1	ab92777	Rabbit polyclonal	Abcam
βcatenin	MA1-301	Mouse monoclonal	Thermo Fisher Scientific
AMH	sc6886	Goat polyclonal	SCBT
βactin	4967L	Rabbit polyclonal	CST, Danvers, MA
GFP	632381	Mouse monoclonal	Clontech
Vasa	ab13840	Rabbit polyclonal	Abcam

100 μL was added to each slide. The slides were incubated for 30 min at RT. This was followed by washing the slides with PBS (twice; 5 min each). DAB components were combined just prior to use. Five microliter of DAB substrate 20× Buffer was added to 95 μL of deionized water. 5 μL of the DAB 20X Chromogen and 5 μL of Hydrogen Peroxide 20X was added to the above solution. Add 100 μL of DAB solution was added to each slide and incubated until a light brown colored background developed. The slides were rinsed several times with deionized water followed by counterstaining with Haematoxylin. The sections were mounted in ProLong Gold antifade reagent (Life Technologies) and observed under light microscope. Number of apoptotic germ cells were recorded from ten random fields of the testicular sections of wild-type and transgenic mice. The average number of apoptotic germ cells per field were calculated. This was repeated for three wild-type and three transgenic mice.

2.10 | Histology

Testes obtained from wild type and transgenic mice were dissected out and fixed in 10% formalin solution for 18 h at 4°C. After fixation, the tissues were embedded in paraffin and blocks were made using the embedding centre. Sections (5 μm thick) were cut with a Reichert Jung microtome 1640 and transferred onto albumin-coated slides. Sections were stained with Hematoxylin and Eosin in order to assess the status of spermatogenesis.

2.11 | Immunohistochemistry

The testicular sections of wild-type and transgenic mice were deparaffinised by keeping them in xylene, twice for 20 min each followed by xylene-alcohol (in 1:1 ratio) for 10 min. Exposure to series of ethanol (100%-70%-50%) was then given to the sections, holding at each solvent for 10 min. After this, the sections were hydrated with distilled water. Antigen unmasking solution (H3300, Vector Labs, Inc., Burlingame, CA) was used to carry out antigen retrieval. 15 mL antigen unmasking solution was added to 1.6 L of distilled water and the sections were immersed in it. The antigen retrieval was carried out by boiling the solution for 10 min. After this, the sections were kept in deionized water followed by washing in 1X PBS (pH 7.4; HIMEDIA), three times for 5 min each. Three washes of 1X PBST (1X PBS, 1% Triton X 100), 5 min each, were given, for cell permeabilization. The sections were blocked with 2% goat serum (S1000, Vector Lab, Inc.). The sections were then incubated with primary antibody diluted with 1X PBS and kept in a moist chamber for 12-16 h. The sections were incubated with secondary antibody diluted with 1× PBS and kept for 2-4 h at RT. This was followed by washing of sections with 1× PBS, three times for 5 min each. Slides were stained with Hoechst for 5 min followed by three washes of 1× PBS. The sections were mounted in ProLong Gold antifade reagent (Life Technologies) and were observed under fluorescence microscope (Nikon, Tokyo, Japan) with suitable filters. In order to stain GFP protein, mouse monoclonal anti-GFP antibody (Clontech) diluted with 1× PBS (1:250) was used as primary antibody and goat anti-mouse IgG conjugated with Alexa fluor 488 (Molecular Probes, Invitrogen, Carlsbad, CA) diluted with 1× PBS (1:500) was used as secondary antibody. In order to stain β-catenin protein, mouse monoclonal anti-Catenin beta (Thermo Fisher Scientific) diluted with 1× PBS (1:250) was used as primary antibody, and goat anti-mouse conjugated with Alexa flour 488 (Molecular Probes, Invitrogen) diluted with 1X PBS (1:500) was used as secondary antibody. In order to stain Vasa protein, rabbit polyclonal DDX-4 (Abcam, Cambridge, UK) diluted with 1X PBS (1:250) was used as primary antibody and goat anti rabbit conjugated with Alexa flour 546 (Molecular Probes, Invitrogen) diluted with 1× PBS (1:500) was used as secondary antibody. The list of antibodies used in this study is given in Table 3.

2.12 | Testicular weight, sperm count, and fertility assessment

Testes weight of wild-type, and transgenic male mice were recorded. The mice were sacrificed by cervical dislocation and testes were dissected out and weighed. The number of epididymal sperm was analyzed. Both the epididymis were dissected out and kept in 1 mL of 1× PBS. Whole epididymis was punctured at several sites and shaken in petri dish containing PBS in order to release the sperm. Punctured epididymis were kept at 37°C for 10-20 min. This facilitated the release of sperms.[27,28] Total numbers of sperm were then counted using a Hemocytometer (Polyoptics GmBH, Kleve, Germany). Transgenic mice were bred with wild-type females. Pups obtained from at least two different females were recorded for each of the transgenic mice.

2.13 | Data analysis

Data were plotted using statistical tool, GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA). Data were generated from at least three different transgenic animals (biological triplicates) and three different sets of cell cultures. Histograms represented data as Mean ± SEM. Unpaired Student's *t*-test was used to calculate significance variance among the result values. P-values < 0.05 were considered as statistically significant

3 | RESULTS

3.1 | Differential expression of *Nor-1* in infant and pubertal mice Sc

The microarray data of differentially expressed genes from infant and pubertal Sc of rat was previously generated in our lab (Accession no: GSE48795). We found that expression of Nuclear orphan receptor 1 (*Nor-1*) was up regulated in pubertal rat Sc as compared to infant rat Sc. Since the functional studies pertaining to the *Nor-1* were planned to be undertaken using transgenic mice, the expression pattern of *Nor-1* (selected from rat microarray) was checked in mice Sc. The mRNA expression level of *Nor-1* was found to be significantly higher ($P < 0.05$) in pubertal (20-day-old) mice Sc as compared to infant (5-day-old) mice Sc (Figure 1A).

3.2 | Generation and analysis of NOR-1 knockdown mice

In order to study the role of NOR-1 in spermatogenesis, transgenic mice were generated by selectively knocking down the expression of NOR-1 in pubertal Sc as described by us previously.[23] The transgene cassette expressing shRNA targeted to *Nor-1* was driven by Pem promoter which is active specifically in Sc from puberty onwards.[20–22] The expression of *Nor-1* shRNA was restricted to Sc at and after puberty. The progeny sired by *Nor-1* shRNA bearing males were screened for the presence of transgene integration using a radio labelled probe against GFP (Figure 1B) by slot blot analysis (Figure 1C). Control transgenic animals expressing shRNA targeted to LacZ were also generated using similar strategy (Supplementary Figure S1A). Such male progeny which were positive for the presence of transgene are henceforth referred to as LacZ KD.

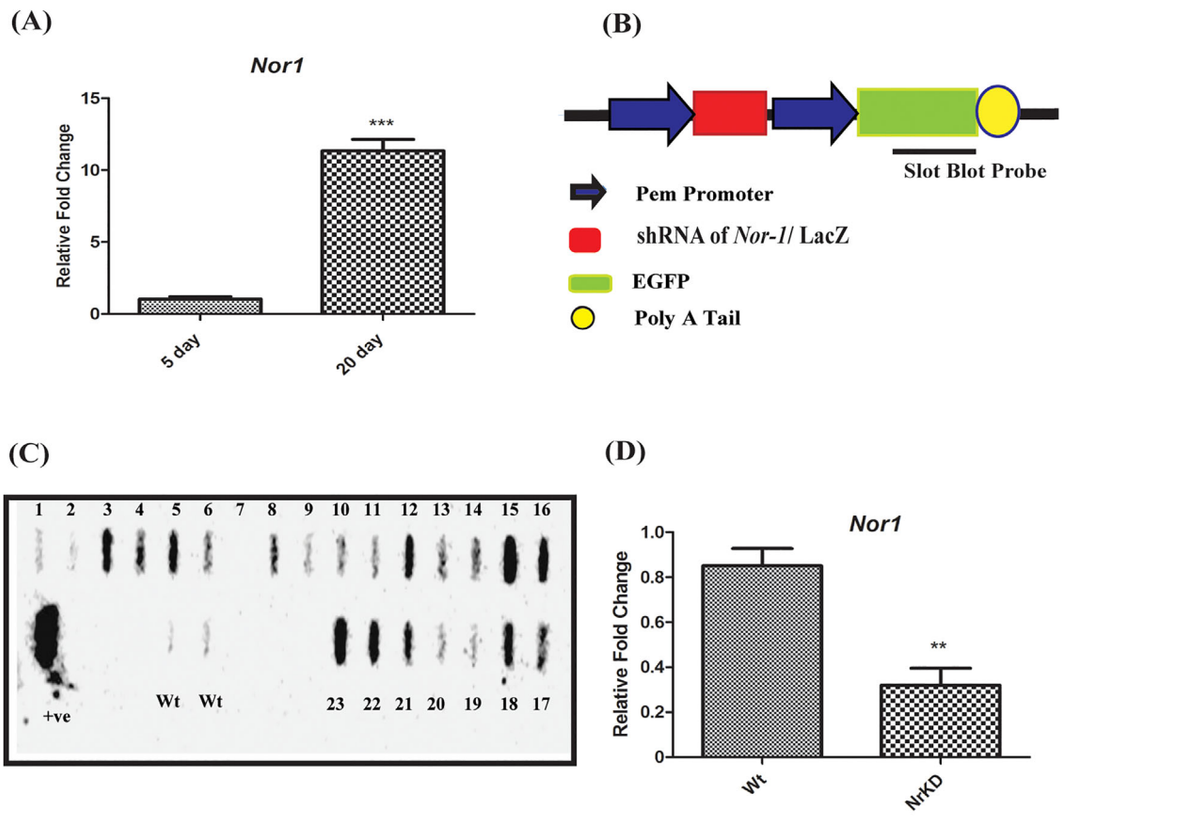

FIGURE 1 Validation of *Nor-1* expression in Sc and generation of transgenic mice with perturbed *Nor-1* expression in pubertal Sc. A, The relative mRNA expression of *Nor-1* was higher in 20-days cultured mice Sc as compared to 5-day mice Sc as shown by quantitative real-time PCR. Histogram depicts mean ± SEM, $n = 3$. ***indicates $P < 0.001$ by unpaired Student's *t*-test. B, Construct bearing *Nor-1* or *LacZ* shRNA. Pem promoter was used to drive expression of shRNA and EGFP. Radio-labeled probe for slot blot was generated from construct as shown in diagram. C, Genotyping of progeny sired by *Nor-1* shRNA bearing mice using slot blot analysis. Slot 1-23 represents the gDNA isolated from tail snips of the pups. Wt indicated gDNA isolated from wild type male mice. +ve indicates positive control (Dual Pem-EGFP cassette bearing plasmid). A single representative slot blot is shown. D, The relative mRNA levels of *Nor-1* were reduced in testicular tissue of transgenic mice (NrKD) as compared to that of wild type (Wt). Sixty-day old mice were used for the study. Histogram depicts mean ± SEM, $n = 3$. **indicates $P < 0.01$ by unpaired Student's *t*-test

Levels of *Nor-1* mRNA were found to be significantly ($P < 0.01$) lower in case of the transgenic mice as compared to wild type mice (Figure 1D). Approximately 60-70% decrease in mRNA levels of *Nor-1* was observed in these transgenic mice. These *Nor-1* knockdown transgenic animals are henceforth referred to as NrKD. No significant change in the levels of *Nr4a1* and *Nr4a2* in transgenic mice was observed when compared with age matched wild-type control, thus confirming the specificity of the shRNA (Supplementary Figure S2).

Immunohistochemistry of testicular sections of NrKD mice showed Sc specific GFP staining since *Egfp* was used as a marker. Such Sc specific GFP expression was absent in the testicular sections of age matched wild-type mice (Supplementary Figure S3A and B). This further confirmed the presence of transgene and its expression specifically in Sc. There was no significant change in the levels of *Nor-1* mRNA expression in testicular tissue of wild-type and LacZ KD mice (Supplementary Figure S1B). The epididymal sperm count of LacZ KD mice was also found to be similar to age matched wild-type mice (Supplementary Figure S1C). Hematoxylin and Eosin staining of the testicular sections of LacZ KD mice showed no change in tissue architecture as compared to wild type mice (Supplementary Figure S1D and S1E). Therefore, for further studies, we used wild-type mice as control.

3.3 | Expression levels of *Tgfβ3*, *tgfβ 1*, and *Smad3* in NOR-1 knockdown mice

NR4A family of nuclear receptors, which include NOR-1 negatively regulate the expression of Tgfβ3 and Smad3.[29] In accordance with this report, we found significant increase ($P < 0.001$) in the mRNA levels of *Tgfβ3* in case of NrKD mice as compared to age matched wild-type mice (Figure 2A). Interstingly, we found that the expression of *Tgfβ1* was also enhanced in NrKD mice as compared to age matched wild type controls, although the increase in *Tgfβ1* was not as high as that of *Tgfβ3* (Figure 2B). The mRNA levels of *Smad3* were also elevated significantly ($P < 0.05$) in case of NrKD mice as compared to the age matched wild type control animals (Figure 2C). The increased transcripts levels of *Smad3 Tgfβ3* and *Tgfβ1* in NrKD mice confirmed the downstream effects of knocking down NOR-1 in these animals.

3.4 | Levels of β-catenin in NOR-1 knockdown mice

The signalling components of Tgf-β and Wnt/β-catenin pathways interact at various levels and control several developmental processes.[30,31] Tgf-β and Smad molecules are reported to increase the protein stability and nuclear accumulation of β-catenin.[32–34]

Nuclear receptors (like NOR-1) are also known to regulate the expression of β-catenin at both mRNA and protein levels.[35] In line with the published literature, NrKD mice showing higher transcript levels of both *Tgfβ3*, *Tgfβ1*, *and Smad3*, showed significantly higher ($P < 0.01$) mRNA expression of *β-catenin* compared to age matched wild type control animals (Figure 3A). This was also confirmed at protein level by immunoblot analysis (Figure 3B). Immunostaining studies on the testicular sections further confirmed the higher abundance of β-catenin in the testes of NrKD mice as compared to age matched wild-type control (Figures 3F and 3G, Supplementary Figure S4). The expression levels of β-catenin target genes like *Axin2, Pitx2*, and *Gja1* were also assessed and found to be higher in case of of NrKD mice as compared to age matched wild type controls (Figure 3C-E).

3.5 | NOR-1 mediated regulation of sertoli cell maturation

Constitutively activated β-catenin in pubertal murine Sc interfers with its maturation.[36] Adult Sc with activated

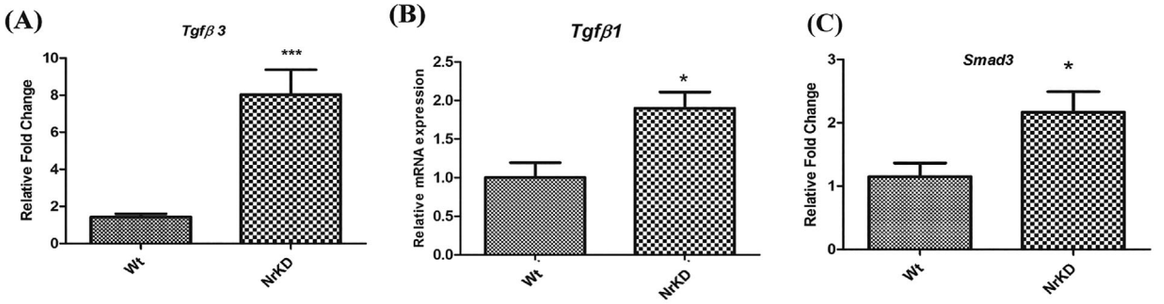

FIGURE 2 Expression of *Tgfβ3, Tgfβ1*, and *Smad3* in NOR-1 knockdown mice. The relative mRNA levels of (A) *Tgfβ3*, (B) *Tgfβ1*, and (C) *Smad3* were higher in testicular tissue of NrKD mice as compared to that of age matched wild-type control (Wt). Histogram depicts mean ± SEM, $n = 3$. ***indicates $P < 0.001$ by unpaired Student's *t*-test. *indicates $P < 0.05$ by unpaired Student's *t*-test. Sixty-day old mice were used for the study

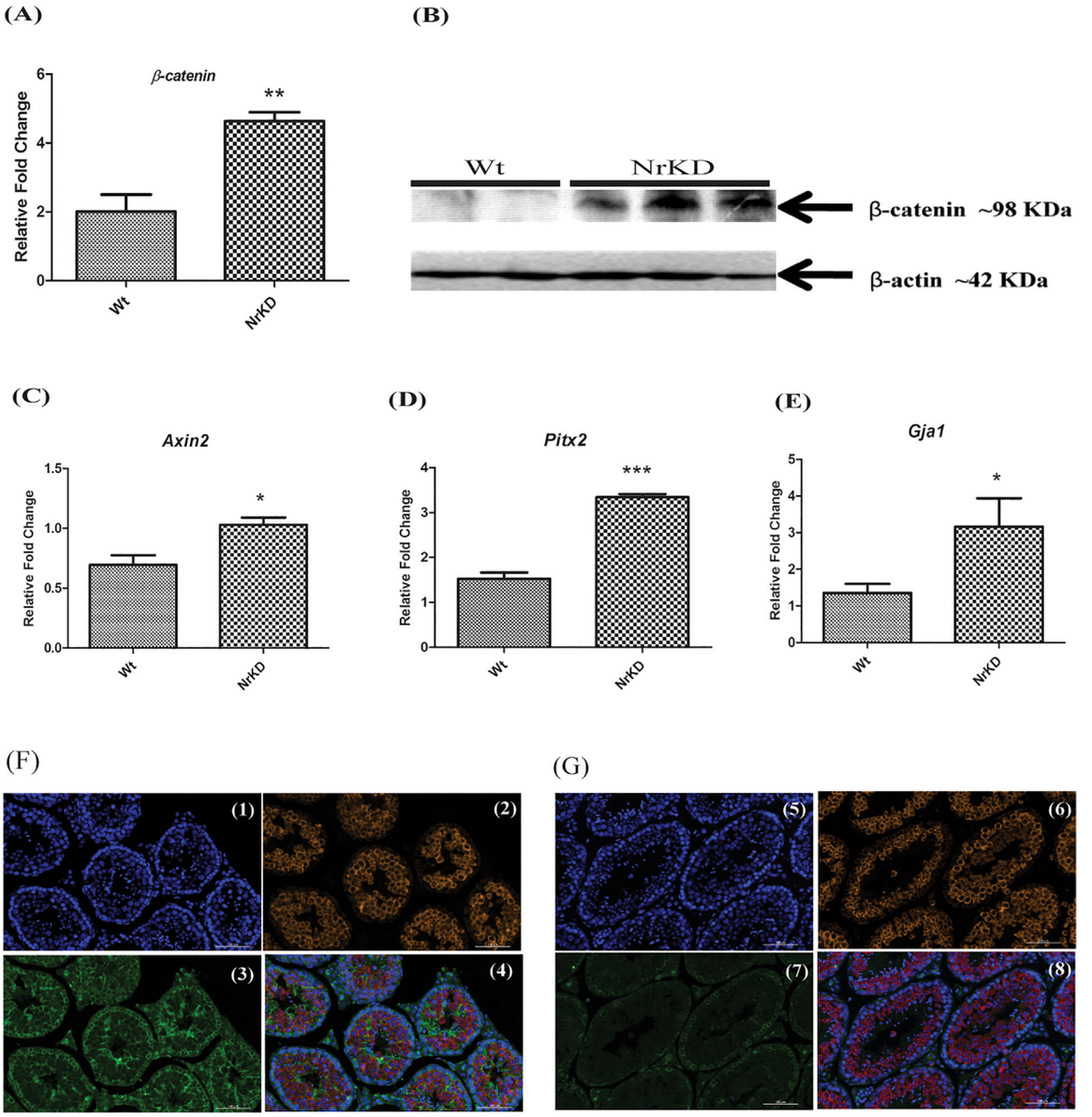

FIGURE 3 Effects of NOR-1 knockdown on β-catenin expression. A, The relative mRNA levels of *β-catenin* were higher in NrKD mice as compared to wild-type (Wt). Histogram depicts mean ± SEM, $n = 3$. **indicates $P < 0.01$ by unpaired Student's *t*-test. B, Immunoblot analysis showed higher β-catenin protein expression in NrKD mice as compared to wild type (Wt) mice. β-actin was used as loading control. A single representative blot is shown. (C-E) The relative mRNA levels of β-catenin targets like (C) *Axin2*, (D) *Pitx2,* and (E) *Gja1* were higher in NrKD mice as compared to wild type (Wt). RNA and protein isolation were performed from testicular tissue of adult wild type and NrKD mice. Histogram depicts mean ± SEM, $n = 3$. ***indicates $P < 0.001$ by unpaired Student's *t*-test. *indicates $P < 0.05$ by unpaired Student's *t*-test. (F&G) Immunohistochemical detection of β-catenin in testicular sections of (F) NrKD mice and (G) wild type mice. (1,5) Shows Sc and Gc nuclei stained with Hoechst as observed under Blue/cyan filter, (2,6) Shows Gc cytoplasm stained with anti-Vasa antibody as observed under TRITC filter, (3,7) Shows cells stained with anti-β-catenin antibody as observed under FITC filter, (4,8) Shows the merged image of NrKD and wild type testicular sections respectively. Testicular section of NrKD mice shows higher expression of β-catenin as compared to that of wild-type. Scale bar represents 100 μm. Sixty-day old mice were used for the study

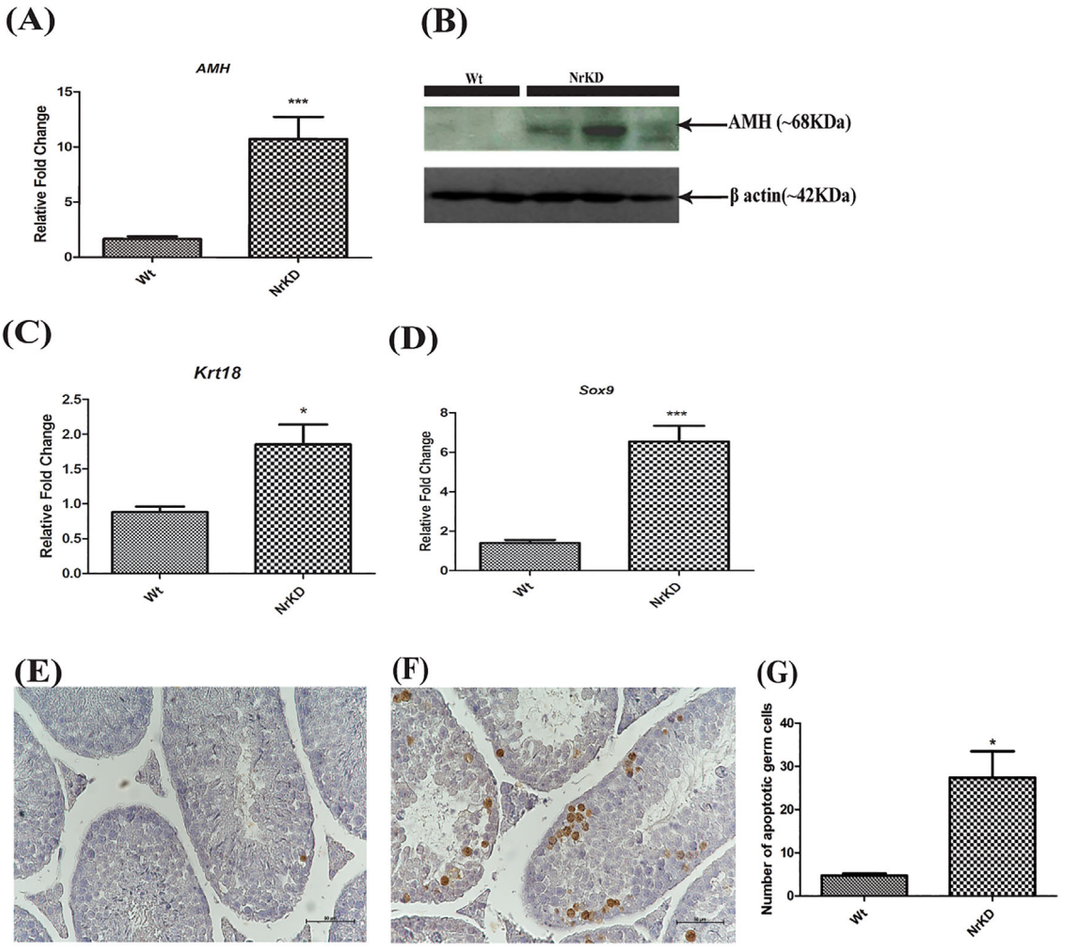

FIGURE 4 Status of Sc and Gc in NOR-1 knockdown mice. The relative mRNA levels of (A) *Amh* were higher in NrKD mice as compared to wild-type. (B) Immunoblot analysis showed higher AMH protein expression in NrKD mice as compared to wild type (Wt) mice. β-actin was used as loading control. A single representative blot is shown. The relative mRNA levels of (C) *Krt18* and *(D) Sox9* were higher in NrKD mice as compared to wild-type. RNA and protein isolation were performed with testicular tissue of adult wild type and NrKD mice. TUNEL assay of testicular sections of (E) wild-type and (F) NrKD mice showed higher rate of germ cell apoptosis in NrKD mice. Scale bar represents 50 μm. (G) The average number of apoptotic germ cells were higher in NrKD mice as compared to wild-type. Histogram depicts mean ± SEM, $n = 3$. ***indicates $P < 0.001$ by unpaired Student's *t*-test. *indicates $P < 0.05$ by unpaired Student's *t*-test. Sixty-day old mice were used for the study

Wnt/β-catenin signalling are reported to express markers of immaturity.[37] Since adult NrKD mice showed stabilization of β-catenin, we checked the expression levels of *Anti-Mullerian Hormone* (*Amh) and* Cytokeratin 18 *(Krt18)* which are infant Sc markers. The expression levels of *Amh* and *Krt18* were higher in NrKD mice as compared to age matched wild type controls (Figures 4A and 4C). Such higher expression of AMH was confirmed by immunoblot analysis (Figure 4B). We also found that the expression of Sox9 which is reported to be a target of beta catenin, was also elevated in NrKD mice as compared to age matched wild type controls (Figure 4D).[38]

3.6 | NOR-1 mediated regulation of germ cell apoptosis

Proper maturation of Sc is crucial for Gc development and differentiation.[3] Since Sc of NrKD mice showed elevated expression of Sc immaturity markers, we checked the status of germ cells in such mice. TUNEL Assay was performed on testicular sections of wild type (Figure 4E) and NrKD mice (Figure 4F). The number of germ cells undergoing apoptosis were more in NrKD mice as compared to wild type mice (Figure 4G). This indicated that diminished NOR-1 expression compromised Sc maturation leading to improper germ cell development and eventually leading to Gc apoptosis.

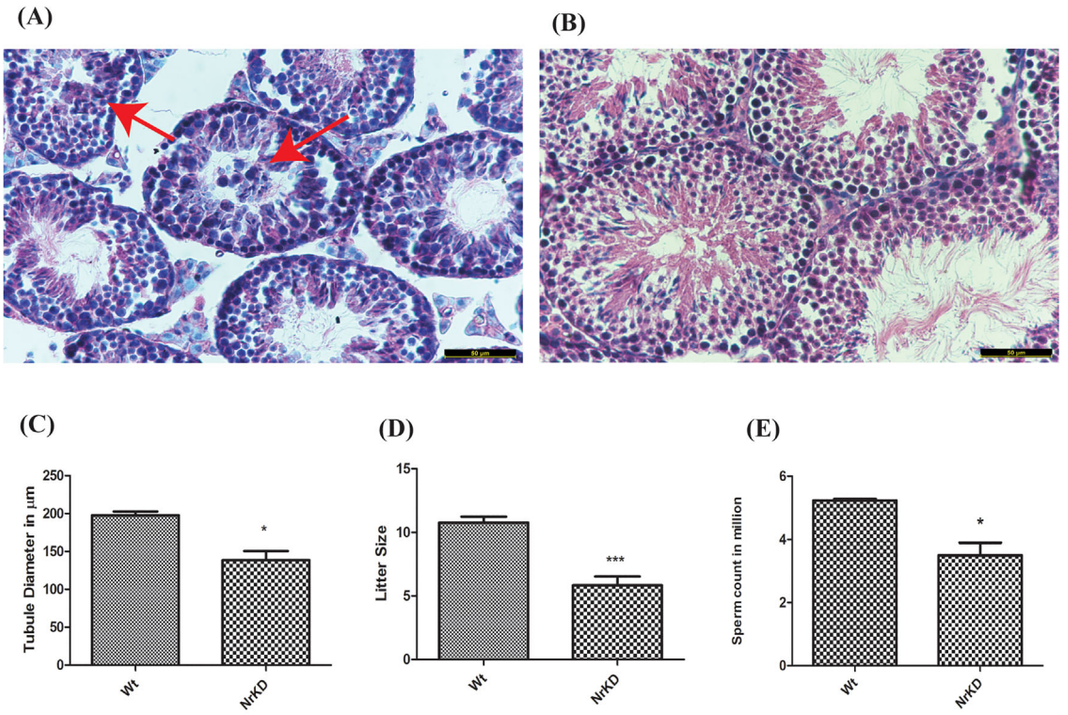

FIGURE 5 Compromised fertility in NOR-1 knockdown mice. Hematoxylin and eosin staining of testicular sections shows reduced spermatogenesis and tubular diameter in (A) NrKD mice as compared to (B) wild-type. Red arrow denotes sloughing off germ cells. Scale bar represents 50 µm. Reduction in (C) tubule diameter, (D) litter size, and (E) epididymal sperm count was seen in NrKD mice as compared to age matched wild type mice (Wt). Histogram depicts mean ± SEM, $n = 3$. ***indicates $P < 0.001$ by unpaired Student's *t*-test. *indicates $P < 0.05$ by unpaired Student's *t*-test. Sixty-day old mice were used for the study

3.7 | Testicular architecture of NOR-1 knockdown mice

The testicular sections of NrKD mice (Figure 5A) showed sloughing off of germ cell (denoted by red arrow) as compared to wild-type mice (Figure 5B). Testis of NrKD mice showed reduced tubular diameter when compared to age matched wild-type control animals (Figure 5C). All these observations suggested that the Sc of NrKD mice are functionally compromised owing to maturational defects and were less efficient in supporting spermatogenesis. However there was no significant change in testis weight of NrKD mice when compared to wild-type mice (Supplementary Figure S5).

3.8 | Sperm count and fertility

Fertility studies revealed significant ($P < 0.05$) decrease in the litter size of NrKD mice as compared to that of age matched wild-type control mice (Figure 5D). The NrKD mice showed a decline in the sperm count as compared to that of age matched wild-type control mice as seen after sacrifice of these males, post siring (Figure 5E). Thus it appears that expression of NOR-1 in pubertal Sc is crucial for maintaining normal sperm count and thus fertility. Low sperm concentrations are strongly associated with low likelihood of pregnancy.[39]

4 | DISCUSSION

Sertoli cell maturation is an essential step in regulation of spermatogenesis. Infant Sc fail to support germ cell differentiation into mature sperm. Sc attain the ability to interpret the hormonal signals and secrete paracrine factors only at puberty. A wide array of genes expressed during puberty may have a role in Sc maturation and thus in control of spermatogenesis. The microarray analysis of transciptome from infant and pubertal rat Sc, previously done in our lab, showed differential expression of many genes. Expression of *Nor-1* was found to be up regulated in pubertal rat Sc. Similar trend was also observed in pubertal mice Sc. The functional relevance of a particular gene can be studied using a transgenicmice by selectively knocking down its expression specifically in Sc in age specific manner. Promoter of Pem (Rhox5) gene is which is active specifically in mice Sc from puberty onwards.[20–22] The shRNA cassette targeting *Nor-1* in mice Sc was cloned under Pem promoter.

In vivo expression of shRNA successfully reduced levels of *Nor-1* mRNA, specifically in Sc of adult transgenic animals as compared to wild-type age matched controls. Approximately 60-70% knockdown was observed in NrKD mice. Immunohistochemistry of testicular sections of NrKD mice showed Sc specific expression of EGFP. This confirmed the integration and expression of transgenic cassette.

NR4A family of nuclear receptors to which NOR-1 belongs, negatively regulate the expression of Tgfβ3 and Smad3, a key signal transducer of Tgfβ signalling.[29] Sequence analysis of the regulatory regions of *Tgfβ3* and *Smad3* have shown that these genes possess a NBRE-related octanucleotide sequence to which Nr4A family of nuclear receptors can bind and regulate their expression.[29] We found an increased expression levels of *Smad3* and *Tgfβ3* in NrKD mice which confirmed the shRNA mediated NOR-1 knockdown effects in these animals. We also found that the expression of *Tgfβ1* was also elevated in NOR-1 knockdown mice.

Co-operation between TGF-β and Wnt/β-catenin pathways is known to control several developmental processes.[30,31] Tgfβ and Wnt signalling components interact at various levels. Reports have shown that Smad 2, 3, and 4 bind at LEF1 and synergistically activate the Xtwn promoter in Xenopus.[40]

Cross-talk between Wnt/β-catenin and TGF signalling pathways have been reported during chondrogenesis of mesenchymal cells.[41] TGF-β stimulates the expression of Wnt2, Wnt4, Wnt5a, Wnt7a, Wnt10a, and Lrp5.[33] TGF-β is also known to increase the protein stability and nuclear accumulation of β-catenin in human mesenchymal progenitor cells and human bone marrow stromal cells.[32,33] Smad3/4, which acts downstream of TGF β interact with β-catenin and form a protein complex which protects β-catenin from proteosomal degradation and transports it to nucleus which then leads to β-catenin mediated transcriptional activity.[34] Smad3 is also known to physically interact with Dishevelled 1.[34] In line with the published literature, NrKD mice showing higher transcript levels of both *Tgfβ3* and *Smad3*, showed high protein levels of β-catenin. Immunohistochemistry also confirmed the higher expression of β-catenin in testicular sections NrKD mice in as compared to that of age matched wild type control.

Some reports suggest that NR4A receptors repress β-catenin mediated transactivation in osteoblasts.[42] β-catenin expression can be regulated either at mRNA or at protein levels. Nuclear receptors are known to regulate the expression of β-catenin at both the levels.[35] This might explain the reason for higher levels of *β-catenin* at transcript level as well as protein level in NrKD mice. Since β-catenin expression was elevated the expression of β-catenin target genes (like *Axin2, Pitx2, Gja1*) were also found to be enhanced in the testes of NrKD mice. Interestingly, it was also observed that the expression of male sex differentiation marker Sox9, which is reported to be a target of β-catenin [38] was also up regulated in the testes of NrKD mice.

Constitutive expression of activated β-catenin by Sc prevents its maturation during puberty.[36,43–45] Sustained Wnt/β-catenin signalling in Sc disrupts their ability to support spermatogenesis and leads to germ cell loss. Such Sc also remain immature and show high expression of markers of immaturity.[37] In normal scenario, elevated levels of AMH are found in infant rodent Sc (upto 7 days of age) which is indicative of Sc immaturity.[46,47] We found increased transcript levels of *Krt18* and *Amh*, which are infant Sc markers, in Sc from adult NrKD mice. Protein levels of AMH were also found to be up regulated. This suggested impaired maturation of Sc in NrKD mice due to β-catenin stabilization.

Sertoli cell maturation is an important phenomenon for spermatogenesis. Proper maturation of Sc is crucial for Gc development and differentiation.[3] Any impairment in the process of Sc maturation will hamper its function. Hence, such Sc (which underwent defective maturation) will be less efficient in supporting the process of spermatogenesis. This is evident from the TUNEL Assay in testicular sections of NrKD mice. The number of germ cells undergoing apoptosis was more in the testis of NrKD mice as compared to wild-type mice. Testicular sections also showed compromised spermatogenesis. Tubular diameter was reduced and germ cells sloughed off and filled the lumen. Significant decrease in the litter size and sperm count was observed in such mice. It is important to note that Rhox5 (Pem) promoter is reported to also express in the epididymis. Hence, the involvement of defects in sperm maturation (due to NOR-1 knockdown in the epidiymis) in the observed decline in litter size warrants further investigation. High CTNNB1 and AMH provide substantial basis to suggest defects in Sertoli cells. Moreover, changes in epididymis may not affect sperm count which is significantly low in transgenic mice here. The testicular defects of NrKD mice suggested that knock down of NOR-1 in Sertoli cells is associated with maturational defect which led to compromised spermatogenesis in NrKD mice.

Our study suggests that aberrant gene expression in Sc may hamper its maturation process leading to idiopathic infertility in some cases due to failure of Sc to support Gc differentiation. Thus it is important to study the functions of such Sc specific genes which may have a role in spermatogenesis. We found *Nor-1* as one of such Sc specific gene expressed in age dependent manner which has a role in spermatogenic output. Based on our observations from in vivo Sc-specific age-restricted knockdown studies, we propose that NOR-1 may be associated with Sc mediated regulation of robust Gc division and differentiation during onset of puberty. It may be reasonable to suggest that perturbations in NOR-1

functioning may lead to reduced sperm count which might be one of the several unidentified causes of male idiopathic infertility.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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