A switch in Sertoli cell responsiveness to FSH may be responsible for robust onset of germ cell differentiation during prepubertal testicular maturation in rats

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FSH AND TESTOSTERONE (T) are known to act synergistically to regulate spermatogenesis (61). However, actions of FSH and T on the male germ cells (Gc) are indirect and mediated via testicular Sertoli cells (Sc), which express receptors for these hormones. Despite sufficient circulating levels of FSH and T postnatally, predominant appearance of spermatogonia B and spermatocytes is not discernible until 11 and 18 days of postnatal age, respectively, in rat testes. In an attempt to explore the underlying causes, we cultured Sc from neonatal (5- and 9-day-old) and prepubertal (12- and 19-day-old) rat testes and compared the status of FSH receptor (FSH-R) and androgen receptor (AR) signaling. Protein and mRNA levels of FSH-R and AR remained uniform in cultured Sc from all age groups. Androgen binding ability of AR was similar, and T-induced nuclear localization of AR was discernible in Sc from all age groups. Binding of FSH to FSH-R, subsequent production of cAMP, and mRNA of stem cell factor (SCF) and glial cell line-derived neurotrophic factor (GDNF), known to be essential for the robust differentiation of repopulating spermatogonia, were significantly augmented in prepubertal Sc compared with those in neonatal Sc. However, treatment of neonatal Sc with chloroquine or forskolin, which stimulate cAMP production bypassing FSH-R, demonstrated a concomitant rise in SCF and GDNF mRNA expression, which was similar to the FSH-mediated rise observed in prepubertal Sc. These observations suggested that, during prepubertal Sc maturation, the ability of FSH-R to respond to FSH is significantly augmented and is associated with the robust differentiation of repopulating spermatogonia, and such a switch in Sc from FSH-resistant to FSH-responsive mode during prepubertal development may underlie the initiation of robust spermatogenesis.

spermatogenesis; follicle-stimulating hormone receptor; androgen receptor; infertility; Sertoli cells

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drogen-mediated transcriptional events (69). In addition to Sc, testicular Leydig cells (Lc) and peritubular cells (PTc) also express AR (61). However, AR-mediated action in Sc plays the key role in male fertility by regulating the completion of the meiotic division of Gc (9, 14, 52). The action of T on Sc has been reported in prenatal (59) and neonatal (4) rats. However, a comparative study of the components of T signaling in neonatal and prepubertal Sc has not been investigated thoroughly (6–7).

In the present study, we evaluated developmental changes in Sc responsiveness toward FSH and T with respect to the status of Gc differentiation in testes of 5- and 9-day-old (neonatal) and 12- and 19-day-old (prepubertal) rats. Primary cultures of rat Sc from various age groups were employed for this purpose to dissect out independent actions of FSH and T on Sc necessary for initiating robust differentiation of Gc.

MATERIALS AND METHODS

Animals and reagents. Wistar rats (Rattus norvegicus) were obtained from the Small Animal Facility of the National Institute of Immunology (New Delhi, India). All animals were housed and used as per the national guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals. Ovine (o)FSH, rat FSH (NIAMD-rat-FSH-I-6), and anti-cAMP antibody were obtained from National Hormone and Pituitary Program (NHPPI), National Institutes of Health (NIH; Torrance, CA). All other reagents, unless stated otherwise, were procured from Sigma Chemical (St. Louis, MO).

Tissue histology. Tissue histology was done as described by us previously (18). Briefly, after castration, testicular tissue samples of rats were fixed in Bouin’s solution at room temperature for 24 h. Dehydration of tissues was done in a series of ascending concentrations of ethanol for 1 h in each grade of ethanol. The tissues were embedded in paraffin, and 4-μm sections were cut. Sections were stained with hematoxylin and eosin and were examined for evaluating the status of Gc differentiation.

Isolation of Sc. Testes were obtained from rats of various postnatal ages 5 and 9 (neonatal) or 12 and 19 days old (prepubertal). Sc were isolated using a sequential enzymatic digestion that has been previously described by us (18), with little modifications (74). Isolated Sc clusters were exposed to 20 mM Tris-HCl (pH 7.4) for 3–5 min to destroy Gc (25) and treated with TRIzol before storage at −80°C for subsequent RNA extraction. Data generated from freshly isolated Sc were considered as closer to in vivo.

Long-term culture. On day 1 of culture, isolated Sc clusters were counted under an inverted phase contrast microscope (Nikon, DIAPHOT 300, under ×20 magnification) and were seeded at a density of 0.25 × 10^5 cell clusters per well ml for 5- and 9-day-old rats, and 0.5 × 10^5 cell clusters per well ml for 12- and 19-day-old rats, as previously reported by us (18). Cultures were continued in DMEM-nutrient mixture F-12 Ham (DMEM-F12 HAM) containing 1% FCS for 24 h in a humidified 5% CO2 incubator at 34°C. Note that neonatal Sc divide faster than prepubertal Sc. Next day, cells were washed with prewarmed medium (DMEM-F12 HAM) and cultured further in serum replacement growth factor medium (GM medium) 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 (25). Sc were then washed twice to remove dead Gc, and the culture was continued further in GF medium. On day 4 of culture, one portion of Sc of each age group was treated with TRIzol and stored at −80°C for RNA extraction (0 h), and the rest were given various treatments. A detailed experimental work plan is given in Fig 1.

Purity of culture. Cells were cultured on coverslips for 4 days (in GF medium without hormone supplementation) and stained with vimentin antibody (Abcam, USA, Ab8978) to detect Sc as described previously (74). PTc and Lc contamination in the culture was identified by determining the alkaline phosphatase and the 3β-HSD activity, respectively (10, 36).

Evaluation of immunoactive FSH-R protein in permeabilized Sc by flow cytometry analysis. For this study, Sc were isolated from the four different age groups on the same day and were cultured for 4 days. Sc were dislodged by scraper, washed in PBS, fixed in 2% paraformaldehyde (PFA) for 15 min at 4°C, and then washed with cell-permeabilizing buffer (1× PBS containing 0.1% saponine and 1% BSA). Total FSH-R protein expression inside Sc was detected by COOH-terminal-specific anti-rabbit polyclonal antibody (Abcam, USA, Ab65975-100 in 1:100 dilution) incubated with permeabilized Sc for 2 h at 4°C followed by immunostaining with FITC-labeled anti-rabbit.
IgG raised in goat (Alexa 488; Molecular Probes, Invitrogen, A-11001) for 1 h at 4°C. The cells were subsequently washed with PBS and analyzed by flow cytometry (FACS caliber, BD Biosciences). Cells were gated using forward scatter vs. side scatter to exclude dead cells and debris. Fluorescence, detected in an FL-1 filter of 25,000 cells/age group, was acquired in logarithmic mode for visual inspection of the distributions. Acquisition of signal in linear mode was used to quantify the expression of FSH-R by calculating the mean fluorescence intensity. Data were analyzed by the software WinMDI v. 9.0. Splenocytes that do not express FSH-R were used as negative control in this study.

**FSH binding ability of FSH-R.** FSH binding to FSH-R was performed using radioactive FSH in intact (unpermeabilized) and permeabilized Sc. O-FSH was iodinated as described before (13). Briefly, iodination of 5 μg of o-FSH was carried out with 50 μCi of 125I-Na (PerkinElmer, specific activity 17.4 Ci/mg) by the iodogen method. On day 4 of culture, Sc were dislodged by scraper and then washed and suspended in PBS before 2 million Sc from each age group were incubated with 100,000 cpm of 125I-o-FSH, alone or in the presence of increasing concentrations of excess (ranging from 100 ng to 4 μg) cold FSH for 2 h at 34°C. Detectable, specific FSH binding sites, i.e., the approximate number of FSH-Rs present on the surface of each Sc, were calculated using GraphPad radioactivity calculator (http://www.graphpad.com/quickcalcs/radcalcf orm.cfm). Total (both intra- and extracellular) FSH binding sites were detected by incubating Sc with 0.1% saponine (for permeabilization) in the presence of radioactive FSH (100,000 cpm of 125I-O-FSH) and 1,000-fold more cold FSH for 2 h at 34°C. Data were represented as specifically bound FSH (in terms of cpm) per million Sc.

**Cyclic AMP assay.** On day 4 of culture, media from Sc treated with 1) GF medium containing 0.1 mM IBMX (vehicle), i.e., Control (C); 2) vehicle containing o-FSH (25, 50, and 100 ng/ml); 3) vehicle containing cholera toxin (CT, 100 ng/ml); and 4) vehicle containing forskolin (10 μM) for 1/2 h were used to determine cAMP by radioimmuno assay (RIA) as reported by us previously (18).

**Androgen binding ability of AR.** An AR binding assay was performed as previously described (23). Briefly, on day 4 of culture, Sc were incubated for 4 h with either 100 nM radioactive [3H]T (specific activity 115 Ci/mmol; PerkinElmer) in the presence or absence of cold 10,000 nM T. The Sc were also incubated with 5 nM radioactive [3H]R1881 (specific activity 82 Ci/mmol; PerkinElmer) in the presence or absence of 5 μM cold R1881, which is a nonaromatizable form of T. The cells were washed five times with ice-cold PBS. Specifically bound [3H]T or [3H]R1881 from cytoplasm and nucleus were extracted by incubation of washed cells with 1 ml of ethanol for 1/2 h at room temperature. Radioactivity in an aliquot of ethanol (0.9 ml) was measured in a liquid scintillation counter to determine bound radioactivity. The remaining ethanol was evaporated, and the cells were treated with cell lysis buffer for protein estimation. Bound androgen levels were expressed in terms of femtomoles per milligram of protein by Bradford protein assay reagents from Sigma per the manufacturer’s instructions.

**Immunoblot analysis of AR.** Nuclear extracts from cultured Sc of each age group after 1 h of T treatment were isolated using a NePER kit (Thermo Scientific) according to the manufacturer’s protocol. Protein concentration was determined using the Bio-Rad Protein Assay. Immunoblots were detected using the EZ-ECL chemiluminescent substrate kit (Biological Industries). CREB was used as an internal control, since it is reported to be persistently localized inside the nucleus of different ages of Sc (20).

**Localization of AR by confocal immunofluorescence.** Sc were cultured on coverslips for 4 days and treated with 100 nM T or vehicle (ethanol) for 60 min and then washed in PBS before being fixed in 2% parafomaldehyde for 15 min. Cells were permeabilized in 0.1% Tween 20 prior to blocking with goat serum and then incubated with rabbit antisera against AR (Abcam, ab3509, 1:100 dilution) for 12 h at 4°C. Bound primary antisera were detected by secondary antibodies tagged with Alexa 488 (Molecular Probes, Invitrogen; A-11001). Sc nuclei were stained with DAPI. All the images were captured on confocal microscope (Nikon, A1Rs). Gain in intensity of DAPI and FITC filter were fixed at the uniform value in all groups. Ten random fields from multiple coverslips were analyzed to determine the pattern of AR localization in Sc.

**In vitro treatments for gene expression.** On day 4 of culture, Sc were treated either for 2 h or for 24 h with 1) GF medium alone or 2) GF medium with o-FSH (50 ng/ml), 3) CT (100 ng/ml), 4) forskolin (10 μM), 5) B8r-cAMP (0.5 mM), and 6) T (100 nM). At the end of treatment, cells were treated with TRIzol and stored at −80°C for mRNA analysis. The dose of T used here was previously reported (23), and o-FSH was previously standardized by us and found to be bioactive in rat Sc (18).

**qRT-PCR.** qRT-PCR amplifications were performed using the Realplex3 (Eppendorf) in a total volume of 10 μl (1 or 2 μl of cDNA, depending upon the abundance of the transcripts), 0.5 μM of each primer, and 5 μl of Power SYBR Green Master Mix (Applied Biosystems). Primers for each gene (target gene as well as internal control Ppiα) were validated by a standard curve calculated from the C_T values of real-time amplification from serial dilutions of the cDNAs. Primers with an efficiency of 1 ± 0.2% were used. The qRT-PCR reaction started with melting of cDNA at 95°C for 15 min followed by 40 amplification cycles (30 s at 95°C, 45 s at 60°C, and 45 s at 65°C). Melting curve analyses for each gene were performed to detect a specific amplification peak for each gene. The expressions of mRNA of the target genes (FSH-R and AR) were evaluated by the efficiency-corrected ΔC_{T} method [method = (efficiency + 1) ^ {−ΔC_{T}}] as described previously (74). Hormone (FSH or T) and other signaling agents (CT, forskolin, or 8Br-cAMP) -mediated augmentation of SCF, GDNF, and claudin 11 were calculated by relative fold changes using the 2−ΔΔC_{T} method as described before (68). The means (±SE) of at least three individual experiments were evaluated for each treatment group for the target gene. Primers used are described in Table 1.

**Data representation and statistical analysis.** One treatment group comprised three wells within one culture set. At least three such sets of cultures for each age group (performed on different calendar dates) were used to interpret the data. Testes from about 25–30, 20–25, 10–15, and 6–10 male rats were pooled for 5-, 9-, 12-, and 19-day-old rat Sc cultures, respectively. One-way ANOVA followed by Dunnnett’s test using the InStat v. 3.0 statistical program (Graphpad Software, San Diego, CA) was used for statistical analyses of the data.

**RESULTS**

Age-dependent change in the status of gc differentiation in rat testes. Histological analyses of testicular sections demonstrated the presence of spermatogonia A in the seminiferous tubules of 5-day (Fig. 2A), 9-day (Fig. 2B), 12-day (Fig. 2C), and 19-day-old rats (Fig. 2D). However, the presence of spermatogonia B was discernible only in the tubules of 19-day-old rats (Fig. 2D). Spermatogenesis was evident on day 19.
found to be increased with age, and a distinct lumen was visible at postnatal 19 days (Fig. 2D).

Purity of Sc cultures. In all cultures, more than 95% of the cells were positively stained for vimentin (data not shown). There were no Lc in culture, and contamination of PTc was ≤2% (data not shown).

Expression of AR mRNA and protein. Expression of AR mRNA in freshly isolated Sc (Fig. 3A) and in cultured Sc (Fig. 3B) as evaluated by qRT-PCR was found to be uniform in all age groups. No significant change in the expression of the AR protein was detected in Sc from all four age groups by immunoblot analysis (Fig. 3, C and D).

Androgen binding ability of AR. The binding ability of either aromatizable T (Fig. 3E) or nonaromatizable R1881 (Fig. 3F) remained uniform in Sc cultured from all age groups.

Intracellular localization of AR after T treatment. Confocal analysis of Sc with and without T treatment revealed that the majority of AR was diffused in the cytoplasm when exposed to vehicle (ethanol) for 1 h in 5-, 9-, and 12-day-old rats, and T-induced AR influx in the nucleus was seen in all of them (data not shown). In contrast to the younger ages, the majority of AR was located in the nucleus of 19-day-old Sc (data not shown), and T treatment resulted in nuclear localization of sparsely located AR in cytoplasm, causing a denser nuclear presence of AR (data not shown).

T-mediated augmentation of SCF, GDNF, and claudin11 mRNA. No significant changes in the expression of SCF and GDNF mRNA were detected upon treatment with T in Sc from all four age groups by qRT-PCR analysis (Fig. 4, A and B). The relative fold rise in claudin 11 mRNA expression upon T

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FSH-R, FSH receptor; AR, androgen receptor; SCF, stem cell factor; GDNF, glial cell line-derived neurotrophic factor.

Histological cross sections of rat testes

![Histological cross sections of rat testes](image)

Fig. 2. Histological cross section of 5-day-, 9-day- (A and B), 12-day, and 19-day-old (C and D) rat testes. Small arrows indicate spermatogonia A; large arrows indicate spermatogonia B. Arrowheads indicate spermatoocytes. Magnification, ×60.
treatment was significantly (P < 0.05%) higher only in 19-day-old as Sc compared with the rest of the age groups studied (Fig. 4C).

Expression of FSH-R mRNA. qRT-PCR revealed that the FSH-R mRNAs were uniformly expressed in freshly isolated Sc obtained from different postnatal ages of rats (Fig. 5A). Similarly, there was no age-related difference in the level of FSH-R mRNA expressed by cultured Sc (Fig. 5B).

Age-dependent expression of immunoactive FSH-R protein by Sc. In this analysis, binding of both primary and secondary antibody was found to be specific, because very low fluorescence (4.03 and 1.77% of total cells) was detected in splenocytes, which do not express FSH-R and with secondary antibody alone in rat Sc (negative control for primary antibody), respectively (data not shown). Total fluorescence was measured by FL-1 filter per equal number of Sc (e.g., 25,000 cells) obtained from various postnatal age groups. This fluorescence-based data indirectly represented the total immunoactive FSH-R protein present in Sc. Histogram analysis indicated that, of 25,000 cells, about 87% of the population in 5-day-old (Fig. 6A), 81% of the population in 9-day-old (Fig. 6B), 85% of the population in 12-day-old (Fig. 6C), and 88% of the population in 19-day-old rats (Fig. 6D) had detectable fluorescence intensity in FL-1 filter. Therefore, no significant difference in FSH-R protein present in Sc from various age groups was detected. For each age group, <0.04% cells of a total of 25,000 cells showed autofluorescence (Fig. 6, A–D).

Ligand binding ability of FSH-R. Two million Sc from each age group were incubated with iodinated α-FSH in the absence and presence of cold FSH ranging from 100 to 4,000 ng, and

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Fig. 3. Expression of androgen receptor (AR) mRNA and protein and androgen binding ability of postnatal rat Sertoli cells (Sc). AR mRNA expression in freshly isolated Sc obtained from 5-, 9-, 12-, and 19-day-old rat testes (A). AR mRNA expression in Sc obtained from 5-, 9-, 12-, and 19-day-old rats after 4 days of culture (B). Nuclear fractions of primary Sc cultured from 5-, 9-, 12-, and 19-day-old rats were evaluated by Western blot analysis for AR and CREB (as internal control) protein (C). Densitometric analysis of AR protein normalized by CREB protein (D). Androgen binding ability of cultured Sc obtained from 5-, 9-, 12-, and 19-day-old rats was detected either by T (E) or nonaromatizable R1881 (F). Immunoblots provided (for each age group of rats) are representative of 3 sets of independent experiments. For androgen binding assay, Sc were isolated from various age groups on the same day. The mean ± SE of 3 triplicates of each age was analyzed, and data shown here are representative of ≥3 independent experiments.
specificity of FSH binding to rat Sc was discernible with the displacement of iodinated FSH by increasing the dose of cold FSH (Fig. 7A). Specific FSH binding sites i.e., probable FSH-Rs on the surface of Sc, were found to be significantly lower in unpermeabilized 5- and 9-day-old Sc compared with that of the 12- and 19-day-old Sc (Fig. 7B). Compared with their respective extracellular binding, total specific binding of FSH (after permeabilization of Sc) was found to be elevated in all age groups (Fig. 7, C and D). A twofold increase in FSH binding upon Sc membrane permeabilization was observed at 5 and 9 days of age (Fig. 7C), but such a rise was not seen at 12 and 19 days of age (Fig. 7D). However, specific total binding of radioactive FSH to Sc obtained from permeabilized cells from 5- and 9-day-old rats were still about fourfold lower with that of 12- and 19-day-old rat Sc (Fig. 7, C and D).

FSH-mediated cAMP production by postnatal rat Sc. FSH-mediated augmentation of cAMP production was significantly higher in 12- and 19-day-old rat Sc than Sc at 5 and 9 days of age (Fig. 8, A–D). Increasing doses of o-FSH ranging from 25 to 100 ng/ml failed to augment cAMP production by Sc from 5 and 9 days of age (8, A and B). However, the dose (of o-FSH) -dependent rise in cAMP production was discernible in Sc from 12 and 19 days of age (Fig. 8, C and D).

Basal expression of SCF and GDNF mRNA in freshly isolated Sc of postnatal rat testes. Expressions of SCF and GDNF mRNAs were significantly higher in freshly isolated Sc obtained from 12- and 19-day-old postnatal rat testes than those of the 5- and 9-day-old rats (Fig. 9, A and B).

FSH-mediated SCF and GDNF mRNA expression. As a result of FSH treatment, relative fold changes in the expression of SCF and GDNF mRNAs were significantly higher in freshly isolated Sc obtained from 12- and 19-day-old rats compared with that of 5- and 9-day-old Sc (Fig. 9, A and B).

Fig. 4. T-mediated stem cell factor (SCF), glial cell line-derived neurotrophic factor (GDNF), and claudin 11 mRNA expression in postnatal Sc. Relative fold change of SCF (A), GDNF (B), and claudin 11 (C) mRNA expression upon 24 h of T treatment evaluated by qRT-PCR. Each bar represents mean ± SE from ≥3 individual experiments. *P < 0.05, one-way ANOVA followed by Dunnett’s posttest.

Fig. 5. Expression of FSH receptor (FSH-R) mRNA in postnatal Sc as evaluated by qRT-PCR. FSH-R mRNA expression in freshly isolated Sc obtained from 5-, 9-, 12-, and 19-day-old rat testes (A). FSH-R mRNA expression in Sc obtained from 5-, 9-, 12-, and 19-day-old rats at 4 days of culture (B). The mean ± SE of ≥3 independent experiments for each age group was used for statistics.
of SCF and GDNF mRNAs were significantly ($P < 0.05$%) higher in 12- and 19-day-old Sc compared with those in 5- and 9-day-old Sc (Fig. 9, C and D, respectively).

cAMP production by bypassing FSH-R. Unlike FSH, treatment with CT or forskolin increased cAMP production by Sc of 5 and 9 days of age (Fig. 10, A and B, respectively). However, CT- or forskolin-mediated cAMP production by Sc obtained from rats of 12 and 19 days of age were comparable to those mediated by FSH (data not shown).

SCF and GDNF mRNA expression by bypassing FSH-R. Unlike that by FSH, treatment with CT, forskolin, or 8-Br-cAMP significantly ($P < 0.05$%) augmented SCF and GDNF mRNA expression in Sc cultured at 5 and 9 days of age (Fig. 10, C–F). In Sc obtained at 12 and 19 days of age, CT-,

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**Fig. 6.** Histogram of immunoactive FSH-R protein expressed in postnatal rat Sc as quantified by flow cytometry. Total of 25,000 cells from each age group were analyzed on the same day to quantify FSH-R expression pattern in postnatal Sc. Around 87% of the population from 5-day-old Sc showed specific fluorescence intensity detected in FL-1 (A). Autofluorescence of Sc was detected only in 0.01% of population where no antibody was used (A). Around 81% and 0.04% of population in 9-day-old (B), 85% and 0% population in 12-day-old (C), and 88% and 0% population in 19-day-old (D) Sc showed specific fluorescence intensity and autofluorescence, respectively. Histogram is representative of ≥3 independent experiments.
forskolin-, or 8-Br-cAMP-mediated augmentations of SCF and GDNF mRNA expression were similar to that by FSH (data not shown).

**DISCUSSION**

We examined age-dependent changes in the responsiveness of rat Sc to FSH and T with respect to the differentiation status of male Gc in the testes. Five-day-old neonatal rats represent proliferative Sc (12, 74) with the establishment of the spermatogonial stem cell niche on the basement membrane within the seminiferous epithelium (15); 9-day-old rats represent testes with proliferative Sc (46) and undifferentiated spermatogonia A (22); 12-day-old rats represent differentiating Sc facilitating the robust generation of spermatogonia B (22); and

![FSH binding activity in postnatal rat Sc](image1)

**Fig. 7.** FSH binding ability in postnatal rat Sc. Two million Sc from each age group were incubated with iodinated FSH in absence and presence of cold FSH ranging from 100 ng to 4 μg on the same day (A). Detectable specific FSH binding sites or probable number of FSH-Rs present on per individual Sc surface (B). Specific intracellular FSH binding sites were estimated using permeabilizing Sc from each age group (C and D). Up, unpermeabilized Sc; P, permeabilized Sc.

![FSH mediated cAMP production by postnatal rat Sc](image2)

**Fig. 8.** FSH-mediated cAMP production by postnatal rat Sc. Increasing doses of o-FSH-mediated cAMP production by cultured rat Sc obtained from 5 (A), 9 (B), 12 (C), and 19 days (D) of age. Three different doses of o-FSH were used, i.e., 25, 50, and 100 ng/ml. Note that 50 ng/ml o-FSH was found bioactive in terms of maximal production of cAMP, and 100 ng/ml o-FSH was found to be saturated in terms of cAMP production in prepubertal rat Sc (12 and 19 days of age, respectively).
We have demonstrated that the binding of FSH upon permeabilization of FSH-R from cytoplasm to plasma membrane. FSH-R protein inside Sc may stem from the diminished expression in Sc of all age groups, we found an increase in the protein was comparably expressed in Sc of all age groups, we found an increase in the expression in Sc of all age groups. In cultured Sc, previous reports regarding FSH-R expression of FSH-R mRNA across freshly isolated Sc or across cultured Sc. Data generated from freshly isolated Sc in the present study were considered closer to in vivo as reported before (73–74).

There were no interage group variations in the basal expression of FSH-R mRNA across freshly isolated Sc or across cultured Sc. Previous reports regarding FSH-R expression during postnatal testicular development in rats are controversial (5, 26, 32, 33, 35, 53, 55, 67, 70, 73). Since the number of FSH-Rs present on the membrane of Sc are very low (5), it is difficult to detect the FSH-R protein accurately by immunoblot analysis from the preparation of membrane fractions of Sc. We therefore evaluated the age-specific expression of immunoactive FSH-R protein in cultured Sc by flow cytometry using there was limited response of 5- and 9-day-old Sc to FSH in terms of post-ligand-binding downstream signaling and transcriptional events in these Sc at 5 and 9 days of age. The ability of FSH to augment cAMP production by Sc was minimal. Observation of limited response of 5- and 9-day-old Sc to FSH in terms of cAMP production was strengthened by the inability of FSH to augment SCF and GDNF transcription in Sc from these age groups. However, unlike prepubertal Sc (12 and 19 days of age), increasing doses of o-FSH to neonatal Sc (5 and 9 days old) augmented SCF and GDNF transcription in Sc from these age groups. The rise in FSH binding upon permeabilization was twofold higher in 5- and 9-day-old Sc compared with barely minimal rise in such binding seen in 12- and 19-day-old Sc. This indicated that, although FSH-R capable of binding to FSH was generated by 5- and 9-day-old Sc (as also supported by our mRNA and protein data), their membrane localization was impaired.

In postnatal rat testes, binding affinity of FSH to FSH-R is known to remain unaltered (5). We therefore measured the post-ligand-binding downstream signaling and transcriptional events in these Sc at 5 and 9 days of age, the ability of FSH to augment cAMP production by Sc was minimal. Observation of limited response of 5- and 9-day-old Sc to FSH in terms of cAMP production was strengthened by the inability of FSH to augment SCF and GDNF transcription in Sc from these age groups. However, unlike prepubertal Sc (12 and 19 days of age), increasing doses of o-FSH to neonatal Sc (5 and 9 days of age) failed to induce a dose-dependent increase in cAMP production and further authenticated the limited binding ability of FSH-R in these age groups. In contrast, treatment with CT or forskolin, which directly activate either G alpha subunit or AC, respectively, bypassing FSH-R, augmented cAMP production by Sc obtained from those at 5 and 9 days of age, indicating that G alpha subunit or AC were functional from the early post-
nental period. Most importantly, an increase in intracellular cAMP levels achieved upon treatment with CT, forskolin, or 8Br-cAMP successfully augmented SCF and GDNF mRNA expression in 5- and 9-day-old Sc. This indicated that insufficient expression of these important genes, despite FSH treatment in neonatal (5- and 9-day-old) rat Sc, stems from the limited cAMP response as a result of low FSH binding to FSH-R and not because of the compromised intracellular signaling in Sc of this age group.

It is important to note that, in patients suffering from idiopathic male infertility, hormonal therapy fails to initiate spermatogenesis (58), which is a situation similar to 5- and 9-day-old rat testes as observed by us and by others (22, 41, 43). Under a clinical situation, a hypophysectomized man was found to be fertile due to an activating mutation of FSH-R with enhanced cAMP production (30). Keeping this in mind, it is reasonable to believe that augmentation of cAMP, bypassing FSH-R and treatment in vitro, using a biopsy of testicular tissue from infertile individuals may potentially lead to generation of advanced Gc, which can be used for assisted reproduction.

Interestingly, in studies with immature rats, FSH has been shown to govern the differentiation of repopulating spermato-
gonial cells (39, 45). This action of FSH is further supported by the spermatogenic delay in FSH-R KO mice observed at the postnatal ages of 7 and 21 days (37). In hypogonadal (hpg) mice, FSH treatment for 7 days or more (51, 63, 64) or transgenic expression of human FSH (2, 3, 31) has been demonstrated to induce significant expansion of spermatogonial cells associated with the appearance of meiotic Gc in the absence of LH or T, confirming further that FSH alone can remarkably enhance the differentiation of repopulating spermatogonia to enter meiotic division, which may be considered as first wave of spermatogenesis. However, it is important to note that FSH action is restricted to the proliferation of Sc only during neonatal age, whereas FSH regulates spermatogonial differentiation in later stages of development both in rats (39) and in hpg mice (51). Moreover, the c-kit-SCF system has been demonstrated to play a crucial role in the differentiation of spermatogonia A (16, 22). Therefore, from our own observation and earlier reports, we conclude that a “switch” in the ability of rat Sc to exert optimal FSH action in terms of induction of Gc differentiation is an age-dependent phenomenon and is characterized by the upregulation of expression of genes like SCF and GDNF by Sc, which are known to induce the onset of robust spermatogenesis in vivo. Elevated basal expression of SCF and GDNF in freshly isolated Sc (closer to in vivo) obtained from 12 and 19 days of age, compared with those in Sc from younger ages, further strengthens this notion. Our histological observation also supports a previous report of the predominant appearance of spermatogonia B and pachytene spermatocytes in rat testes after 11 and 19 days of age, respectively (22, 43).

Like FSH-R, there was no interage group-related variation in the expression of AR across freshly isolated Sc or across cultured Sc. The immunoblot analysis and androgen binding ability of cultured Sc indicated that AR mRNAs were uniformly translated to proteins in all age groups and were functionally comparable in terms of ligand binding ability. To the best of our knowledge, this is the first demonstration of AR mRNA and protein expression in Sc during postnatal development. AR is reported to be transported to the nucleus of 26-day-old rat Sc culture after androgen treatment (56, 57). Generation of mice having Sc-specific ablation of the DNA binding domain of AR by Lim et al. (42) demonstrated that the genomic pathway of T signaling is indispensable for male fertility. To investigate the age-dependent mode of T signaling in Sc, nuclear localization of AR upon T treatment was evaluated. We found that a fraction of AR was distributed in the cytoplasm of Sc, and treatment of T resulted in nuclear localization of the majority of AR in Sc from all age groups. This result suggested that AR-mediated genomic signaling is probably operational from the neonatal age in rats. Our observation is consistent with the recent studies in 5-day-old mice with Sc-specific ablation of the DNA binding domain of AR (42) or in neonatal Sc-specific AR knockout mice (SCARKO) (17, 71, 72), which were sterile. Unlike FSH, T-mediated augmentations of SCF and GDNF expression were not discernible in cultured Sc of all the age groups. This observation indicated that age-dependent differentiation of premeiotic Gc is probably not dependent on T, as was also noticed in SCARKO mice (9, 14). This in vitro observation was supported by in vivo findings where T failed to induce spermatogonial differentiation when injected to neonatal rats even in the presence of endogenous FSH (38). Claudin 11 is one of the major components of testicular TJs (48) that maintains the integrity of the BTB (44), Sc polarity, and male fertility (44). This gene has been demonstrated to be regulated by T in vivo (17, 71, 72) and in vitro (24, 34). In our study, claudin 11 was found to be significantly (P < 0.05%) upregulated by T only in 19-day-old rat Sc. Taking all these together, we conclude that, although AR could localize to the nucleus upon binding to its ligand in all age groups, one of its activities manifests at 19 days of age in terms of a measurable augmentation in claudin 11 expression. It is noteworthy that claudin 11, which is needed for the establishment of the BTB (48), has been found by us to be expressed in Sc from neonatal age; however, a remarkable rise in its expression upon T treatment was observed only at 19 days of age, when the TJ formation has been shown to reach its peak (21, 24, 34).

A very recent comparative study by O’Shaughnessy and coworkers (50) with mice lacking either FSH-R or AR or both in the Sc, have demonstrated that, although FSH and T act synergistically to optimize maximal spermatogenic output, action of FSH is more conspicuous on Sc and premeiotic Gc during early testicular development, whereas the action of T become more prominent at the stage of meiosis during puberty. Observation from our study generated substantial evidence suggesting that the compromised FSH-R activity (but not that of the AR), during neonatal age of rats probably puts a hold on the robust differentiation of repopulating spermatogonial cells. A “developmental switch” in rat Sc, from an FSH-resistant to an FSH-responsive state during prepubertal testicular maturation around 12 days of age onward in rats might underlie the age-dependent onset of robust spermatogenesis, which is evident from our histological testicular sections and others’ (22). cAMP-mediated augmentation of SCF and GDNF expression in 5- and 9-day-old rat Sc suggests that intracellular events necessary to induce Gc differentiation usually remain potently functional from the early neonatal period; however, efficient binding of FSH to FSH-R is necessary for turning those events on in vivo. Studies of in vivo neutralization of GnRH followed by FSH treatment in neonatal rats (from day 1 of age) may be necessary to substantiate this notion that appropriate FSH responsiveness of Sc is a major determinant of robust onset of spermatogenesis at around 11–12 days of age. Future studies are also required to examine why biologically active FSH-Rs increase with age despite constant levels of FSH-R mRNA and immunoactive protein.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

I.B., K.S., and S.S.M. conception and design of research; I.B., B.S.P., and M.G. performed experiments; I.B., B.S.P., S.B., and S.S.M. analyzed data;
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