Chapter I

Comparison Of The Integrity Of FSH And T Signal Transduction Cascades In Sertoli Cells Obtained From Testes Of 9 Days (Immature) And 40 Days (Mature) Old Rats
INTRODUCTION

The classical endocrine control of spermatogenesis depends on the release of GnRH by hypothalamus, which in turn stimulates the secretion of FSH and LH by pituitary. LH controls the production and secretion of T by testicular Lc. Both FSH and T receptors are located on the Sc (Baccetti et al., 1998). It is believed that the combined action of FSH and T on Sc stimulates these cells to secrete peptides promoting Gc differentiation (paracrine regulation) and develop specialized Sertoli-germ cell junctions (structural membrane specializations) that create an ‘isolated environment’ and mediate differentiating Gc migration towards the lumen of the seminiferous tubule (Baccetti et al., 1998). Due to these factors, Gc development is solely dependent on Sc and interaction of FSH and T with Sc is crucial in the initiation and maintenance of spermatogenesis. However, even in the presence of sufficient amount of LH, FSH and T, spermatogenesis is not initiated in the early stages of postnatal development in rats. Presence of primary spermatocytes cannot be detected in the rat testis until 15 days of postnatal age even though there is no appreciable change in the circulating levels of LH, FSH and T from birth to adulthood (Dohler and Wuttke, 1975; Yang et al., 1990).

It is difficult to define infantile period in rats since there is no distinct phase of hormonal development. However, up to postnatal day 9, only spermatogonial population is observed in testicular sections in the phase of appreciable hormone levels, which we consider as spermatogenetically inactive testis (Dym et al., 1995). By postnatal day 35, round spermatids can be seen in the testis (Yang et al., 1990). Testis from the 40 days old rat, with round as well as elongated spermatids, may be considered as the spermatogenetically active testis.

In rat, FSH can be seen in as early as 15-19 days old fetus and FSHR activity, in terms of Sc proliferation, can be detected by 17.5 days of gestation. Also, the LH receptor can be detected by 15.5 days of gestation and the levels of testicular T are high at birth (Plant, 1994). These facts suggest functionality of both FSH and T even before or at least at the time of birth. Sertoli cell proliferation, a parameter directly dependent on FSH, has also been found to be maximum between 1-10 days of post natal age, indicating the capability of the Sc to recognize and respond to FSH (Orth, 1982; Orth, 1984a). The serum levels of FSH in infant rats are high and are almost comparable to those found in adult, especially during the neonatal period (Dohler and Wuttke, 1975). Overt expression
of the androgen receptor in rat Sertoli cells can be seen from day 5 of postnatal age (You and Sar, 1998). In case of T, the serum levels are found to be high at birth and stay so up to 19 days of age, followed by a slight decline in the serum T levels which again increases around 35-40 days of age (Dohler and Wuttke, 1975). However, the germinal epithelium remains unstimulated during early phase of postnatal development. From day 1-10 postpartum, only spermatogonia can be detected in the testis with spermatogonia B also appearing around 11 days postpartum (Dym et al., 1995). Primary spermatocytes cannot be detected until 15 days of age, whereas the round spermatids appear in the testis by approximately 30 days of age in rats (Yang et al., 1990). Evaluation of the biochemical and molecular basis of this paradox during infancy merits consideration in the light of its importance in increasing our understanding about idiopathic male infertility, a situation similar to that in infants (normal or high hormone but no spermatogenesis), and its diagnosis. To this end, we compared the status of androgen and FSH receptor signaling in immature and mature rat Sc.

**MATERIALS**

Animals and materials used are described in appendix I. All buffers and solutions are described in appendix II.

**METHODS**

**Histochemical Analysis of Testes**

**Tissue Processing**

For assessing GC development, testes from rats were fixed in Bouin’s fixative (saturated picric acid: formaldehyde: acetic acid in the ratio of 15:5:1) for 24hrs. Subsequently, the tissues were processed by standard procedures. Briefly, two pieces from each tissue were taken and washed under running water for 3 to 4hrs to remove excess fixative. The tissues were then processed through ascending grades of ethanol (50-70-95%) for 1hr at each grade, and then subjected to two changes of 100% ethanol for 1hr each, following which they were transferred to xylene-ethanol (50% each, v/v) for 30 min. Tissues were finally processed in 100% xylene for 1hr. The xylene was replaced with xylene-paraffin (50% each v/v) solution followed by further infiltration with melted paraffin (at 58-60°C). Three changes of paraffin of 1hr each were made. The tissues were then embedded in
paraffin and blocks were made using the embedding centre (Reichert Jung embedding centre). Sections (4μm thick) were cut with a Reichert Jung microtome 1640 and transferred onto albumin-coated slides.

**Staining of Tissue Sections**

The tissue sections were deparaffinized in xylene and processed through descending grades of ethanol (100-70-50%). Subsequently, the sections were hydrated with distilled water and stained with Harri's hematoxylin for 10 min at room temperature (RT). Excess stain was removed with 1% acid ethanol (1% HCl in 95% ethanol) and washed briefly under running tap water. For staining with eosin, the slides were processed in ascending grades of ethanol and then stained with 1% eosin (1% ethanolic eosin) for 30 sec. Excess stain was removed with ethanol and the slides were treated with xylene for 5 min. After mounting in DPX mountant, the sections were studied under bright field illumination with a Nikon Optiphot upright microscope.

**Isolation of Sertoli Cells from 18 Days Old Rats**

Sertoli cells from 18 days old Wistar rats were isolated using modified procedure of Welsh and Wiebe (1976). Testes from 6 rats (for one culture) were decapsulated and washed with HBSS. The testes were minced and resuspended in HBSS (12 testes/12ml). The tissue resuspension was shaken mechanically and kept at unit g for 2-3 min. The supernatant (which contained RBC and free Lc) was decanted and the tissue was resuspended again in HBSS. The process was repeated 3 times. After these 3 washes, the tissue was subjected to first enzyme digestion in 25ml of HBSS containing 3400U collagenase for about 30 min in a shaking water bath (160 oscillations per min) at 34°C. At the end of this digestion, an aggregate mass of tissue was removed leaving pieces of tubules. The tubules were collected by centrifugation at 600 rpm (72g) for 5 min. The pellet was washed 3 times in 20ml HBSS per washing. Then, the washed tubular fragments (pellet) were treated with 6 mg pancreatin in 20ml HBSS for approximately 5 min at 34°C in a shaking water bath (160 oscillations per min). This step was monitored very closely till a single aggregate mass consisting mainly of PTe was formed. Immediately, the aggregate was removed, 5% fetal calf serum (FCS) was added and the solution was chilled on ice to quench the reaction. The cell suspension was centrifuged at 1000 rpm (100g) for 4 min, the enzyme solution was removed by decanting and the pellet (containing Sc clusters and Gc) was resuspended in 20ml HBSS with 1% FCS. The cells
were washed twice in 15ml HBSS and then filtered through an 80μm stainless steel filter to remove remaining aggregates of PTC. The cell suspension was then kept at unit g, the pellet containing Sc clusters was collected and the supernatant comprising mostly of Gc was discarded. Such separation of the Gc from the cell suspension by sedimentation at unit g was repeated till maximum possible amount of Gc were removed. Finally, the cell suspension, consisting mainly of Sc clusters with a few Gc, was washed twice in culture media (DMEM/F12 HAM). Then the cell clusters were resuspended in 40ml culture media with 1% FCS before plating 1ml each (~1000 clusters) in 24 well cell culture plates. All the procedures were performed at 4°C except when the temperature is specified.

**Culture Conditions**

The cells were cultured in DMEM/F12 HAM media containing 1% FCS for first 24 hr in a humidified, 5% CO₂ incubator at 34°C. The next day (Day 2), cells were washed twice with media and cultured in media containing serum replacement factors (5 μg/ml of Sodium selenite, 10μg/ml of insulin, 5μg/ml of transferrin and 2.5ng/ml of EGF). This media was designated as GF media. On day 4, the residual Gc, if any, were removed by hypotonic shock (Galdieri *et al.*, 1983). For this purpose, cells were incubated with 20 mM Tris-HCl, pH 7.4 for 3 min at 34°C, and then cells were washed thrice with culture media to remove dead Gc and the culture was continued in GF media.

**Hormone Treatment**

On day 5, 24hrs after hypotonic shock, cells were treated with media alone, or different concentrations of ovine (o) FSH (50ng, 100ng/ml), T (10⁻⁷M), or a combination of both oFSH (50ng/ml) and T (10⁻⁷M). Cells were also treated with 8-Bromo-cAMP (150μM, 300μM, 600μM) or Cholera toxin (1U/ml, 10U/ml). To measure the amount of cAMP produced by these cells in response to these treatments, media were collected 30 min post-treatment and incubated for 5 min in a boiling water bath to destroy endogenous phosphatase activity before storing at -70°C. After removal of media for cAMP assay, the cells were further treated with respective reagents for 23.5hrs. Media were collected and stored after 24hrs of culture to measure lactate and E₂.

**Cell Counting**

After the media were collected, Sc were washed with 0.5ml pre-warmed (34°C) HBSS. Then, cells were digested with 200μl of trypsin-EDTA (0.2 % trypsin, 0.05mM EDTA) solution at 34°C for 3 min (Janecki and Steinberger, 1987). The cells were removed from the wells and chilled on ice. The remaining cells in the wells were collected by washing...
with ice cold HBSS and total volume was made up to 1ml. Cell suspension was shaken manually and 1% FCS was added. A fraction (10µl) of cell suspension from each well was used for counting cells on a hemocytometer under 10X magnification. Rest of the cells were pelleted (805g, 4°C, 4min), resuspended in 500µl ice cold HBSS and centrifuged again. The cell pellets resulting from each well were suspended in Trizol and stored at -70°C for RNA isolation.

**Isolation of Sertoli Cells from Infant (9 Days Old) Rats**

The procedure to culture Sc from the infant rat is similar to the one described for 18 days old rat with a few modifications. Briefly, decapsulated testes were minced and washed with HBSS. Further isolation of Sc was carried out in three steps as described below.

**Step I:** Tissue was digested in 2700U collagenase/20ml HBSS solution (for 30 testes from 15 animals) for approximately 25 min at 34°C in a shaking water bath (160 oscillations per min) until a major mass of cell aggregate with few small ones were formed. These cell aggregates were discarded and remaining cell suspension was centrifuged at 1200 rpm (285g) for 4 min; the pellet was washed with HBSS before resuspending in 15ml HBSS. A drop of the suspension was observed under a phase contrast microscope. The cell suspension consisted of tubular fragments, Sc clusters and few single cells probably Gc or Lc. To separate Sc clusters, the cell suspension was settled at unit g for 10 min. The supernatant from this step was collected and centrifuged at 800 rpm (127g) for 5 minutes to collect the clusters of Sc. This fraction of Sc clusters (Fraction-I) was kept on ice till other steps were completed. The pellet (containing tubular fragments) was used for the next step (Step II).

**Step II:** The pellet (tubular fragments) from previous step was digested using collagenase solution (1300U/10ml) for 10 min at 34°C in a shaking water bath (160 oscillations per min). The aggregate of cells formed at the end of the reaction was removed and the cell suspension was processed as described in Step I. Small clusters of Sc were collected as Fraction-II.

**Step III:** The pellet obtained after the above enzyme digestion step was treated with 5-10 ml of pancreatin solution in HBSS (1300U pancreatin/10ml HBSS), depending on the amount of tissue. The solution was shaken at 160 oscillations per min in a shaking water bath at 34°C. The digestion was continued till a single cluster of cell aggregate was formed (~10 min). The reaction was stopped by placing the solution on ice and adding
5% FCS. The cell aggregate was discarded and the suspension was centrifuged at 1200 rpm (285g) for 4 min and pellet was resuspended in 10ml HBSS containing 1% FCS. The cells were collected at 1000 rpm (200g) for 5 min and resuspended in 10ml HBSS. The solution was filtered through 80µm filter mesh to remove any cell aggregates formed. The filtrate was centrifuged at 1000 rpm for 5 min; the pellet was resuspended in HBSS (Fraction-III). Fraction-I, II and III were pooled. The cells were pelleted at 1000 rpm (200g) for 5 min. The cell pellet was washed twice with culture media and resuspended in 25ml culture media with 1% FCS. The cell suspension was plated in 24 well tissue culture plates (1ml per well).

Culture Conditions
Culture conditions were same as described for 18 days old rat.

Isolation of Sertoli Cells from Adult (40 Days Old) Rat Testes
Since established procedures (Wilson and Griswold, 1979) for isolation and culture of Sc from adult rats are very limited, we used a procedure modified from that described by Majumdar et al., (1995b) for culture of Sc from the adult hamster testes. Briefly, testes from 40 days old Wistar rats were decapsulated and washed with HBSS. Decapsulation was done carefully to avoid rupturing of the tubules, and then isolation procedure was carried out in 3 steps.

Step 1: Decapsulated testes (6 testes from 3 rats) were digested in pre-warmed collagenase solution (4086Ucollagenase/30ml HBSS/6 testes) for 3 min at 34°C in a shaking water bath (150 oscillations per min). The solution was chilled on ice and the enzyme solution was decanted. The tissue was washed thrice with 15ml HBSS per wash, resuspended in 25ml of HBSS and put for mechanical shaking at 150 oscillations per min at 34°C in a shaking water bath for 7 min. This resulted in the removal of most of the interstitial cells. After the mechanical shaking, the tissue was allowed to sediment at unit g and the supernatant was discarded. The tissue (mostly tubules with relatively very less interstitial cells) was now minced mildly and resuspended in 40ml of HBSS and distributed into two tubes. The tissue suspensions were shaken and settled at unit g for 2-3 min, and then the supernatants (containing Gc and some interstitial cells) were discarded. This process was repeated for at least 4 times to remove maximum amount of RBC and free Lc; this process also resulted in the removal of a small amount of Gc.
Step II: The pellet obtained from the final wash (Step I) was resuspended in collagenase solution (4086U collagenase/30ml HBSS). The reaction was carried out at 37°C in a shaking water bath (160 oscillations per min) for 15 min. Aggregates of cell mass were obtained (unlike in infants where a single aggregate appeared). The solution was kept on ice to quench the enzyme reaction. Maximum amount of cell aggregates were removed by a pair of forceps and remaining aggregates were removed by filtering through an 80μm filter mesh. The filtrate was centrifuged at 600 rpm (72g) for 5 min, and the pellet so obtained was resuspended in 30ml HBSS and centrifuged at 600 rpm (72g) for 5 min. The pellet, containing pieces of tubules, big clusters of Sc (small fragments of tissue), small clusters of Sc (which can be cultured directly), and various populations of Gc was resuspended in 20ml HBSS, each, in two tubes and kept at unit g for 5 min. A big pellet obtained after 5 min (Pellet-I), containing mostly tubular fragments and big clusters of Sc, was kept on ice for further digestion (Step III). The supernatant, containing small Sc clusters and various population of Gc, was decanted and allowed to settle down further at unit g for 5 min. The pellet, obtained by this unit g sedimentation, was resuspended in 20ml HBSS and a drop was observed under the microscope. The resuspension, containing mostly Sc clusters with relatively less amount of Gc, was kept further at unit g for about 5 min to collect the Sc clusters at the bottom of the tube. The supernatant (1), containing small sized Sc clusters with a large population of Gc, was decanted and the clusters were allowed to settle down further at unit g for 5min. The resulting pellet was resuspended in 20ml HBSS. This resuspension after the unit g step contained lesser number of Gc as compared to the supernatant (1). Such unit g steps were repeated till maximum number of Sc clusters (A) with fewer Gc could be obtained. This usually could be achieved after three unit g steps.

Step-III: Pellet-I (containing pieces of tubules and big Sc clusters) was treated with collagenase solution (1360U collagenase/10ml HBSS) for 9 min at 37°C in a shaking water bath at 150 oscillations per min. The reaction was stopped by adding 2% FCS and the cell suspension was kept on ice for 2-3 min. The cell aggregates formed, at the end of the digestion were removed by forceps or filtration through 80μm filter mesh. The cell suspension was centrifuged at 800 rpm (127g) for 4 min and the enzyme solution (supernatant) was removed. The pellet was resuspended in 15ml of HBSS and the suspension was centrifuged at 600 rpm (71g) for 5 min. The pellet was resuspended in
15 ml of HBSS and allowed to settle at unit g for approximately 7 min to obtain Sc at the bottom (B). The supernatant consisting mainly of Gc was discarded.

The fractions of Sc clusters, A and B obtained from Step-II and Step-III were resuspended in 5 ml HBSS each and pooled. Clusters of Sc were pelleted at 500 rpm (49g) for 4 min. The supernatant was discarded and clusters of cells were washed twice using DMEM/ F12 HAM media (resuspending in 10 ml and centrifuging at 500 rpm for 4 min for each wash). Finally, the pellet (obtained by processing 6 testes) was resuspended in 30 ml DMEM/F12 HAM media containing 1% FCS and plated 1 ml in each well of a 24 well tissue culture plate.

**Culture Conditions**

*In vitro* culture conditions were same as described for 18 days old rats.

**Cytochemical Evaluation of the Cultured Cells**

**Cell Viability**

Viability of the cultured Sc was estimated by staining the cells with trypan blue (0.04% in 10 mM PBS) for 10 min. Cells were washed with PBS and observed under microscope for blue cells (non-viable cells).

**Purity of the Culture**

3β-Hydroxy-Steroid Dehydrogenase Activity of the Cultured Cells

Leydig cells in the culture were identified by demonstration of 3β-hydroxy-steroid dehydrogenase (3βHSD) activity (Klinefelter et al., 1987). Briefly, the cells in the well or 15 μl of the cell suspension on a glass slide were allowed to dry 1-2 hr at RT. The dried cells were exposed to the staining solution (1:1 mix of Solution 1 and 2 where Solution 1: 2 mg of nitro blue tetrazolium in 0.6 ml of etiocholanolone stock solution (1 mg/ml in DMSO) and Solution 2: 10 mg of NAD+ in 9.8 ml PBS) for 1 hr and rinsed with distilled water before fixing and mounting. Cells were observed for blue colored product indicating 3βHSD positive cells.

Alkaline Phosphatase Activity of the Cultured Cells

Peritubular cell contamination in the culture was identified by determining the alkaline phosphatase activity of the cultured cells as described by Chapin et al., (1987). The cells were covered with the staining solution (2.5 mg of Fast blue RR added in mix of solution 1 and 2, where Solution 1: 2.5 mg of naphthol ASB1 phosphate dissolved in 10 μl DMSO.
Solution 2: 2.5 ml of 1:1 distilled water diluted AMP buffer) and kept in the dark at RT for 10 min. The staining solution was removed and cells were washed with diluted AMP buffer. Cells were observed under the microscope for blue color indicating alkaline phosphatase activity of the cells.

**Presence of Lipid Droplets in the Cultured Cells**

Lipid droplets in the cells were stained by Oil-Red'O. Cells, presoaked with propylene glycol, were incubated overnight with Oil-Red'O solution (0.5 % of Oil-Red'O crystals in propylene glycol) at RT. The staining solution was removed; cells were washed with PBS and observed under the microscope for the presence of red colored lipid droplets.

**Biochemical and Immuno Assays**

**Lactate Assay**

Lactate present in the culture media was measured as described in the lactate assay kit with some modifications. The reaction mixture comprised of 10 mg NAD\(^+\), 2ml glycine buffer and 100U lactate dehydrogenase. Standard curve for lactate was obtained in the range of 0.66 to 6.34\(\mu\)g. The reaction mixture (100\(\mu\)l/ 500\(\mu\)l reaction volume) was added to the lactate standards and samples, incubated at 37\(^\circ\)C water bath for 15 min and the absorbance was measured at 340nm.

**Testosterone assay**

Testosterone present in the serum of 9 and 40 days old rats was estimated using WHO matched reagent and protocol described for human. Briefly, 50\(\mu\)l of the plasma was transferred to each of two tubes of 5 ml capacity. Freshly purified diethyl ether (2ml) was added to each tube. The content was vortex mixed for one minute and allowed to settle. The ether supernatant was transferred to a clean dry tube for evaporation. The extraction was repeated once more. The ether from both the extractions was evaporated in a fume hood. When the ether extracts dried, 500\(\mu\)l of buffer S was added to each sample tube. The solution was vortex mixed allowing the buffer to roll around the tube. The solution was allowed to stand for 5-10 min and then vortex mixed again to ensure that the extracts were properly dissolved before going to next step. To this 500\(\mu\)l of extract solution, 100\(\mu\)l of tracer (100nCi/ml) and 100\(\mu\)l of working concentration of antiserum were added. The solution was incubated at 4\(^\circ\)C, overnight. Next day, 200\(\mu\)l of charcoal reagent was added to each tube. This step was done very rapidly so that the time of the incubation medium in contact with charcoal did not vary across the assay. To ensure equal dispensing, the
charcoal reagent was stirred continuously during the addition process. The tubes were vortex mixed and allowed to stand for 30 min at 4°C, following which the tubes were centrifuged at 500g for 10 min. The supernatant was carefully decanted into scintillation vials. To each tube, 4ml of scintillation cocktail was added and allowed to equilibrate overnight at RT before counting in a β-counter. A standard assay with known concentration of T was run along with the unknown samples. The concentrations of the samples were calculated against this standard curve. The intra- and inter-assay coefficients of variation were <7% and <10% respectively.

**Cyclic AMP Assay**

Cyclic AMP concentrations in culture medium were analyzed by radioimmunoassay using \[^{125}I\]cAMP-TME (2′-0′ monosuccinyl cAMP tyrosine methyl ester) and anti-cAMP antibody in accordance to the instructions provided by the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health).

Briefly, cAMP-TME was iodinated by chloramine-T method and the iodinated molecule was separated by loading on a Sephadex G-10 (Amersham Pharmacia) column and eluting with phosphate buffered saline (PBS). Various dilutions of cAMP standard as well as the samples (250μl each) were acetylated by adding 7.5μl acetylation mix (2:1 mix of triethylamine and acetic anhydride) before incubating overnight with the tracer and anti-cAMP antibody. Next day, 1% anti-rabbit gamma globulin was added to the tubes. The tubes were then incubated at 4°C for 30 min to precipitate the antigen-antibody complex. Finally, 25% polyethylene glycol was added before pelleting the suspension at 3000 rpm (1800g), 4°C for 20 min. The supernatant was discarded and the counts in the pellet were subsequently detected using a gamma counter. The concentration of cAMP in the sample was calculated from a standard curve plotted using known concentrations of cAMP run in parallel, along with unknown samples.

**Estradiol Assay**

Estradiol was estimated using the protocol provided by WHO Matched Reagent Program. Briefly, 200μl of aliquots of culture media was transferred to glass tubes and 2ml of diethyl ether was added to each tube. The solution was vortex mixed for 1 min and allowed to settle. The ether extract was transferred to assay tubes and evaporated under a fume cupboard. After evaporation, 500μl of buffer S was added to each sample and vortex mixed to completely dissolve the extracts. To this 500μl of sample solution, 100μl
of working tracer (100nCi/ml of E$_2$) and 100 µl of working dilution of antiserum were added. The mixture was incubated overnight at 4°C. Next day, 200µl of the charcoal reagent was added rapidly to each tube, while the charcoal reagent was continuously stirred. The tubes were vortex mixed and allowed to stand for 30 min at 4°C. The solution was centrifuged at 500g for 10 min. The supernatant was decanted carefully into scintillation vials. This step was done very fast to avoid delay between centrifugation and decanting the supernatant. Scintillation cocktail mixture (4ml) was added to each vial. The suspension was equilibrated at RT for overnight and the radioactivity in each sample was counted using a β-counter. The concentration of the E$_2$ in the sample was calculated from a standard curve plotted using known concentrations of E$_2$ run in parallel, along with unknown samples. The intra- and inter-assay coefficients of variations were <5% and <9% respectively.

**Androgen Binding Assay**

On day 5 of culture, at least 3-4 wells of Sc from the 9 and 40 days old rats were incubated for 4 hrs with 10,000cpm/ml $[^3]$H T (200µl/ well of 24 well culture plate). After the incubation, cells were washed 5 times with ice cold HBSS and 1ml of ice-cold ethanol was added to each well. The cells were incubated for 30 min at RT to extract bound T. At end of the incubation period, 800µl of ethanol was collected in a scintillation vial for measuring the amount of radioactivity in a β-counter. Excess of cold T ($10^{-4}$ M and $10^{-2}$ M) was added in some wells along with the radioactive hormone to ensure specific binding. Excess of ethanol remaining in the wells was evaporated and cells were collected in lysis buffer for protein estimation. Results are expressed in terms of specific T bound in fmol/mg protein.

**Isolation of RNA from the Samples Stored in Trizol**

Phase separation to isolate total RNA from the Trizol treated samples was carried out by adding 0.2ml chloroform per ml Trizol. The tubes were shaken vigorously by hand for 15 seconds and incubated at RT for 2 to 3 minutes. The samples were centrifuged at 12,000g for 15 min at 4°C. The upper aqueous phase so obtained was transferred to a fresh tube and further purification of RNA was carried out as per instructions provided along with the RNAqueous-4PCR Kit (Ambion). Briefly, an equal amount of 64% ethanol was added to the aqueous phase, mixed and applied to the filter provided in the kit. Subsequent
washing steps were carried out using the wash solutions provided as per the kit protocol. The RNA was finally eluted in the elution solution. The eluted RNA was treated with DNase I to remove trace DNA contamination. At the end of the treatment, DNase I was removed from the RNA using DNase I inactivation solution provided in the kit. The RNA so obtained was quantitated spectrophotometrically and stored at -70°C.

**Analyses of the mRNA Expression of the Hormone Regulated Genes by RT-PCR**

Total RNA (1 µg) isolated from each treatment group was first reverse transcribed using Reverse Transcription System (Promega Corp., USA) with AMV reverse transcriptase and oligo (dT)₁₅ for the single-strand cDNA synthesis. Subsequent PCR reactions (10 µl reaction volume) were carried out using 0.5 µl of the RT reaction for checking expression of each gene. In cases where the expression of the gene was undetectable, higher volumes (1, 2 or 3 µl) of the RT reaction were used in subsequent PCR to confirm our findings. The list of genes probed for, along with the primer sequences used and the expected product sizes is given in TABLE 2.

**Data and Statistical Analyses**

In all experiments, 3-4 wells comprised one treatment group within one culture set. At least 3 such sets of cultures were used to interpret the data. One culture of the 9 days old rat comprised of testes pooled from 20-30 animals, one culture of the 18 days old rat comprised of testes pooled from 8-10 animals whereas one Sc culture from the 40 days old rat was comprised of testes pooled from 3-4 animals. Densitomeric analysis of the gels for quantitation of expression level of the genes was carried out using the ImageJ software (ImageJ 1.33u) provided by the National Institutes of Health, USA in their public domain [http://rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij). Each gel photograph provided is a representative of three analyses carried out on three different culture sets of each age group. One-way ANOVA followed by Dunnett’s post test was used for statistical analysis of the data.
Table 1. Primer Sequences and product sizes used for RT-PCR analyses of Sertoli cell mRNA from 9 and 40 days old rat

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHR</td>
<td>ATTGACTGGCAAAACAGGAGCA</td>
<td>TTGGCAATCTTTGCTGGTCGC</td>
<td>416</td>
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<tr>
<td>AR</td>
<td>TGCAAGTGGCAAAGAAACAGGAC</td>
<td>TTGGTTGCAACACACAGACAGT</td>
<td>408</td>
</tr>
<tr>
<td>Transferrin</td>
<td>CCACATGGAAACCGACCTCTC</td>
<td>AACTGCCCAGAAGAAACTGG</td>
<td>401</td>
</tr>
<tr>
<td>InhibinβB</td>
<td>AGCGCGTCTCTGAGATCATCA</td>
<td>TCGGATGCGATGCTTGCTATC</td>
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</tr>
<tr>
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<td>ACAAGTTTCTGATCCCTTGGC</td>
<td>TCCATCTTGGTCCCTTTGGGCTC</td>
<td>521</td>
</tr>
<tr>
<td>StAR</td>
<td>TGGTGGATGGGTACAGTCC</td>
<td>GCTCAGGCATCTCCCCAAGGT</td>
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<tr>
<td>Cyclophilin A</td>
<td>TCACCATTTCCGACTGTTGAC</td>
<td>ACAGGACATTGGCGCAGGATG</td>
<td>120</td>
</tr>
</tbody>
</table>
RESULTS

Viability and purity of the Sc cultured from 18 days old rat

To check the viability and purity of Sc cultured from these three age groups trypan blue staining, staining of Sc lipid droplets and demonstration of 3β-HSD (in Lc) as well as alkaline phosphatase activity (in PTe) was undertaken. Viability of the cells on day 6 in Sc cultures from the 18 days old rat was found to be more than 98%. The cultured cells from this age group were negative for 3β-HSD activity indicating absence of Lc in the cultures (Fig. 1A). As a positive control, Lc stained positive for 3β-HSD activity are shown in Fig. 1B. Staining for assaying the alkaline phosphatase activity of the cultured cells revealed about 2% PTe contamination in the cultures from 18 days old rats (Fig. 1C). Lipid droplets (stained by Oil-Red’O) were detected in more than 95% of the cells in this culture confirming that at least 95% of the cells were Sc (Fig. 1D). These results are summarized in the Table 2.

Cyclic AMP production by Sc Cultured from the 18 Days Old Rat in Response to FSH and Other Treatment Agents

Sertoli cells from the 18 days old rat were taken as a test system to confirm the bioactivity of the agents used in further experiments because Sc from this age group are most sensitive to such stimulation. The Sc from this age group were also used for standardizing the dose of the treatment agents. Treatment of Sc isolated from the 18 days old rat with oFSH (50ng/ml) resulted in a significant (p<0.05) rise in cAMP production by these cells. Similar results were observed in wells treated with a combination of oFSH (50ng/ml) and T. Cells treated with T alone did not show any change in the basal cAMP production (Fig. 2).

Lactate Production by Sc Cultured from the 18 Days Old Rat in Response to FSH and Other Treatment Agents

Sertoli cells cultured from the 18 days old rat produced lactate, which was significantly (p<0.05) augmented by oFSH (50ng/ml, 100ng/ml). Treatment with cholera toxin (1U/ml, 10U/ml) and 8-Br-cAMP (150µM, 300µM, 600µM) also resulted in augmentation of lactate production by the Sc (Fig. 3AB). On the basis of above
Fig. 1 Cytochemical evaluation of the purity of 18 days old rat Sertoli cell culture. A Absence of Leydig cells in the culture as shown by 3β-HSD negative staining; B 3β-HSD positive purified Leydig cells as positive control; C Less than 2% peritubular cells in the culture as shown by alkaline phosphatase staining; D More than 95% of the cells stained positive for lipid droplets confirming at least 95% of the cells are Sertoli cells.
Table 2. Cytochemical Evaluation of Sertoli Cells Cultured from 9, 18 and 40 Days Old Rats

<table>
<thead>
<tr>
<th>Age of rats (days)</th>
<th>3β-HSD positive cells (%)</th>
<th>Alkaline phosphatase positive cells (%)</th>
<th>Oil-Red'O positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0</td>
<td>≤3</td>
<td>95</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>~2</td>
<td>95</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>~2</td>
<td>95</td>
</tr>
</tbody>
</table>
Fig. 2 Cyclic AMP production by Sc cultured from the 18 days old rat in response to hormone treatment. oFSH = 50ng/ml of oFSH; T = $10^{-7}$ M of testosterone; oFSH + T = 50ng/ml of oFSH and $10^{-7}$ M of T. Each panel denotes one set of Sc culture. * p<0.05
Fig. 3 Lactate production by Sc cultured from the 18 days old rat in response to FSH and other treatment agents. A Lactate production in response to oFSH (50ng/ml; 100ng/ml) and cholera toxin (1U/ml; 10U/ml); B Lactate production in response to 150µM, 300µM and 600µM 8-Br-cyclic AMP. Graph is a representative of 3 such sets of experiments. * p<0.05
observations 50ng/ml of oFSH, 150μM of 8-Br-cAMP and 1U/ml of cholera toxin were used for further Sc studies.

**Aromatization of T to Estradiol by Sc Cultured from the 18 Days Old Rat**

In the absence of T as a substrate, the aromatization ability Sc from the 18 days old rat could not be detected. The Sc from this age group were not able to produce significant amounts of E2 when provided with T as a substrate. Treatment of these Sc with FSH, in the presence of T, significantly (p< 0.05) augmented the aromatization of T to E2 (Fig. 4).

**Histological Analysis of 9 and 40 Days Old Rat Testes**

Microscopic examination of the testis section of the 9 days old rat revealed presence of Sc and spermatogonia A in the seminiferous tubules with no lumen (Fig. 5A); occurrence of spermatogonia B was extremely rare (one or two per 30-40 tubular sections). In contrast, the cross section of 40 days old rat testis showed advanced Gc including a few sperm heads in the seminiferous epithelium (Fig. 5B).

**Plasma T Levels of the 9 and 40 Days Old Rats**

The circulating levels of T in 9 and 40 days old Wistar rats from NII breeding colony were similar (Fig. 6).

**Viability and purity of the Sc cultured from 9 and 40 days old rat testes**

To check the viability and purity of Sc cultured from these three age groups trypan blue staining, staining of Sc lipid droplets and demonstration of 3β-HSD (in Lc) as well as alkaline phosphatase activity (in PTc) was undertaken. Viability of the cells on day 6 in both cultures was found to be more than 98%. A monolayer of Sc cultured from 9 and 40 days old rat is shown in Fig. 7AB. The cultured cells from both age groups were negative for 3β-HSD activity indicating absence of Lc in the cultures (Table 2). As a positive control, Lc stained positive for 3β-HSD activity are shown in Fig. 1D. Lipid droplets (stained by Oil-Red’O) were detected in more than 95% of the cells in the cultures from both age groups (Fig. 7CD) confirming, at least 95% of the cells were Sc. Staining for assaying the alkaline phosphatase activity of the cultured cells revealed about 2% PTc contamination in the cultures from 40 days old rats, whereas the cultures from 9 days old
Fig. 4 Aromatizing ability of the Sc cultured from the 18 days old rat in response to FSH and T. oFSH = 50ng/ml of oFSH; T= $10^{-7}$ M of testosterone; oFSH + T = 50ng/ml of oFSH and $10^{-7}$ M of T. Graph is a representative of 3 such sets of experiments. * p< 0.05

Fig. 6 Plasma T levels of the 9 and 40 days old rat
Cross section of the testis from the 9 days old rat

Cross section of the testis from the 40 days old rat

Fig. 5  Cross section of 9 and 40 days old rat testes. Note the presence of spermatogonia A (blue arrow) and Sertoli cells in the seminiferous tubules of 9 days old rat. Also note the presence of a few elongated spermatids (yellow arrow) in the tubule of 40 days old rat. Both sections are at same magnification.
Fig. 7  Sertoli cells cultured from 9 days old rats A and 40 days old rats B. More than 95% of the cells stained positive for lipid droplets confirming at least 95% of the cells are Sertoli cells in both age groups C and D; Alkaline phosphatase activity of the cells cultured from the infant E and pseudoadult F monkeys. Arrow indicates the alkaline phosphatase positive (blue) cells.
rats had less than 3% PTe contamination (Fig. 7EF). These results are summarized in the Table 2.

**Cyclic AMP Production by Sc cultured from the 9 and 40 Days Old Rat in response to Hormone Treatment**

Within 30 min of treatment with FSH, Sc from 9 days old rat produced significantly (p<0.05) high amounts of cAMP as compared to basal. Treatment of these cells with a combination of FSH and T yielded similar results. Rise in the basal cAMP levels was not observed when the Sc from the 9 days old rat were treated with T alone (Fig. 9).

Similar to the response of the 9 days old rat, the Sc from the 40 days old rat also exhibited a significant (p<0.05) rise, as compared to basal, in the levels of cAMP due to treatment with FSH as well as a combination of FSH and T. Treatment with T alone did not result in an increase in the basal amount of cAMP produced by the Sc from the 40 days old rat (Fig. 10). However, the levels of cAMP, produced in response to FSH or FSH and T, by the Sc from the 40 days old rat were significantly (p<0.05) higher than that produced by the Sc from the 9 days old rat (Fig. 11). It is important to note that the basal levels of cAMP produced by the Sc from both the age groups were similar.

**Aromatizing Ability of Sc Cultured from the 9 and 40 Days Old Rat**

Unlike our observations in the Sc from the 18 days old rat (Fig. 4), addition of T as a substrate to the Sc cultures resulted into a significant (p<0.05) rise in production by the Sc cultured from the 9 as well as 40 days old rat; addition of FSH to such T treated wells did not enhance the E2 production further (Fig. 12, 13).

**Lactate Production by Sc Cultured from the 9 and 40 Days Old Rat in Response to FSH and T**

In the 9 days old rat, although treatment with FSH or a combination of FSH and T resulted in a significant rise in basal cAMP levels of the Sc, no change in basal lactate levels was observed due to these treatments. Treatment of the Sc from the 9 days old rat with T alone also did not modulate the production of lactate by these cells (Fig. 14).

In contrast to the observations in the 9 days old rat, treatment with FSH or a combination of FSH and T resulted in a significant (p<0.05) augmentation in the amount
Fig. 9  Cyclic AMP production by Sc cultured from the 9 days old rat in response to hormone treatment. oFSH = 50ng/ml of oFSH; T = 10^{-7} M of testosterone; oFSH + T = 50ng/ml of oFSH and 10^{-7} M of T. Each panel denotes one set of Sc culture. * p< 0.05.
Fig. 10  Cyclic AMP production by Sc cultured from the 40 days old rat in response to hormone treatment. oFSH = 50ng/ml of oFSH; T= 10^{-7} M of testosterone; oFSH + T = 50ng/ml of oFSH and 10^{-7} M of T. Each panel denotes one set of Sc culture. * p< 0.05
Fig. 11  Cyclic AMP production by Sc cultured from the 9 and 40 days old rat in response to hormone treatment. Open bars represent Sc from the 9 days old rat and hatched bars represent Sc from the 40 days old rat. oFSH = 50ng/ml of oFSH; T = 10^{-7} M of testosterone; oFSH + T = 50ng/ml of oFSH and 10^{-7} M of T. Graph represents the mean ± SEM of three sets of Sc cultures from each age group. a, b and c are significantly different p< 0.05
Fig. 12  Aromatizing ability of Sc cultured from the 9 days old rat in response to FSH and T. oFSH = 50ng/ml of oFSH; T = $10^{-7}$ M of testosterone; oFSH + T = 50ng/ml of oFSH and $10^{-7}$ M of T. Each panel denotes one set of Sc culture. * p< 0.05
Fig. 13  Aromatizing ability of Sc cultured from the 40 days old rat in response to FSH and T. oFSH = 50ng/ml of oFSH; T= 10^{-7} M of testosterone; oFSH + T = 50ng/ml of oFSH and 10^{-7} M of T. Each panel denotes one set of Sc culture. * p<0.05
Fig. 14 Lactate production by Sc cultured from the 9 days old rat in response to FSH and T. oFSH = 50ng/ml of oFSH; T = 10^-7 M of testosterone; oFSH + T = 50ng/ml of oFSH and 10^-7 M of T.* p< 0.05. Each panel denotes one set of Sc culture
of lactate produced by the Sc from the 40 days old rat. Treatment with T alone, however, did not effect the production of lactate by the Sc from this age group (Fig. 15).

**Lactate Production by Sc Cultured from the 9 and 40 Days Old Rat in Response to FSH Analogs**

Similar to the effect of FSH, treatment of the Sc from the 9 days old rat with the FSH analogues, cholera toxin and 8-Br-cAMP, also did not result in augmentation in lactate production. (Fig. 16).

Treatment of the Sc from the 40 days old rat with the same FSH analogues resulted in a significant (p<0.05) rise in lactate production. This rise in lactate production induced due to treatment with cholera toxin and 8-Br-cAMP was equivalent to that observed in case of treatment with FSH to the Sc from the 40 days old rat (Fig. 17).

**Expression of the ABP mRNA in Sc Cultured from the 9 and 40 Days Old Rat in Response to Treatment with FSH and T**

Expression level of the ABP mRNA was high in the 40 days old rat Sc as compared to that in the Sc from the 9 days old rat (Fig. 18 lanes 1 and 5). In the Sc from the 40 days old rat, ABP mRNA declined to undetectable levels in response to treatment with FSH or a combination of FSH and T (Fig. 18 lanes 6 and 8). Treatment with T alone did not result in any change in the levels of the ABP mRNA in this age group (Fig. 18 lane 7).

In contrast, treatment of the Sc from the 9 days old rat with FSH, T or a combination of FSH and T did not alter the expression levels of ABP mRNA (Fig. lanes 1 to 4).

**Expression of the Inhibinβ8 gene in Sc Cultured from the 9 and 40 Days Old Rat in Response to Treatment with FSH and T**

Expression of mRNA of inhibinβ8 could be detected in the Sc from both the age groups (Fig. 19 lanes 1 and 5). In the 9 days old rat, treatment of the Sc with FSH, T or a combination of FSH and T did not alter the levels of inhibinβ8 (Fig. 19 lanes 1 to 4).

In contrast, the treatment of the Sc from the 40 days old rat with FSH or a combination of FSH and T led to a decline of the expression of inhibin β8 mRNA (Fig.
Fig. 15 Lactate production by Sc cultured from the 40 days old rat in response to FSH and T. oFSH = 50ng/ml of oFSH; T = 10^{-7} M of testosterone; oFSH + T = 50ng/ml of oFSH and 10^{-7} M of T. Each panel denotes one set of Sc culture. * p< 0.05
Fig. 16  Lactate production by Sc cultured from the 9 days old rat in response to cAMP and cholera toxin. oFSH = 50ng/ml of oFSH; cAMP= 150μM of 8-Br-cyclic AMP; CT = 1U/ml of cholera toxin. Each panel denotes one set of Sc culture
Fig. 17  Lactate production by Sc cultured from the 40 days old rat in response to cAMP and cholera toxin. oFSH = 50 ng/ml of oFSH; cAMP = 150 μM of 8-Br-cyclic AMP; CT = 1 U/ml of cholera toxin. Each panel denotes one set of Sc culture. * p< 0.05
Fig. 18  RT-PCR analyses of mRNA expression in the Sc from the 9 and 40 days old rat. A Expression of the ABP mRNA in Sc the from 9 (lanes 1-4) and 40 days old rat (lanes 5-8). B mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 9 (lanes 1-4) and 40 days old rat (lanes 5-8). For both panels lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 50ng/ml of oFSH; lanes 3 and 7 represent Sc well treated with 10^{-7}M of T; lanes 4 and 8 represent Sc well treated with a combination of 50ng/ml of oFSH and 10^{-7}M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture (n=3).
Fig. 19  RT-PCR analyses of mRNA expression in the Sc from the 9 and 40 days old rat. **A** Expression of the InhibinβB mRNA in Sc from 9 (lanes 1-4) and 40 days old rat (lanes 5-8). **B** mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 9 (lanes 1-4) and 40 days old rat (lanes 5-8). For both panels lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 50ng/ml of oFSH; lanes 3 and 7 represent Sc well treated with 10⁻⁷M of T; lanes 4 and 8 represent Sc well treated with a combination of 50ng/ml of oFSH and 10⁻⁷M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture (n=3).
19 lanes 6 and 8). Treatment with T alone did not alter the inhibin βB expression (Fig. 19 lane 7).

**FSHR Expression in Sc Cultured from the 9 and 40 Days Old Rat**

Analysis by RT PCR of FSHR mRNA expression in the 9 days old rat Sc revealed the presence of FSHR mRNA transcripts in this age group. Treatment of these Sc with FSH, T or a combination of FSH and T did not alter the FSHR expression in the 9 days old rat (Fig. 20).

In case of the Sc from the 40 days old rat, FSHR expression was higher than that observed in the Sc from the 9 days old rat. Treatment of the Sc from the 40 days old rat with FSH resulted in a decrease in the levels of FSHR mRNA. A further decline in the FSHR mRNA levels was observed when these Sc were treated with a combination of FSH and T. Treatment with T alone did not alter the FSHR mRNA level in the Sc from this age group (Fig. 21).

**Time Course Analysis of FSHR Expression in Response to FSH in Sc Cultured from the 9 and 40 Days Old Rat**

In the 9 days old rat, expression of FSHR mRNA increased slightly due to differences in the length of FSH treatment to the Sc from this age group (Fig. 22).

In contrast to that observed in case of the 9 days old rat, variation in the length of exposure to FSH led to a distinct modulation of the FSHR expression in the Sc from the 40 days old rat. The FSHR expression increased in the first 6hrs of FSH treatment and further treatment with FSH resulted in a decline of the FSHR mRNA transcripts in the Sc from the 40 days old rats. (Fig. 23).

**Expression of AR mRNA in Sc Cultured from the 9 and 40 Days Old Rat**

Expression of the AR mRNA could be detected in the Sc from both the age groups (Fig. 24, 25). In the 9 days old rat, treatment with FSH, T or a combination of FSH and T led to a slight increase in the expression of the AR mRNA (Fig. 24).

In the 40 days old rat treatment with FSH or a combination of FSH and T resulted in a decline of the expression AR mRNA to undetectable levels (Fig. 25 lanes 2 and 4). Treatment with T alone did not alter the AR mRNA expression in the Sc from the 40 days old rat (Fig. 25 lane 3).
RT-PCR analyses of mRNA expression in the Sc from the 9 days old rat. A Expression of the FSHR mRNA in Sc the from 9 days old rat B mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 9 days old rat. For both panels lane 1 represents control Sc wells; lane 2 represents Sc well treated with 50ng/ml of oFSH; lane 3 represents Sc well treated with 10^{-7} M of T; lane 4 represents Sc well treated with a combination of 50ng/ml of oFSH and 10^{-7} M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture (n=3).

RT-PCR analyses of mRNA expression in the Sc from the 40 days old rat. A Expression of the FSHR mRNA in Sc the from 40 days old rat B mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 40 days old rat. For both panels lane 1 represents control Sc wells; lane 2 represents Sc well treated with 50ng/ml of oFSH; lane 3 represents Sc well treated with 10^{-7} M of T; lane 4 represents Sc well treated with a combination of 50ng/ml of oFSH and 10^{-7} M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture (n=3).
Fig. 22 Time course study of the expression of the FSHR mRNA in the Sc from the 9 days old rat by RT PCR. A Expression of the FSHR mRNA in response to 0, 4, 6, 8, 12 and 24hr of FSH treatment. B Expression of the housekeeping gene, cyclophilin, from the same samples. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture (n=3).
Fig. 23  Time course study of the expression of the FSHR mRNA in the Sc from the 40 days old rat by RT PCR. A Expression of the FSHR mRNA in response to 0, 4, 6, 8, 12 and 24hr of FSH treatment. B Expression of the housekeeping gene, cyclophilin, from the same samples. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture (n=3).
RT-PCR analyses of mRNA expression in the Sc from the 9 days old rat. A Expression of the AR mRNA in Sc the from 9 days old rat B mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 9 days old rat. For both panels lane 1 represents control Sc wells; lane 2 represents Sc well treated with 50ng/ml of oFSH; lane 3 represents Sc well treated with $10^{-7}$M of T; lane 4 represents Sc well treated with a combination of 50ng/ml of oFSH and $10^{-7}$M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture ($n=3$).

RT-PCR analyses of mRNA expression in the Sc from the 40 days old rat. A Expression of the AR mRNA in Sc the from 40 days old rat B mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 40 days old rat. For both panels lane 1 represents control Sc wells; lane 2 represents Sc well treated with 50ng/ml of oFSH; lane 3 represents Sc well treated with $10^{-7}$M of T; lane 4 represents Sc well treated with a combination of 50ng/ml of oFSH and $10^{-7}$M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture ($n=3$).
Androgen Binding Ability of the Sc Cultured from the 9 and 40 Days Old Rat

To compare the number of AR present in the Sc from the 9 and 40 days old rats, androgen binding assay was carried out in the Sc cultured from both the age groups. The specific androgen binding ability of the Sc from the 40 days old rat was significantly (p< 0.05) higher than that of the Sc from the 9 days old rat (Fig. 26).

Expression of StAR mRNA in Sc Cultured from the 9 and 40 Days Old Rat

Sertoli cells from the 9 as well as 40 days old rat expressed StAR (Fig. 27 lane 1 and 5). Treatment of the Sc from the 9 days old rat with FSH, T or a combination of FSH and T did not alter the expression of StAR mRNA in these cells (Fig. 27 lanes 1 to 4).

In the 40 days old rat, treatment with FSH or a combination of FSH and T resulted in a decline in expression of the StAR mRNA. This decrease was more evident in the Sc treated with a combination of FSH and T (Fig. 27 lanes 5 and 8). Treatment with T alone led to an increased expression of StAR mRNA in the 40 days old rat (Fig. 27 lane 6).

Expression of the Transferrin Gene in Sc Cultured from the 9 and 40 Days Old rat in Response to FSH and T

In the Sc from the 40 days old rat, basal amounts of transferrin mRNA were detectable. Treatment with FSH or a combination of FSH and T led to the down regulation of this mRNA to undetectable levels in the Sc from the 40 days old rat, whereas treatment with T alone did not alter the transferrin mRNA expression (Fig. 28 lanes 5 to 8).

The transferrin mRNA transcripts were not detectable in the 9 days old (Fig. 28 lanes 1 to 4). Please note that the absence of transferrin mRNA was confirmed by repeating PCR analyses using higher volumes of the RT reaction.

DISCUSSION

Preliminary Studies Using Sc from the 18 days old rat

The primary hormonal regulation of spermatogenesis involves the action of FSH and T on Sc. Most of such studies, examining the actions of hormones, have been carried out on Sc cultured from 18-21 days old rats (Jutte et al., 1983b; Meroni et al., 2003; Riera et al., 2003). The Sc from rats of this age can be easily cultured, also these cells respond
Fig. 26 Androgen binding ability of Sc from the 9 and 40 days old rat. Each bar denotes mean ±SEM of 3 sets of cultures from respective age group. * p< 0.05
Fig. 27  RT-PCR analyses of mRNA expression in the Sc from the 9 and 40 days old rat. A Expression of the StAR mRNA in Sc the from 9 (lanes 1-4) and 40 days old rat (lanes 5-8). B mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 9 (lanes 1-4) and 40 days old rat (lanes 5-8). For both panels lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 50ng/ml of oFSH; lanes 3 and 7 represent Sc well treated with 10^{-7}M of T; lanes 4 and 8 represent Sc well treated with a combination of 50ng/ml of oFSH and 10^{-7}M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture (n=3).
Fig. 28  RT-PCR analyses of mRNA expression in the Sc from the 9 and 40 days old rat. A Expression of the Transferrin mRNA in Sc the from 9 (lanes 1-4) and 40 days old rat (lanes 5-8). B mRNA expression of the housekeeping gene, cyclophilin, in Sc from the 9 (lanes 1-4) and 40 days old rat (lanes 5-8). For both panels lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 50 ng/ml of oFSH; lanes 3 and 7 represent Sc well treated with $10^{-7}$ M of T; lanes 4 and 8 represent Sc well treated with a combination of 50 ng/ml of oFSH and $10^{-7}$ M of T. Numbers on the right represent the size of the PCR product (in base pairs). The graph depicts the expression of the FSHR mRNA relative to cyclophilin expression in respective treatment groups in three sets of cultures. The gel picture is a representative of such analyses carried out in three sets of culture ($n=3$).
maximally to hormone stimulus (Heckert and Griswold, 2002b). Since we proposed to evaluate the response of infant and adult Sc to hormone stimulus, it was important to establish that the hormone responsiveness in our culture system did not differ from those reported previously. To this end, we cultured Sc from testes of 18 days old rat and measured three functional parameters of the Sc, namely cAMP production, lactate production and E₂ production, in response to treatment with hormones or their analogues. These parameters are known to be regulated by FSH and T (Jutte et al., 1983b;Newton et al., 1992;Steinberger et al., 1978). The Sc from this age group also served as a test system to check the bioactivity as well as optimum dose of various agents used in further studies. Follicle stimulating hormone induces an increase in cAMP production by Sertoli and granulosa cells (Means et al., 1980). Previous studies also demonstrated that the rat FSH receptor does not exhibit species specificity and is able to bind FSH from different species (Means and Vaitukaitis, 1972;Simoni et al., 1997). Our observation of a rise in the cAMP production by the Sc cultured from the 18 days old rats due to treatment with oFSH was in concurrence with earlier reports (Jutte et al., 1983b) and also demonstrated the biological activity of the oFSH. Cholera toxin has been reported to replicate the effect of FSH in Sc by activating the adenylyl cyclase via ribosylation of a guanine nucleotide component (Gₐₐ) of the G protein complex (Gorczynska and Handelsman, 1991;Moss and Vaughan, 1979). Augmentation of cAMP production in the Sc from the 18 days old rats due to treatment with cholera toxin confirmed that FSH and cholera toxin use the same signaling pathway in the Sc. Since an increase in cAMP production was observed in all the doses of the treatment agents, oFSH at a concentration of 50ng/ml and cholera toxin at a concentration of 1U/ml was used for further studies reported here. The failure of T to augment cAMP production, above basal levels, further corroborated the G protein coupled receptor pathway specific production of cAMP in the Sc. Hence, we established the specificity, bioactivity as well as dose of the treatment agents with respect to cAMP production in Sc from the 18 days old rat. As mentioned earlier, cAMP production by the Sc is an event that occurs subsequent to the hormone-receptor binding and thus lies upstream in the hormone-signaling cascade (Simoni et al., 1997). Due to this fact, an increase in cAMP production may not necessarily translate into an intact signal transduction at the level of transcription and translation. To ensure that the treatment agents were able to trigger the FSH signal transduction cascade to its functional end point, it was necessary to check the effect of these treatment agents on the Sc factors which are
known to be regulated by FSH. One of the key functions of Sc is to supply the spermatogenic Gc with energy substrates. There is now a general agreement that postmeiotic Gc preferentially use lactate produced by Sc as their major energy substrate (Grootegoed et al., 1989). Importance of lactate for Gc survival is demonstrated by Trejo et al (1995) who reported a reduction in viability of male Gc in rats due to lactate deprivation (Trejo et al., 1995). Also injection of lactate in cryptorchid testis of adult rats was shown to result into a significant rise in sperm production (Cortes et al., 1987). Sertoli cells produce large amounts of lactate from glucose and it has been shown that FSH stimulates this lactate production (Jutte et al., 1983b; Meroni et al., 2003). Another factor important for Gc survival is E2 (Carreau et al., 2003; Pentikainen et al., 2000b). The necessity of E2 for male fertility is well established. Mice in which the P450 aromatase gene is knocked out display abnormalities in postmeiotic Gc, which is associated with an increase in apoptosis indicating a major role of E2 in Gc survival (Fisher et al., 1998; Robertson et al., 1999). Also male mice with estrogen receptor (ER) α KO are infertile (Eddy et al., 1996). In male rodents, Sc are asserted as the major source of estrogens in the immature testis (Carreau et al., 2003). Previous reports have demonstrated a FSH-mediated enhancement in the ability of Sc to aromatize T to E2 (Boitani et al., 1989; Newton et al., 1992; Phamantu et al., 1995; Verhoeven, 1980). In light of the above facts, it was reasonable to designate lactate and E2 as the FSH regulated Sc factors essential for the development of Gc and production of normal sperm. The augmentation of lactate production by Sc from the 18 days old rat due to treatment with oFSH, cAMP and cholera toxin, in our laboratory conditions and the FSH-mediated augmentation of the aromatizing ability of these cells for converting T to E2, validated the ability of the treatment agents to efficiently trigger the FSH signal transduction cascade at the chosen concentrations. Please note that the same concentrations of the treatment agent had also resulted in an increase in the production of cAMP by the Sc from this age group. These observations were in line with previous reports (Jutte et al., 1983a; Newton et al., 1992).

Having established our culture conditions as well as optimized the dose of treatment agents, we proceeded to study the hormone signaling status of the immature and mature Sc. We defined the "immaturity" of the Sc as its inability to support spermatogenesis as opposed to the "maturity", meaning the ability of these Sc to support spermatogenesis. Sertoli cells from the infant animal lacking spermatogenesis may be
taken as “immature” while those from the adult as “mature”. In case of rats, it is difficult
to define the period of infancy as they do not exhibit an age dependant hormonal profile
as opposed to the monkey and human systems. According to some investigators, the
period of infancy in rat is defined from 8-21 days of age (Ojeda and Ubranski, 1994).
Also, there are a few reports which state that abundance of spermatogonia B in the rat
seminiferous tubules is discernable at/or after the age of 11 days (Dym et al., 1995) and
the spermatocytes are apparent in the tubules of 15 days old rats (Yang et al., 1990).
These reports indicate that spermatogonial differentiation in the rat is discernible at the
age of about 11 days. In agreement with these reports, the histological examination of the
testicular sections of 9 days old rat from our colony revealed the presence of only
spermatogonial population in the seminiferous tubules. A similar histological examination
of the testicular sections of the 40 days old rat revealed the presence of differentiating Gc
and spermatids. Since, the aim of our study was to undertake a comparative analysis of
FSH and T receptor mediated signaling in Sc from spermatogenetically inactive and
active testis, these two ages of rats (9 days old and 40 days old) were selected for further
analyses. Please note that the reason for choosing the 40 days old rat in our study was
that the Sc from this age group can be easily isolated (as they are devoid of large numbers
of advanced Gc) and unlike adult Sc, they attach and divide efficiently in culture. To
establish this fact and also to validate the previous reports of the adult-like hormonal
drive in the infant rat, we assessed the circulating T levels of the 9 and 40 days old rats of
our colony (Dohler and Wuttke, 1975). The serum T concentrations of the 9 and 40 days
old rats were equivalent in these two age groups indicating a similar androgenic drive to
the testis. Sertoli cells were then isolated and cultured from the testes of 9 and 40 days old
rats. Contamination of PTC was marginally more in case of 9 and 40 days old rat as
compared to the 18 days old rat Sc culture; however, PTC contamination in cultures of
both the age groups was less than previously reported by others (Kissinger et al., 1982).
Skinner and Fritz (1985) have demonstrated that upto 5% of PTC contamination does not
influence the Sc functions in vitro (Skinner et al., 1985). Since, the proportion of PTC was
less than 5%, in the Sc cultures from all three age groups, we assumed that the influence
of PTC in these cultures would not be remarkable. The status of hormone receptor
signaling in the two developmental stages was then assessed in the respective Sc cultures.
**FSH Mediated Signaling in the Sc from the 9 and 40 Days Old Rats**

Following the hormone-receptor interaction, the primary response to the FSHR signaling is the production of cAMP (Simoni et al., 1997). The production of cAMP in response to FSH by the Sc from 9 as well as 40 days old rats indicated the ability of these cells to respond to the hormone. This observation also demonstrated the active status of FSHR in the Sc 9 and 40 days old rats. The significantly higher stimulation of cAMP by FSH in the 40 days old rat as compared to the Sc from the 9 days old rat contrasted with the report of Eskola et al. (1993) who have shown, using testicular homogenates, that the cAMP response of the rat testes is low at birth and at day 30 post partum; and a maximal cAMP response was observed at around day 10 post partum (Eskola et al., 1993). These contrasting observations may be attributed to the lack of influence of other testicular cells in our Sc cultures as compared to the testicular homogenates used in the study of Eskola et al. (1993). van Sickle et al. (1981) attribute the loss of cAMP response after the post natal peak in rats, as observed by Eskola et al. (1993), to the appearance of an interfering substance acting at the level of either adenylyl cyclase-related GTPase or the receptor signal generation (Van Sickle et al., 1981). Adenylyl cyclase is the only enzyme known to synthesize cAMP in cells (Means et al., 1980). Likewise, cyclic nucleotide-dependent phosphodiesterase (PDE) is the only enzyme involved in the degradation of cAMP (Means et al., 1980). The report of Means et al. (1979) establishing, kinetically, that a change in the Vmax of adenylyl cyclase is equal to an equivalent change in the Km of PDE, which is inversely related to the Vmax of PDE, indicate dependency of the steady-state concentration of cAMP upon the activity of PDE present in the cells (Means et al., 1979). This is highlighted by reports of a large increase in the PDE activity in the Sc with a corresponding curtailment of cAMP accumulation in the Sc as the age of the rat increases to 40 days or more (Griswold, 1993; Means et al., 1976). Heckert and Griswold (2002) suggested that response to FSH in Sc from the adult might be measurable only in the presence of a PDE inhibitor. Follicle stimulating hormone has been reported to cause the inhibition of PDE (Fakunding et al., 1976; Means et al., 1978). However, reports of the change in the isoforms of PDE, as the rat matures, might explain the failure of the ability of FSH to inhibit PDE activity in the adult animal (Means et al., 1980). In the rat, FSHR is detectable since infancy and the number of FSHR per cell seems to increase during the ontogenic development (Fakunding et al., 1976; Thanki and Steinberger, 1976). Additional components of the cAMP-dependent pathway may also cause these
fluctuations in response of the Sc. Intracellular cAMP levels are regulated at the level of synthesis via regulation of adenylyl cyclase activity and at the level of degradation by PDEs (Conti et al., 1991). Therefore, a change in adenylyl cyclase or PDE activity may also play a role in the fluctuation in cAMP levels during the different stages of the cycle. Morena et al (1995) evaluated the stage-dependent expression of the cAMP-specific PDEs (cAMP-PDEs) and the sensitivity of the FSH responses to PDE inhibitors specific for the cAMP-PDE in adult rats (Morena et al., 1995). They demonstrated the accumulation of FSH-dependant cAMP in the tubules of the adult rats in the presence of PDE inhibitors. In another study, Morris et al (1994) have demonstrated the presence of a spermatid factor which inhibits cAMP signaling in Sc (Morris et al., 1994). Welsh et al (1979) have demonstrated that addition of guanyl nucleotide, GMP-P(NH)P (another substrate for PDE), led to an increase in the levels cAMP in Sc homogenates upon FSH stimulation. They have also shown that if homogenates were centrifuged to separate the cellular components in supernatant from those in pellet, the cAMP levels upon FSH stimulation in the supernatant increased remarkably (Welsh et al., 1979). Alternatively, just dilution of the homogenates (resulting in the dilution of the factor inhibiting cAMP levels) also resulted into the production of relatively higher amount of cAMP in response to FSH. Observation that plasma membrane fractions of the Sc from the 18 as well as 35 days old rat responded to FSH treatment with an increase in cAMP production suggests that the FSHR-adenylyl cyclase coupling mechanism is functional in isolated Sc membranes regardless of age (Welsh et al, 1979). The absence of a cAMP response in the testicular homogenates of the 30 days old rat as shown by Eskola et al (1993) could be due to the low concentration of the PDE inhibitor (10mM as compared to 100mM used by us) used by them. For the first time, we are demonstrating the increase in cAMP production by the Sc from the 40 days old rat due to treatment with FSH.

Although Sc from 9 and 40 days old rats exhibited an increase in cAMP production by the due to treatment with FSH, the observation of aromatization of T to E₂ in the absence of a FSH stimulus by these Sc was surprising. It is known that the formation of the blood-testis-barrier in the rat occurs around day 15-18 of postnatal age (Bergmann and Dierichs, 1983; Russell and Peterson, 1985). This results in a sequestration of the developing Gc in the adluminal compartment of the seminiferous tubule. The seminiferous tubule fluid composed, mainly of Sc secretions, plays a pivotal role during this time for Gc development and differentiation. In the rat, the formation of these inter-Sc tight junctions is more or less coincident with period of maximal FSH
responsiveness (Sharpe, 1994a). The FSH independent conversion of T to E₂ by the Sc from 9 days old rat, in our study, might be attributed to the differential response of the cells from this age group to FSH before the formation of the blood-testis barrier. The aromatizing ability of Sc from the 10 days old rat has been shown to be under FSH regulation by Rosselli and Skinner (1992). This coincides with a high cAMP response of the 10 days old rat testis as reported by Eskola et al (1993). The cAMP response of the Sc from the 9 days old rat (as observed in this study) differed from that reported previously for the Sc from the 10 days old rat. This might be a reason for the FSH independent action of the Sc from the 9 days old rat as compared to the FSH dependency of the Sc in the 10 days old rat reported by Roselli and Skinner (1992). The testis of the 10 days old rat is on the verge of spermatogonial differentiation as presence of appreciable numbers of differentiated spermatogonia can be detected in the testis by day 11 (Dym et al., 1995). This may also attribute to the differences in the hormone response of the Sc from the 9 and 10 days old rat. Maturation of the Sc in the rat involves a progressive switch from being mainly FSH regulated (10-30 days of postnatal age) to be mainly T modulated (Sharpe, 1994a). As the responsiveness of the Sc to FSH declines during early puberty, its responsiveness to T increases, this is reflected by the modulation of the components of the seminiferous tubular fluid, ABP and androgen-regulated proteins by T in the adult rat (Jegou et al., 1982; Jegou et al., 1983; Rich et al., 1983; Sharpe et al., 1992). Aromatization of T to E₂ by the Sc from the 40 days old rat, without FSH mediated inputs (in contrast to that found in studies using Sc from the 18 days old rat), may represent this FSH to T shift in hormone responsiveness of the Sc from this age group. Since it was not possible in our study to directly assay the activity of the aromatase enzyme (which is responsible for converting T to E₂ in the Sc) in the absence of T, the possibility of a hormone-independent constitutive activation and/or expression of aromatase in the Sc from the 9 and 40 days old rat may not be ruled out.

Increase in the production of lactate by the Sc from the 40 days old rat due to treatment with FSH, in the presence of the PDE inhibitor, IBMX, observed in our study strengthens the argument of the capability of these cells to exhibit a FSH dependant response in the absence of PDE activity (McLachlan et al., 1996b). The inability of FSH to augment lactate production by the Sc from the 9 days old rat despite the ability of FSH to induce an appreciable elevation in cAMP levels could be either due to a defective response of these Sc to the hormone stimulus downstream of cAMP production or that the cAMP signal generated by these cells is not sufficient to trigger the FSH signaling
pathway lying downstream of cAMP. The failure of exogenous administration of cAMP analogs, 8-Br-cAMP and cholera toxin (both lead to an elevation in the intra-cellular cAMP levels), to increase lactate production by Sc from the 9 days old rat supports the former hypothesis of a deficit lying at the post-cAMP level in this age group. This suggests an absence of the translational manifestation of FSH signaling pathway in the Sc from the 9 days old rat. It is important to note that addition of corresponding concentrations of 8-Br-cAMP and cholera toxin to the Sc from the 40 days old rat (which are conducive to spermatogenesis as revealed by histological studies) resulted in a significant rise in lactate production by these cells, similar to that observed due to FSH treatment. It is well known that the action of FSH on Sc results in an overall stimulation of protein synthesis by them (Means et al., 1978). A specific low molecular weight heat stable molecule has been reported to be synthesized by Sc in response to FSH. This protein, termed as protein kinase inhibitor (PKI), is reported to inhibit the catalytic subunit of cAMP dependant protein kinase (Beale et al., 1977). The expression of PKI correlates negatively with the protein kinase activity in the Sc, thus, PKI may have potential role in the termination of the Sc response to the gonadotropins stimulus (Means et al., 1980; Tash et al., 1979). Sertoli cells isolated from the 10 days old rat contain enough PKI to inhibit 41% of the total catalytic subunit of the protein kinase (Tash et al., 1979). The status of PKI has not been studied in less than 10 days old rats; however, the low FSH response (as compared to that in the 40 days old rat), in terms of cAMP production, in the Sc from the 9 days old rat coupled with a probable constitutive production of PKI in these cells (considering Sc from the 9 days also have high PKI activity like the 10 days old rats) may be responsible for the failure of exogenous cAMP and cholera toxin to augment the lactate production by the Sc from this age group. However, either absence or inactivity of the molecules involved in FSH signaling, post-cAMP, in the Sc from the 9 days old rat may not be ruled out. Between 19 and 43 days of age, the amount of catalytic subunit of protein kinase increases 10-fold, but the specific activity of PKI does not increase (Means et al., 1980; Tash et al., 1979). Thus, even in the absence of hormone, the mature Sc has more amounts of free catalytic subunit of the protein kinase available than the FSH exposed immature Sc. Therefore, the reported diminished sensitivity of the mature cells to FSH may be due in part to the fact that phosphorylation events normally regulated by FSH in immature cells proceed constitutively in the older cells (Means et al., 1980). This is highlighted by the
observation of higher basal levels of lactate, as compared to that in the Sc from the 9 days old rat, produced by the Sc from the 40 days old rat.

Follicle stimulating hormone is reported to upregulate the expression of a number of proteins. These include ABP (Joseph et al., 1988), aromatase (O'Donnell et al., 2001b), insulin-like growth factor binding protein 3 (Rappaport and Smith, 1995), the c-fos protooncogene (Jia et al., 1996), tissue-type plasminogen activator (Nargolwalla et al., 1990), transferrin (Migrenne et al., 2003), α-subunit of inhibin (Pineau et al., 1990) and the βB-subunit of inhibin (Anawalt et al., 1996). Thus, modulation in the expression of these FSH-regulated protein(s) in the Sc from 9 and 40 days old rat due to treatment of FSH may reflect an intact FSH signal transduction in these Sc. To this end, we checked the mRNA levels of ABP and inhibinβB in response to FSH in Sc from 9 and 40 days old rat. Androgen-binding protein is a product of Sc, secreted into the tubular lumen and transported into the epididymis; it is present in the testis of all mammals examined (Joseph, 1994). Testosterone and FSH control the synthesis and secretion of ABP (Hansson et al., 1973). The hormonal requirements for ABP synthesis are very similar to those for normal spermatogenesis (Anthony et al., 1984a;Anthony et al., 1984b), hence, it is considered as a biological marker for Sc function (Gunsalus et al., 1981). Apart from the established role of ABP as a binding/carrier protein for sex steroids (Joseph, 1994), it has also been suggested that, in a variety of experimental models, ABP might regulate fertility and spermatogenesis (Anthony et al., 1984a;Anthony et al., 1984b;Holland et al., 1987;Huang et al., 1991b;Huang et al., 1992;Pogach et al., 1993). Germ cells are capable of internalizing ABP and its homologous protein, sex hormone-binding globulin (Gerard et al., 1991;Gerard et al., 1994). Specific cell surface receptors for ABP have been described in several cell types, including Gc (Felden et al., 1992a;Felden et al., 1992b;Nakhla et al., 1994). It has been proposed that activation of these receptors by ABP complexed with steroids may mediate the actions of ABP (and perhaps testosterone) on spermatogenesis (Danzo, 1995;Gerard, 1995). The observation that the Sc from the 40 days old rat, in response to treatment with FSH, were capable of modulating the expression of the ABP mRNA confirmed the active status of the FSH-mediated signaling in this age group. The lack of FSH-mediated regulation of ABP expression in the Sc from the 9 days old rat contributes to the hypothesis of a deficit in the FSH signaling pathway in this age group. These observations, however, were in contrast to the report of Rich et al (1983) who demonstrated an increase in the rate of ABP secretion in rats from birth to 20
days of age in contrast to a decreased ABP secretion by the older animals (30-35 days post partum). This ABP secretion was not dependant on hormone additions in the 7-10 days old rat whereas the Sc from the 30-35 days old rat exhibited a mild responsiveness, in terms of ABP secretion, to hormone treatment (Rich et al., 1983). Our observations of the regulation of ABP mRNA in the Sc from infant and 40 days old rats may explain the observation of Rich et al (1983). A low level constitutive production, unaffected by any hormone treatment, of ABP mRNA in the infant may translate into higher amount of ABP production; whereas a hormone induced decline in ABP mRNA in the Sc (from the 40 days old rats in this study) might be the phenomenon responsible for the decreased ABP production by the Sc from the 30-35 days old rat as shown by Rich et al (1983). However, differences in mRNA transcription, stability, translation and/or post-translational modifications in the Sc from these two age groups may also contribute to the above findings and requires further investigation. The decline in FSH stimulated ABP mRNA levels in the adult rat, though unexpected, might be due to the time period (24hrs) of FSH treatment to the Sc. Under physiological conditions, FSH stimulus to the testis is pulsatile in nature (Sharpe, 1994b; Simoni et al., 1997). Continuous exposure to hormone leads to a decreased response by a process termed as desensitization (Simoni et al., 1997). The incubation of Sc with FSH has been reported to cause in the desensitization of adenylyl cyclase resulting in the decrease of cAMP production leading to loss of FSHR in these cells (Le Gac et al., 1985). Thus, FSHR desensitization (due to 24hrs exposure to FSH in vitro) coupled with a differential transcriptional and/or translational status of the mature rat may underlie the failure of FSH to augment ABP protein levels in the 30-35 days old rat as reported by Rich et al (1983) in the past. However, a significant decline in ABP mRNA due to addition of FSH indicated the presence of intact FSHR-mediated signal transduction in the CS from the 40 days old rat.

Inhibin was first identified as a gonadal hormone that potentially inhibits pituitary FSH synthesis and secretion (de Kretser and Robertson, 1989). Inhibin was shown to be produced in two forms through dimeric assembly of an α subunit (18 kDa) and one of two closely related β subunits (βA and βB, approximately 14 kDa) (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985; Robertson et al., 1985). Dimers of α and βA and α and βB subunits form inhibinA and inhibinB, respectively. Results of in situ hybridization studies indicate that Sc express both the βA- and βB-subunits, which, taken
together with prior studies showing expression of the α-subunit in these cells, indicates the capacity of Sc to produce inhibin A and B (Buzzard et al., 2004). It is now clear that inhibinB is the main inhibin secreted in the male rat and its levels relate to Sc number and activity (Anawalt et al., 1996; Buzzard et al., 2004; Illingworth et al., 1996). The presence of inhibinβB mRNA in the Sc from the both age groups parallels the earlier reports demonstrating the presence of circulating inhibinB in rat since birth (Buzzard et al., 2004). Serum inhibinB levels in the rat rise progressively from early postnatal life to reach a peak, at day 40 in the study by Buzzard et al and at day 20 in the study by Sharpe et al., both demonstrating a progressive decline beyond day 40. This decline parallels and correlates with declining FSH concentrations (Buzzard et al., 2004; Sharpe et al., 1999). The elevated levels of inhibinB and A in the immediate postnatal period parallel the period of Sc activity in male infants as shown by elevated inhibinB levels during the first year of human life (Andersson et al., 1998). The stimulus for this period of Sc activity may result from the perinatal elevation of FSH noted in man and identified in the rat by earlier studies (Au et al., 1986; Lee et al., 1975). Although the testicular concentrations of inhibinB decline dramatically (due to dilution by the Gc populations, which are believed not produce inhibin), the total testicular content of inhibinB rises progressively from postnatal day 25 in concert with rising FSH levels and plateaux by 50 days of age in rat (Bhasin et al., 1989). The report of Buzzard et al (2004) demonstrating a peak in the levels of inhibinB in the 40 days old rat contrasted with the observation of a FSH-induced decline in the levels of inhibinβB mRNA in response to FSH treatment in the 40 days old rat in this study. This might again be due to the receptor desensitization leading to the down regulation of the FSHR coupled signal transduction cascade. The slight rise observed in the levels of inhibinβB mRNA in this age group due to treatment of the Sc with a combination of FSH and T (as compared to the expression due to FSH treatment alone) indicates a role of T in regulating the production of inhibinB. Such a dependency of inhibinB production on T instead of FSH has been suggested in the infant primate where a neonatal surge in LH levels leads to an increase in the circulating inhibinB levels (McKinnell et al., 2001; Plant and Marshall, 2001). Effect of T on the cell functions has been suggested to modulate the mRNA and/or the protein stability rather than the gene transcription in the cell (Sar et al., 1993). The persistence of inhibinβB mRNA in the Sc from the 40 days old rat treated with a combination of FSH and T in contrast to the decline in inhibinβB expression in the Sc treated with FSH alone indicates the ability of T to
increase the stability of the inhibinβB mRNA in the male rats. In the the hpg male mouse, over expression of the FSH gene (by transgenesis) does not result into a corresponding rise in circulating levels of inhibinB (Allan et al., 2001). However, in the same study, a directly proportional relationship of inhibinB production to FSH expression was observed in the female hpg mouse transgenic for FSH. This study supports our observation of T mediated regulation of inhibinβB gene expression.

Since, the decline in mRNA expression levels of ABP and inhibinβB suggested the phenomenon of FSHR down regulation due to receptor densensitization, RT PCR analysis of FSHR was carried out in the Sc from the 9 and 40 days old rat in response to FSH treatment. Expectedly, a decline in the levels of FSHR mRNA in response to FSH treatment was observed in the Sc from the 40 days old rat. Absence of FSH-mediated modulation of the FSHR mRNA expression in the Sc from the 9 days old rat indicates the inactive status of the FSHR signaling in this age group. A higher expression level of FSHR could also be discerned in the 40 days old rat as compared to that in the 9 days old rat. However, since this analysis was carried out at the later part of the study, quantitative measurements of the FSHR mRNA expression by Real Time PCR could not be carried out due to constraints in time. O'Shaughnessy and Brown (1978) have demonstrated a decrease in cAMP production in 4hrs after intratesticular injection of FSH, but a decrease in FSH binding sites could be observed in their study only after 8hrs of the FSH injection. This indicated a time-dependant modulation of FSHR expression in response to hormone treatment (O'Shaughnessy and Brown, 1978). The observation of a rise in FSHR mRNA at the initial time points of FSH exposure (4 and 6hrs) followed by its decrease at the later time points (12 and 24hrs) in the Sc from the 40 days old rat elucidates the phenomenon of receptor densitization in our study in vitro. In case of the 9 days old rat, FSH treatment did not lead to a decline in the FSHR mRNA levels; rather a rise in the FSHR mRNA expression could be discerned due to the FSH treatment. This could result from the low level of cAMP signal produced in the Sc from the 9 days old rat due to treatment with FSH. This level of cAMP signal might be sufficient for the Sc from this age group to regulate the FSHR expression, but might have failed to reach the signal levels required to mediate the receptor down regulation. It is important to note that, in our study the responses of the Sc to FSH were assayed in the presence of the PDE inhibitor, IBMX, in order to amplify the FSH signal. Hence, observation of the ability of low amount of cAMP produced by the infant Sc to regulate the FSHR expression may be due to the
prolonged availability of cAMP in Sc of our cultures. In the Sc from the 9 days old rat, absence of FSH-mediated effect on the expression of ABP and inhibinβA in contrast to the FSH-mediated increase in FSHR expression indicated that expression of the FSHR is more sensitive to the changes in intra-cellular cAMP than the expression of other FSH-regulated genes. Alternatively, a defect in the translation of FSHR mRNA into the mature FSHR in the Sc from the 9 days old rat might underlie the limitation of these Sc to regulate the expression of ABP and inhibinβA in response to FSH. This is also indicative of the limited ability of the Sc from the 9 days old rat to respond to FSH. Analysis of the expression of the FSH-regulated genes in the cholera toxin and/or 8-Br-cAMP treated wells of the Sc from the 9 days rat would lead to a better understanding of the sensitivity of the Sc from this age group to the intra-cellular changes mediated by the FSH in this age group. However, such analyses could not be carried out in this study due to the constraints of time. The study thus provides evidence for deficit in the Sc from the 9 days old rat at two levels of the FSH signaling pathway, a) low cAMP response due to FSH treatment and b) defective post-cAMP signaling cascade which prevents the further transduction of hormonal signal; both factors contributing to the inability of the Sc from this age group to support spermatogenic progression.

**Androgen Mediated Signaling in the Sc from the 9 and 40 Days Old Rats**

The principal steroidal androgens, T and its metabolite DHT, are thought to mediate their biological effects predominantly through binding to the AR (Keller *et al.*, 1996). Modifications of AR expression have been reported in case of fetal development, sexual development, aging, and malignant transformation (Keller *et al.*, 1996). Regulation of AR levels may occur anywhere along the path from AR gene transcription to its post-translational modification. A variety of factors, including androgens, have been implicated in modulating the AR protein and mRNA expression (Keller *et al.*, 1996). In the rat, castration at 3 days of age does not result in altered AR expression in the rat prostate; in contrast, castration in the adult rat altered AR mRNA and protein levels (Henttu *et al.*, 1992; Husmann *et al.*, 1991; Quarmby *et al.*, 1990; Takeda *et al.*, 1991). These findings suggest that one or more developmentally regulated factor(s) influence the AR expression. These factors also include androgens, which have been shown to decrease the expression of AR mRNA in the rat ventral prostate, a human androgen-responsive prostate carcinoma cell line (LNCaP), and a hepatoma cell (HepG2) line (Quarmby *et al.*, 1990).
Increase in the level of AR mRNA in the Sc is also known to occur due to FSH treatment (Sanborn et al., 1991). In our study, the increase in AR mRNA levels in the Sc from the 9 days old rat due to treatment with FSH indicates this FSH-mediated regulation of AR. The observation of a decline in AR mRNA levels due to FSH treatment in the Sc from the 40 days old rat might be attributed to the FSHR down regulation, and thus shut-down of the FSH signaling cascade, in this age group as demonstrated by the diminished levels of FSHR mRNA in these Sc due to FSH treatment. The report of Quarmby et al (1990) demonstrating the down regulation of AR mRNA in the Sc from 35 days old hypophysectomized rats, which were continually treated with FSH, further corroborates our observations of decline in AR mRNA in the 40 days old rat due to long-term FSH exposure. Studies involving AR localization have reported the absence of AR in the Sc of the 10 days old rat whereas a strong AR signal was reported in the Sc of the sexually mature rat (Sar et al., 1993). Since AR mRNA could be detected in the Sc from the 9 as well as 40 days old rat, we checked for the protein expression as well as function of the AR in these cells. The AR, in common with other members of the nuclear receptor superfamily, functions as a ligand-inducible transcription factor (Heinlein and Chang, 2002b). The binding of T to AR induces receptor dimerization, facilitating the ability of AR to bind to its cognate response element and recruit coregulators to promote the expression of target genes (He et al., 1999; Heinlein and Chang, 2002a; Quigley, 1998; Roy et al., 1999). Thus, this binding of T to AR is prerequisite to its function. The observation of 10-fold higher T-binding ability of the Sc from the 40 days old rat, as compared to that of the 9 days old rat, indicated less number and/or activity of the AR in the 9 days old rat. The low (as compared to the 40 days old rat) expression of AR mRNA in the 9 days old rat was an indication of lesser number of AR in the 9 days old rat. However, due to time constraints a quantitative estimation of the AR mRNA transcripts could not be carried out. Although FSH as well as T increased the AR mRNA expression in the 9 days old rat, this was not reflected in the androgen binding ability of these Sc. Sar et al have reported the absence of AR immunostaining in the Sc of the 10 days old rat, which may indicate defective translation of the AR mRNA in this age group. Also Buzek and Sanborn (1988), using binding assays with $^3$H-dimethylnortestosterone (DMNT) as the ligand, demonstrated a 4-fold increase in rat AR concentrations per Sc between 15 and 35 days of age (Buzek and Sanborn, 1988). These reports corroborate the observations of low T binding ability of the Sc from the 9 days old rat. This also complements our observation of a deficit in the AR signaling capability of the Sc from
In an effort to quantitate the ability of T to trans-activate the expression of target genes, an attempt to transfect the Sc with plasmid or adenovirus vectors containing the luciferase gene driven by a glucocorticoid response element present in the murine mammary tumor virus (MMTV-Luc) was carried out (not included in the Materials and Method section). Thus, binding of androgen to its active receptor would lead to the nuclear translocation of the receptor-ligand complex, where this complex would act as a transcription factor and bind its cognate response element present in the MMTV region of this construct. This would then lead to transcription of the luciferase gene resulting in the production of the enzyme luciferase, whose presence can be assayed.

These attempts for transfection were carried out with an expectation that failure of AR to efficiently induce the reporter (luciferase) activity, in the presence of T, would indicate a defective AR function. However, all our efforts to transfect the primary Sc cultures met with failure. Hence, we looked at the transcription of the genes regulated by T as an alternative for scoring the AR activity in the Sc from the 9 and 40 days old rat. Although the essential role of T in regulating spermatogenesis is well established, only a limited number of androgen-regulated genes in Sc have been characterized (Benbrahim-Tallaa et al., 2002a; Sadate-Ngatchou et al., 2004; Sutton et al., 1998a). We evaluated the mRNA expression of the androgen regulated genes, StAR, transferrin and ABP, in the Sc from the 9 and 40 days old rat. The T-induced increase in the levels of StAR in the Sc from the 40 days old rat contrasted with the report Weiner et al. (1990) demonstrating the decreased expression of StAR in Sc due to treatment with T. However, this is could be due to the fact that Weiner et al. (1990) used the neonatal mice to study the T-mediated regulation of StAR whereas the expression of StAR was studied in the 40 days old rat in this study. The absence of a T mediated regulation of StAR mRNA in the 9 days old rat in contrast to the T-mediated down regulation in the neonatal mice, might be due to the differences in age as well as the model (rat vs mouse) used in our study. The StAR protein regulates the delivery of cholesterol to the site of its first enzymatic conversion that constitutes the rate-limiting and hormonally regulated step in steroidogenesis (Brownie et al., 1972; Clark et al., 1994; Crivello and Jefcoate, 1980; Jefcoate et al., 1987; Privalle et al., 1983; Simpson, 1979). The trophic hormone-induced increase in steroid production in steroidogenic cells is accompanied by rapid increases in StAR mRNA levels (Caron et al., 1997; Clark and Stocco, 1995; Sugawara et al., 1995a). This led to the reports of a cAMP-mediated regulation of the StAR mRNA expression (Caron et al., 1997; Rust et al., 1998; Sugawara et al., 1995a; Sugawara et al., 1995b; Sugawara et al., 1997). Since FSH
induces the production of cAMP in the Sc, FSH may regulate expression of StAR. Indeed the decrease in the levels of StAR mRNA observed in the Sc from the 40 days old rat due to treatment with FSH or a combination of FSH and T, may be attributed to the down regulation of FSHR in these cells due the hormone treatments leading to a down regulation of the hormone-regulated genes, as observed in the case of ABP and inhibinβα expression. The increase in StAR mRNA in the T treated wells of Sc from the 40 days old rat could be a result of the action of T at the non-genomic level, which is reported to increase intracellular cAMP levels in the Sc (Fix et al., 2004). The observation of a greater extent of down-regulation of StAR mRNA in the Sc treated with a combination of FSH and T, as compared to that by FSH alone, at first, seems to be contradict our observations of the effect of FSH or T alone on the expression of StAR. However, this may be due to additive influence of T (through its non-genomic action leading to a rise in intra-cellular cAMP) and FSH (through its receptor mediated action bringing about a rise in intra-cellular cAMP) to down-regulate the FSHR-mediated signaling. This is demonstrated by the higher extent of down regulation of the FSHR in the Sc wells treated with a combination of FSH and T as compared to the down-regulation observed in the Sc treated with FSH alone in the 40 days old rat. These observations also contribute to the previous reports of the synergistic actions of FSH and T during spermatogenesis (McLachlan et al., 2002).

Rat transferrin is a major serum glycoprotein synthesized primarily by the liver and consisting of a single polypeptide chain (Schreiber et al., 1979). It is involved in the transfer of iron from serum to the cells (Wada et al., 1979). In rat seminiferous tubules, Sc are involved in the delivery of transferrin-bound iron to developing spermatids (Huggenvik et al., 1984). Low sperm counts, regions of aspermatogenesis and no sperm in the cauda epididymidis result due to mutations of transferrin mRNA in mice (Griswold, 1995). Thus transferrin synthesis by Sc is essential for normal spermatogenesis. The observation of an absence of transferrin mRNA expression in the Sc from the 9 days old rat was surprising since previous reports have demonstrated the presence of transferrin mRNA in fetal testis and secretion of transferrin has been reported in Sc cultured from the 10 days old rat (Migrenne et al., 2003; Skinner and Griswold, 1980a). In this study transferrin was supplemented in the culture media, the failure to detect transferrin mRNA (even by increasing amounts of cDNA used for PCR) in Sc from this age group could be due to its rapid down regulation in the presence of exogenous transferrin. The presence of
transferrin mRNA in the 40 days old rat, even in the presence of the exogenous transferrin, may suggest a differential response of the Sc from the 9 and 40 days old rat to the intra testicular milieu in vivo. This differential response could be due to the physical and metabolic changes occurring in the Sc at the time of the formation of the blood-testis barrier (Dym and Fawcett, 1970). Failure of T to regulate transferrin mRNA in the 40 days old Sc was surprising. However, Suire et al (1997) have demonstrated that although transferrin mRNA levels, gene transcription rates, and mRNA stability were indistinguishable in Sc from 10 and 17 days old rat, the Sc from the 17 days old rat secreted four times higher amount of transferrin than the 10 days old rats. This increase in transferrin biosynthesis by rat Sc during testicular development was attributed to an increase in translation rate of the transferrin mRNA (Suire et al., 1997). Since the action of T has been reported to lead to increase stability of mRNA and protein rather than its gene expression thus an absence of an effect of T at the level of the gene expression in our study may not reflect inactivity of the AR signaling (Kemppainen et al., 1992; Sar et al., 1993). This also strengthens our findings of an absence of T mediated regulation of the expression of the ABP mRNA in the Sc. In all, our observations identify a deficit in the ability of AR to bind its ligand in the Sc from the 9 days old rat, which along with the deficits in the FSH-meditated signaling pathway might be the primary reason underlying azoospermia in the face of adequate hormonal milieu at this stage of development.

CONCLUSIONS AND FUTURE DIRECTIONS

Our observation on the status of FSH and T signaling pathways in the Sc from the spermatogenically inactive and active testis unfolds the deficits lying in the Sc of the spermatogenically inactive rat. Our observations highlight the role of FSH, rather than T, in regulating the expression of the spermatogenically relevant genes in the 40 days old rat. This indicated that the deficits of the FSH signaling pathway in the 9 days old rat are of primary importance than the deficit lying in the androgen signaling. As mentioned earlier, our studies have been carried out in the presence of a PDE inhibitor. The activity of PDEs is reported to be high in the adult Sc, hence the validity of our reports of a FSH mediated effect on the mature Sc is questionable. Previously, the responses in the mature Sc have been reported to occur in a T dependant rather than in a FSH dependant manner. The high level of PDE activity in the mature Sc might be a key factor contributing to the T dependency of these cells. Our study indicates that the Sc from the mature animals, in presence of a PDE inhibitor in vitro, are capable of displaying a FSH response. Further
studies on synergistic actions of FSH and T and the cross talk between the signaling pathways of these two hormones in the mature Sc are needed to characterize the mechanisms underlying the shift of hormonal dependency of spermatogenesis from FSH to T in the mature animals. Recent reports demonstrate the capability of androgens to trigger several pathways in a non-genomic manner. Hence, it would be interesting to evaluate the capability of androgens (in a genomic and/or non-genomic manner) to regulate the activity PDEs in the Sc from spermatogenically inactive and active stages. This might also account for the absolute necessity of FSH for certain steps of spermatogenesis. The ability of FSH and T to act in a synergistic as well as in an antagonistic manner has been highlighted in our study (see regulation of the expression of ABP, inhibinβB and StAR). The stage-dependant expression of AR in the rat testis may account for the differences in the hormone sensitivity of the steps of the spermatogenic cycle. In this study we demonstrate the synergistic actions of FSH and T in regulating genes essential for spermatogenesis. This synergistic action of FSH and T may translate into the ability of these hormones to compensate for each other; hence controversy regarding the exact role of these hormones exists despite experiments involving a variety of in vivo models possessing a deficit in any one of these hormones. At first glance, our study suggests that a defective/inactive FSH signaling, rather than a deficit in the AR signaling, may be the primary factor limiting the spermatogonial differentiation in the infant testis. However, the main signal necessary for spermatogenesis may originate from the AR signaling pathway, where due to its stage-dependant expression the AR regulates the FSH signaling pathway. This might be possible if activation of the AR signaling pathway leads to the modulation of the PDE activity in the Sc. Studies undertaking the effect of T on the PDE activity in Sc isolated from the sections of seminiferous tubules expressing the AR (collected by transillumination of the tubules to identify the stages of the spermatogenic cycle) in the mature animal are necessary to validate this hypothesis. Sertoli cells cultured from the recently developed mouse model possessing Sc-selective KO of the AR (Sc-ARKO) may be studied to characterize the role of FSH in compensating the absence of T. All together our study suggests that the major role of T might be in increasing the stability of molecules at the levels of transcription and/or translation in the adult Sc. Effects of FSH on the adult Sc detected in the presence of PDE inhibitor, in vitro, may not necessarily occur in vivo. However, the observation of higher level of gene expression in the adult Sc, as compared to that in the immature Sc, in the
absence of hormones indicates a constitutive expression of the FSH regulated genes in the Sc from the 40 days old rat. These findings are supported by the suggestion of the constitutive activation of FSH-regulated phosphorylation events occurring in the mature Sc (Means et al., 1980). This study also highlights the importance of short-term and/or pulsatile exposure to hormones in vitro to efficiently mimic the in vivo conditions. These observations, lack of AR activity in the Sc from the 9 days old rat might be the primary cause of azoospermia in this age group. The reported extremely compromised status of spermatogenesis at the pre-meiotic stage in the testis of the recently described Sc-ARKO mouse model lends weight to our hypothesis (De Gendt et al., 2004b). However, necessity of FSH in regulating other steps of spermatogenesis as well as Sc proliferation, a factor directly correlated to Gc numbers in the testis, may not be ruled out. Hence a combination of a compromised FSH signaling cascade as well as the limited AR activity in the Sc from the 9 days old rat are the factors primarily contributing to the inactivity of the seminiferous epithelium despite the adult-like hormonal drive to the rat testis during this stage of development. Lack of rise in lactate production by addition of cAMP in the Sc from the 9 days old rat as well as an absence of T-mediated modulation on the expression of inhibinβB gene, unlike that in the Sc from the 40 days old rat, suggest a defect in the intracellular signaling in the infant cells and strengthens our hypothesis.