Chapter II

Comparison Of The Integrity Of FSH And T Signal Transduction Cascades In Sertoli Cells Obtained From Testes Of Infant (Immature) And Pseudoadult (Mature) Monkeys
INTRODUCTION

Proliferation and differentiation of male GC is dependent on the gonadotropic hormones, LH and FSH. Whereas FSH acts directly on the GC, LH action is indirect and is mediated by T. In male primates, including humans, the early postnatal period (0-4 months in monkey and 0-6 months in man) is associated with activation of the hypothalamic-pituitary-testicular axis; circulating levels of gonadotropins (LH and FSH) and testicular hormones (T and inhibin) are elevated (Bergada et al., 1999b; Burger et al., 1991; Terasawa and Fernandez, 2001). After the first 6 months of life (juvenile period), circulating levels of FSH, LH, and gonadal steroids are all low, and the hypothalamo-pituitary-gonadal system enters a quiescent stage until the time of puberty. At the onset of puberty, the serum levels of these hormones increase (Chada et al., 2003). Moreover, the LH secreted by the neonatal primate pituitary occurs in a pulsatile manner and is biologically active as reflected by the secretion of adult levels and patterns of testicular T (Plant, 1988). Thus, it is reasonable to propose that the seminiferous tubule of the neonatal boy and male monkey is exposed to an adult-like hormonal drive. Although adult-like hormonal levels in the neonatal period are discernable, the germinal epithelium remains unstimulated during this phase of early postnatal development (Plant, 1988). The GC is the major site of FSH and T action in the testis (Baccetti et al., 1998). According to classical endocrine system, GC, under the influence of FSH and T, produce factors essential for GC development. Specific high affinity binding of FSH to human and monkey fetal testis has been demonstrated (Huhtaniemi et al., 1987). Proliferation of primate GC, a FSH dependant event, occurs in the postnatal as well as pubertal period (Plant and Marshall, 2001). Juvenile GC are also capable of responding to gonadotropins and activating the germinal epithelium (Ramaswamy et al., 2000). In contrast, neonatal GC do not initiate spermatogenesis under the conditions of high endogenous gonadotropins. This led us to hypothesize that the immaturity of the GC is the underlying cause of azoospermia in the neonatal testis. Thus, it is reasonable to evaluate the deficits, if any, in the signaling pathways underlying FSH and/or T action limiting the ability of these cells to activate the seminiferous epithelium. Present study was undertaken to evaluate the competency of the FSHR and AR signal transduction pathways in the infant GC. The functional competency of these "immature" GC was compared to that of the "mature" GC to identify the possible deficits in the hormone signal transduction of the infant GC.
MATERIALS

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

METHODS

Source of the Testis

Testes were surgically removed from 3-4 months old infant and 1½ to 2 year old juvenile rhesus monkeys (Macaca mulatta). Animals were given tetanus toxoid, (0.5 ml, i.m.; Serum Institute of India Ltd., Pune, India) and penicillin benzoate (6,00,000 IU, i.m.; Wyeth Lederle Ltd., Mumbai, India) 24hrs before the surgery. Testes were Surgically removed under sterile conditions, after sedation with ketamine hydrochloride (50mg/kg body weight, i.m.) followed by thiopentone sodium (25 mg/kg body weight, i.v., plus 5 mg supplements, as required; Pentothal, Abbot Laboratory India Ltd., Gujarat, India). Post surgically, each monkey was treated with ampicillin or ampicillin-cloxacillin (5-10 mg/kg body weight, i.m.; Ampiclox, Biochem Pharmaceutical Industries, Mumbai, India) and an analgesic, Diclofenac sodium (0.5 mg/kg body weight, i.m.; Voveran, Novartis India Ltd., Thane, India), twice daily for 5 days. In all, 12 infant and 12 pseudoadult (explained below) monkeys were used for this study.

Preparation of Pseudoadult Monkey

Catheterization and GnRH treatment

Pseudoadult monkeys were prepared by intermittent i.v. infusion of GnRH to 1½ to 2 yr old juvenile rhesus monkeys (Majumdar et al., 1997). This model is shown in Fig. 1. Juvenile monkeys were surgically implanted with chronic indwelling catheters via a femoral or internal jugular vein under sterile conditions. The catheter was exteriorized in the midscapular region. The exteriorized catheter was protected by a nylon jacket and a flexible stainless steel tether (36 in. long, 0.5 in. inner diameter) attached to a swivel device on the top of the cage. This system allowed normal free movement of the monkeys without affecting the continuous access to the venous circulation via the catheter. One side of the swivel (placed on top of the cage) was attached to polyvinyl chloride (PVC) tubing (cage tubing) and this tube was brought to an adjacent room where the remote blood-sampling unit was set up. The cage tubing was then connected to a multi-port
Diagrammatic representation of the pattern of gonadotropins, T and inhibin secretion in the male rhesus monkey from birth to puberty. An adult-like pattern of gonadotropin and T secretion can be induced during the hypogonadotropic juvenile phase of development by treatment with exogenous, pulsatile GnRH (inset). We have termed such adult-like juvenile monkey as "Pseudoadult monkey". The relative increase in the size of testis is indicated by oval cartoon represented at the initiation and completion of the GnRH treatment for 4 weeks.
stopcock A as shown in Fig. 2. Continuous saline, saline flush and GnRH lines were connected to A via the ports, P-1, P-2 and P-3, respectively. The incoming flow rates of continuous saline, saline flush and GnRH solution were adjusted through three pumps (1, 2 and 3 respectively). The pumps, in turn, were operated by a chrontrol (a controller of electric supply to pumps), which can be programmed (not shown in the Fig. 2). The void volume of the cage tubing along with the catheter was approximately 3ml. The flow rate of continuous saline (500ml of saline containing 0.1ml heparin, 1000 IU/ml, Biological E. Ltd., Hyderabad, India) was adjusted at 72ml/24hrs. For pulsatile GnRH treatment, 0.3µg/2ml GnRH solution was released in the cage tubing over a period of 2 min, every 3hrs. This GnRH pulse was immediately followed by a saline flush to deliver the GnRH solution present in the line to the monkey. The flow of the post GnRH saline flush was adjusted at the rate of 3ml/2min. This cycle was repeated every 3hrs. Such intermittent pulsatile GnRH treatment was carried out for 4-6 weeks using pumps governed by chrontrol. The same catheter line was also used for collecting pre- and post-GnRH pulse blood samples.

All the surgical procedures were carried out under sterile conditions, after sedation with ketamine hydrochloride (50mg/kg body weight, i.m.) and anesthesia with thiopentone sodium (30 mg/kg body weight, i.v., plus 5 mg supplements, as required). Post surgically each animal was treated with cefatoxime (50 mg/ kg body weight; Omnatax, Samrudh Pharmaceuticals Pvt. Ltd., Thane, India) twice daily for 5 days and analgesic (Novalgin, 80 mg/kg body weight, Aventia Pharma Ltd., Gujarat, India) twice daily for 5 days.

**Remote Blood Sampling**

To measure the circulating T levels in the GnRH treated juvenile monkeys, venous blood samples, before and after a GnRH pulse, were withdrawn every week. For collecting the blood samples five heparinized syringes were prepared. Two minutes prior to a GnRH pulse, saline present in the tubings (catheter and cage tubing) was withdrawn through the port P-4 of the stopcock (Fig. 2) using a 5ml syringe. Once the saline mixed blood was removed, 1ml of pure blood was collected in the heparinized syringe. The blood sample was transferred to a sterile glass tube and kept at 4°C. The saline collected from the tubings (containing small amount of blood) was flushed back in the tubing. The tubings were flushed further with 3ml of fresh saline. Similarly, blood samples were collected at 20 min, 40 min, 60 min and 120 min post GnRH pulse. Plasma was collected by
Fig. 2 Hormone treatment and remote blood sampling unit. The multi-port stopcock A is attached with 2 three-way stopcock B1 and B2. The continuous saline, saline flush and GnRH lines are connected to ‘A’ via the ports, P-1, P-2 and P-3 respectively, and the flow rates are adjusted through the pumps (1-3). P-5 is connected to monkey cage tubing through a hub. P-4 serves as Blood sampling and injection (medicines) port.
centrifugation at 3000 rpm (1781g) for 5 min at 4°C. The RBCs in all tubes were suspended in 0.5ml of saline, pooled and returned to the monkey via the catheter under sterile condition. The tubings were flushed using fresh saline. The stopcock (B1) and the hub were changed after the blood sample collection and 0.3ml of cefotaxime (125mg/ml) was injected through the line.

**Histochemical Analysis of Testes**

Testes sections from infant, juvenile, and pseudoadult rhesus monkeys were prepared as described for rat testis in Chapter 1. Tissue sections were stained with hematoxylin and eosin as described in Chapter 1.

**Isolation of Sc from Infant Monkey Testes**

Infant monkey Sc were isolated using the procedure of Majumdar et al. (1998) with some modifications (Majumdar et al., 1998). Briefly, testes obtained after castration of infant monkeys were washed in HBSS. The testes were decapsulated and washed twice in HBSS before mincing. Minced tissue from the entire left and right testes was suspended in 12ml of HBSS and distributed into two 15ml Falcon tubes. The suspension was shaken by hand (5-6 times). Seminiferous tubules from the suspensions were recovered after sedimentation at unit $g$ for 5-7 min. The tubules were washed once more in HBSS and pelleted at 100g for 3 min. The pellet, consisting of the seminiferous tubules, was suspended in 15ml of prewarmed collagenase solution (1950U collagenase/15ml HBSS) with 250 Kunitz Units (KU) of deoxyribonuclease (Dnase) and the digestion was carried out at 32°C for 10 min in a shaking water bath at 140 oscillations per min. At the end of this digestion, a suspension comprised of small pieces of the seminiferous tubules was obtained. The tissue suspension was centrifuged for 5 min at 800 rpm (127g). The supernatant was discarded and the pellet was washed twice in 10ml HBSS (by keeping at unit $g$ for 5 min each). The pellet was resuspended in 15ml HBSS and the tubules were dispersed by mechanical shaking (140 oscillations per min) for 3 min at 32°C. The tubules were sedimented at unit $g$ for 5-7 min on ice and the pellet was digested again with 1300U collagenase/10ml of HBSS solution in presence of 250 KU of DNase for 15 min at 32°C. The tissue aggregates formed at the end of the reaction were discarded and the suspension was centrifuged at 100g for 5 min to collect the small pieces of tubules as well as large number of Sc clusters associated with the PTC. The pellet was washed twice
in HBSS (centrifuged at 100g for 5 min each). The pellet, after 2nd wash was suspended in 2.5 mg pancreatin/10ml of HBSS with 250 of KU of DNase at RT for 8-10 min shaking intermittently by hand. The distinctly visible aggregates were discarded and the suspension was kept on ice. Fetal calf serum (5%) was added to cold suspension to quench the action of enzyme. The suspension was then distributed in two 15ml falcon tubes and centrifuged at 100g for 5 min. The pellets were washed twice in 6ml HBSS/tube (centrifuged at 100g for 5 min). The cell pellet was resuspended in HBSS containing 5% FCS. The cell suspension was placed on ice for 5 min and, then filtered through 80μm filter mesh. The filtrate was centrifuged at 180g for 4 min and washed thrice with DMEM/F12 HAM culture media. The pellet was resuspended in 24ml of media with 1% FCS and plated 1ml each in 24 well tissue culture plate, at approximately 0.5 x 10^5 clusters/ml.

Isolation of Sc from Pseudoadult Monkey Testes

Testes, removed surgically using sterile procedures, were placed in cold HBSS. All further steps were carried out on ice or at 4°C unless the temperature was specified. The testes were weighed and washed twice in HBSS and decapsulated. The testes was washed again and minced into 5-6 pieces using a sterile surgical blade. The tissue pieces were washed twice with mild shaking by hand for 30 sec and pelleting at unit g. These preliminary steps were followed by series of enzyme digestion steps.

Step I: The pieces of tissue were digested in prewarmed collagenase solution (3900Ucollagenase/25ml of HBSS) in presence of 500 KU DNase at 33°C for 10 min at 120 oscillations per min in a shaking water bath. Upon digestion, the suspension was kept at unit g for 2 min. The supernatant was discarded and pellet was resuspended in HBSS.

Step II: The pellet from previous step was digested again with collagenase solution (3900Ucollagenase/25ml of HBSS) in presence of 500 KU DNase at 33°C for 13-15 min in a shaking water bath (120 oscillations/min). When the digestion was over, 1% FCS was added to the cell suspension and allowed to settle at unit g for 2-3 min. The supernatant (containing small pieces of tubules) was decanted and marked as "Supernatant I". The pellet was resuspended in 25ml of HBSS containing 3900U collagenase and 500 KU of DNase. The enzyme digestion was carried out at 33°C for 8-9 min in a shaking water bath at 120 oscillations per min. At the end of digestion, 1% FCS was added and the suspension was kept at unit g for 2-3 min. The supernatant was collected and marked as
"Supernatant II". The pellet was digested once more under the same conditions for 5-7 min and suspension was kept at unit g for 2-3 min. The resulting supernatant was collected as "Supernatant III" and the pellet (fibrous cell aggregate) was discarded.

**Step III:** The supernatants (Supernatant I, II and III) were centrifuged at 72 g (600 rpm) for 5 min immediately after respective collections. The pellets obtained were washed twice using 5ml of HBSS/tube/washing (by centrifuging at 72g for 5 min) and were kept on ice. The resulting pellets were resuspended in small volume of HBSS and cell suspensions were finally pooled and total volume was made up to 10ml with HBSS. Pancreatin (2.5 mg) and 250 KU of DNase were added to this cell suspension and enzyme digestion was carried out at RT for 8-10 min with continuous mild shaking by hand till a single cell aggregate was formed. This step was monitored carefully to avoid over-digestion of the cells (jelly-like formation). The reaction was stopped by adding 5% FCS and quenching on ice. The cell aggregate consisting mainly of PTc was discarded and the solution was distributed in two 15ml Falcon tubes. The tubes were centrifuged at 200g for 3 min and the resulting pellets were washed 3 times in 6ml HBSS/tube/washing (by centrifugation at 72g for 5 min). The pellet was resuspended in 3ml HBSS/tube; the cell suspensions from 2 tubes were pooled and filtered through 80µm stainless steel filter. The filtrate was centrifuged at 180g for 4 min and the resulting pellet was washed 3 times with DMEM/F12 HAM culture media (by centrifugation at 72g for 5 min). The cells were resuspended in media containing 1% FCS to make a concentration of 0.5 X 10⁵ cell clusters/ml. 1ml of cell suspension was plated per well of 24 well tissue culture plates.

**Culture Conditions**

Cells were maintained at 34°C in a humidified, 5% CO₂ incubator as described for rat Sc culture in *Chapter I*. On day 4 of culture, cells were exposed to hypotonic solution (20 mM Tris HCl, pH 7.4) to get rid of residual Ga, if any (Galdieri *et al.*, 1983).

**Hormone Treatment**

On day 5, 24 hrs after hypotonic shock, cells were treated with media alone, or different concentrations of recombinant monkey (rm) FSH (5, 10, 20ng/ml), T (10⁻⁷ M), or a combination of both rmFSH (5ng/ml) and T (10⁻⁷ M). Cells were also treated with 8-Br-cAMP (150µM) and Cholera toxin (1U/ml). To measure the amount of cAMP produced by these cells in response to these treatments, media were collected 30 min post treatment, incubated for 5 min in a boiling water bath to destroy endogenous phosphatase activity
and stored at -70°C. The wells were replenished with respective reagents for additional 23.5 hr. At the end, media were collected and stored for lactate and E2 assays.

**Cell Counting**

After the media was collected, Sc were washed with 0.5 ml pre-warmed (34°C) HBSS. Then, cells were digested with 200 μl of trypsin-EDTA solution (0.2 % trypsin, 0.05 mM EDTA)/well 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 1 ml. Cell suspension was shaken manually and 1% FCS was added. A fraction of cell suspension (10 μl) from each well was used for counting cells on a hemocytometer under 10X magnification. Rest of the suspension was centrifuged (805 g, 4°C, 4 min), resuspended in 500 μl of 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.

**Cytochemical Evaluation of the Cultured Cells**

Viability of the cells was checked by trypan blue staining in the culture wells as described in *Chapter I*. Purity of the cells were checked by staining methods as described in *Chapter I*.

1. Contamination of PTc by alkaline phosphatase activity (Chapin et al., 1987).
2. Contamination of Leydig cells by 3β-HSD activity (Klinefelter et al., 1987).
3. Sertoli cells were confirmed by Oil-Red'O staining.

**Biochemical and Immuno Assays**

**Lactate Assay**

Lactate present in the culture media was measured as described in *Chapter I*.

**Testosterone assay**

Plasma T levels of pseudoadult monkeys were measured by RIA as described in *Chapter I*.

**Cyclic AMP Assay**

Cyclic AMP concentrations in culture medium were analyzed as described in *Chapter I*.

**Estradiol Assay**

Estradiol was estimated using the protocol provided by WHO Matched Reagent Program as described in *Chapter I*. 
**Assay of the Bioactivity of the rmFSH Preparation**

To check for bioactivity of the rmFSH preparation used in the study Sc from the 18 days old rat were isolated and cultured as described in Chapter I. On day 5 of culture, cells were treated with plain media, media containing rm FSH (5ng/ml) or oFSH (50ng/ml) and incubated for 24hrs. At the end of the incubation, media was collected and stored for measuring lactate; the cells from each well were recovered by trypsin-EDTA digestion before cell count/ well was measured. The procedures followed for assaying lactate and measuring the cell count were as described in Chapter I.

**Androgen Binding Assay**

Androgen binding capacity of the infant and pseudoadult Sc was assayed on day 5 of culture. The procedure followed is described in Chapter I.

**RNA Isolation**

Total RNA was isolated from the Trizol treated samples as described in Chapter I.

**RT-PCR Analyses**

The RT PCR analyses were carried out as described in Chapter I. The housekeeping gene, Cyclophilin A, was used to check the relative expression of the genes. The list of genes probed along with the primer sequence and product size is given in TABLE 2.

**Real Time PCR Analyses**

Real Time PCR analyses for the quantitative comparison of expression levels of the Sc genes in the infant and pseudoadult was carried out using primers and probes designed using Primer Express (Applied Biosystems). The probes were designed for Taqman assay with FAM and TAMRA fluorescent tags. The housekeeping gene, β-glucuronidase (β-Gus), was used to calculate the relative expression of the genes probed. The validity of each assay was checked by running multiple concentrations of the sample cDNA template (100ng, 200ng and 400ng) in duplicates and calculating the number of cycles required for each concentration to reach the plateau for each gene. Similarly, along with each gene, multiple concentrations of the sample cDNA template were run for the housekeeping gene β-Gus. The sequences of the primers and probes used for our analyses are given in TABLE 3.
Table 2: Primer Sequences and product sizes used for RT-PCR analyses of Sertoli cell mRNA from infant and pseudoadult monkey

<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>AACATCAACCCTGAGGCTTC</td>
<td>ACCTGGCCCTCAGCTTTAA</td>
<td>415</td>
</tr>
<tr>
<td>AR</td>
<td>ACCTGTGTGCCAGCAGAAATG</td>
<td>TCCACGTGTAAGTTGGGAAG</td>
<td>415</td>
</tr>
<tr>
<td>Human Inhibinβa</td>
<td>AGCCTCCAGGATACCAGCAAA</td>
<td>TCAAACGGTCATTGCCCT</td>
<td>421</td>
</tr>
<tr>
<td>ODC</td>
<td>TTCCATGTAGGAAGCGGCTGT</td>
<td>AAGCCACCGCAAATATCAAGG</td>
<td>308</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>TCACCATTTCGACTGAGCC</td>
<td>ACAGGACATTGCGAGCAGA</td>
<td>120</td>
</tr>
</tbody>
</table>
Table 3. Primer and Probe Sequences Used for Real Time PCR Analyses of Sertoli Cell mRNA from Infant and Pseudoadult Monkey

<table>
<thead>
<tr>
<th>NAME</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
<th>PROBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHR</td>
<td>ATGCAGCTGGACGTCAAGG</td>
<td>GCAAAAATCCAGCCCATCAC</td>
<td>56-FAM/TCCGCCATGCTGCCCAGTCA/T/36-TAMTph</td>
</tr>
<tr>
<td>AR</td>
<td>ACATCAAGGAACTCGATCGATCA</td>
<td>AGCTGGTAGAAGCGTCTTGAGC</td>
<td>56-FAM/TGCAATGCAAAAAGAAAAATCCACATCC/36-TAMTph</td>
</tr>
<tr>
<td>β-Gus</td>
<td>CTCATTGGAGATTTTGCCATT</td>
<td>CCGAGTGAAGATCCCCCTTTTA</td>
<td>56-FAM/TGAACAGTCACCGACGAGAGT/GCT/36-TAMTph</td>
</tr>
<tr>
<td>SRC-1</td>
<td>TCCCTCGGTCAATCTAGTATCT</td>
<td>TGGATGGTGCCATGTG</td>
<td>56-FAM/CCAGCTCATGGTGCTCGTCTCA/36-TAMTph</td>
</tr>
</tbody>
</table>
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 and are reported in results. Various sets of experiments were conducted over the period of study (4 years). As the necessity of conducting additional experiments arose, more monkeys of a specific age group were used. Such variability in the source of testis (individual monkey-to-monkey variation within the same age group) is responsible for the variations in the units of the same parameter (assay end point). This is reflected by the differences in the range of the values denoted on the Y-axis of the parameters assayed. One culture of the infant and pseudoadult monkey represents the contribution of two testes of one monkey. In all, 12 infant and 12 pseudoadult juvenile monkeys were used for this study. Student’s T test, at a significance level of $p < 0.05$, was carried out to statistically analyze the data.

RESULTS

Histological Analysis of Infant and Juvenile Monkey Testes

Histological analysis of testis section of infant monkey showed only spermatogonial population of Gc (A dark and A pale) and the Sc in the seminiferous tubules with no lumen (Fig. 3A). A similar situation was found in juvenile monkey testis section except that it had more number of spermatogonia A than those found in infants (Fig. 3B).

Effects of GnRH Treatment to the Juvenile Monkey

Physical observation of the GnRH treated juvenile monkey’s testis revealed enlargement of testes by 2nd week of GnRH treatment. The basal circulating T levels of juvenile monkeys (before GnRH treatment) ranged between 0.27 to 0.72ng/ml. Weekly measurement of plasma T levels, pre- and post-GnRH pulse exhibited an episodic pattern of T secretion (Fig. 4). By the 4th week, plasma T levels in all animals reached that of adult levels (3-7ng/ml). The testicular weight increased from 0.2g in juveniles to 1.1g in pseudoadults.

The histological evaluation of the testis sections of these GnRH treated monkeys confirmed the initiation of spermatogenesis in them. The size of the seminiferous tubules increased as compared to that observed in the juvenile testis and spermatocytes (arrow)
Fig. 3 Cross sections of infant, juvenile and GnRH treated juvenile (pseudoadult) monkey testes. In infant monkey seminiferous tubule, only spermatogonia A (green arrow) and Sertoli cells are present. The diameter of the seminiferous tubules in the juvenile monkey was more than that observed in the infant monkey. The tubular diameter of pseudoadult monkey increased in size and various stages of germ cell differentiation are discernible, including spermatogonia B (blue arrow) and spermatocytes (black arrow). All three sections are at same magnification.
Fig. 4 Augmentation of T production due to pulsatile GnRH administration. The circulating T levels in the 1st, 2nd, 3rd and 4th week of GnRH treatment in three juvenile monkeys. 0 week of GnRH treatment denotes the basal T levels of these monkeys. Arrows indicate the time of GnRH infusion.
were observed in the seminiferous epithelium (Fig. 3C). These monkeys were termed as "Pseudoadult monkeys" which were the source of testis with hormonally responsive Sc.

**Viability and Purity of the Sc Cultured from Infant and Pseudoadult Testes**

A confluent monolayer of infant and pseudoadult monkey Sc in culture is shown in Fig. 5. Viability of the cells on day 6 of the culture was found to be more than 98%. The cultured cells from both the groups were negative for the 3β-HSD activity specific to Lc (Table 4). A confluent monolayer of Sc cultured from the infant and pseudoadult monkeys is shown in Fig. 5AB. Lipid droplets (stained by Oil-Red'O) were detected in more than 95% of the cells in the both the cultures (Fig. 5CD) confirming, at least 95% of the cells were Sc. Detection of the alkaline phosphatase activity, specific to the PTe, revealed less than 2% PTe contamination in the cultures from the infant monkey whereas the cultures from the pseudoadult monkey had about 1.5 % contamination of PTe (Fig. 5EF). These results are summarized in the table below.

**Table 4 Cytochemical Evaluation of Sertoli Cells Cultured from the Infant and Pseudoadult Monkey**

<table>
<thead>
<tr>
<th>Age of monkey</th>
<th>% of 3β-HSD positive cells</th>
<th>% of alkaline phosphatase positive cells</th>
<th>% of Oil-Red'O positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant</td>
<td>0</td>
<td>≤ 2</td>
<td>95</td>
</tr>
<tr>
<td>Pseudoadult</td>
<td>0</td>
<td>~1.5</td>
<td>95</td>
</tr>
</tbody>
</table>

**Cyclic AMP Production by Sc Cultured from the Infant and Pseudoadult Monkeys in Response to FSH and T**

In the Sc cultures from the infant monkey, treatment with rmFSH (5ng/ml) resulted in a significant (p< 0.05) increase in the levels of cAMP produced by these cells. Similar results were observed in wells treated with a combination of rmFSH (5ng/ml) and T. No significant rise in cAMP production by Sc isolated from the infant monkey was observed due to treatment of these cells with T alone (Fig. 6).

Similar to the observations in the Sc from the infant monkey, treatment of the Sc cultured from the pseudoadult monkey with rmFSH (5ng/ml) resulted in a significant (p< 0.05) rise in cAMP production by these cells. A combined treatment of rmFSH (5ng/ml)
Fig. 5 Sertoli cells cultured from infant A and pseudoadult B monkey testes stained with hematoxylin. Lipid droplet staining by Oil-Red'O confirmed more than 95% of the cultured cells are Sertoli cells in both the 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.
Fig. 6 Cyclic AMP production by Sc cultured from the infant monkeys in response to FSH and T. rmFSH = 5ng/ml of rmFSH; T= 10⁻⁷ M of T; rmFSH + T = 5ng/ml of rmFSH and 10⁻⁷ M of T.*<0.05
and T to the pseudoadult Sc also resulted in a significant (p < 0.05) rise in cAMP production whereas no change in from the basal cAMP levels was by these cells due to treatment with T alone (Fig. 7).

It is important to note that the levels of cAMP produced in response to treatment with FSH or a combination of FSH and T was significantly (p < 0.05) higher in the pseudoadult monkeys as compared to that in the infant monkeys.

**Aromatizing Ability of the Sc Cultured from the Infant and Pseudoadult Monkeys**

The ability of the Sc cultured from the infant as well as pseudoadult monkey to aromatize T to E2 could be demonstrated by providing T as a substrate to these cells. Further addition of FSH, along with T, did not result in an augmentation of the aromatization ability of the Sc from both the age groups (Fig. 8, 9).

**Lactate Production by Sc cultured from the Infant and Pseudoadult Monkeys in Response to FSH and T**

Treatment of the infant Sc with FSH (5ng/ml) did not lead to a rise in the basal levels of lactate produced by these cells. Treatment with T or a combination of FSH and T also did not augment lactate production by the infant Sc (Fig. 10).

As in case of the infant Sc, treatment of the pseudoadult Sc with FSH did not lead to an increase in the amount of lactate produced by these cells as compared to basal values. Treatment of the pseudoadult Sc with T or a combination of FSH and T also yielded similar results (Fig. 11).

Please note that treatment with FSH or a combination of FSH and T resulted in an increase in the levels of cAMP produced by the infant as well as pseudoadult Sc (Fig. 6, 7).

**Lactate Production by Sc cultured from the Infant and Pseudoadult Monkeys in Response to Increased Doses of FSH**

As observed earlier, treatment of the infant Sc with 5ng/ml FSH did not augment the lactate production in these cells. Treatment with 10 and 20ng/ml of FSH also failed to induce a rise in the amount of lactate produced basally by the Sc from the infant monkey (Fig. 12).
Fig. 7 Cyclic AMP production by Sc cultured from the pseudoadult monkey in response to FSH and T. rmFSH = 5ng/ml of rmFSH; T = 10^{-7} M of T; rmFSH + T = 5ng/ml of rmFSH and 10^{-7} M of T. * p<0.05
Fig. 8 Estradiol production by Sc cultured from the infant monkey in response to FSH and T. rmFSH = 5ng/ml of rmFSH; T = 10^-7 M of testosterone; rmFSH + T = 5ng/ml of rmFSH and 10^-7 M. T * p< 0.05
Fig. 9 Estradiol production by Sc cultured from the pseudoadult monkey in response to FSH and T. rmFSH = 5ng/ml of rmFSH; T = 10^{-7} M of testosterone; rmFSH + T = 5ng/ml of rmFSH and 10^{-7} M. T * p < 0.05
Fig. 10 Lactate production by Sc cultured from the infant monkey in response to FSH and T. FSH = recombinant monkey FSH 5ng/ml; T = testosterone 10^-7 M
Fig 11 Lactate production by Sc cultured from the pseudoadult monkey in response to FSH and T. FSH = recombinant monkey FSH 5ng/ml; T = testosterone $10^{-7}$ M
Fig. 12  Lactate production by Sc cultured from the infant monkey in response to increasing doses of rmFSH. F5= 5ng/ml of rmFSH; F10 =10ng/ml of rmFSH; F20= 20ng/ml of rmFSH.
Similar to our observations in the infant monkey, all concentrations of FSH (5, 10 and 20ng/ml), used to treat the pseudoadult Sc, did not augment the lactate production by these cells (Fig. 13).

**Lactate Production by Sc Cultured from the Infant and Pseudoadult Monkeys in Response to 8-Br-cAMP**

Similar to the effect of FSH treatment, treatment of the Sc from the infant monkey with the FSH analog, 8-Br-cAMP, also did not result in augmentation in lactate production. (Fig. 14).

In the pseudoadult monkey also treatment with 8-Br-cAMP did not induce a rise in the levels of lactate produced by the Sc from this age group, which was equivalent to the lactate produced by these cells in response to treatment with 50ng/ml of oFSH (Fig. 15).

**Bioactivity of the Recombinant Monkey FSH Preparation**

The Sc from the 18 days old rat were used as a test system to check the bioactivity of the rmFSH preparation used in the study. In response to treatment with 5ng/ml of rmFSH these Sc produced significantly (p< 0.05) high levels of lactate (Fig. 16).

**Expression of the InhibinβB Gene in Sc Cultured from the Infant and Pseudoadult Monkeys in response to FSH and T**

The Sc from both infant and pseudoadult monkeys expressed inhibinβB mRNA. The levels of inhibinβB were higher in case of the infant as compared to the pseudoadult (Fig. 17 lanes 1 and 5). Treatment of the Sc from the pseudoadult monkey with T resulted in a decline in the expression level of inhibinβB whereas similar treatment of the infant did not alter the inhibinβB expression (Fig. 17 lanes 3 and 7). Treatment of the pseudoadult Sc with FSH led to an increase in inhibinβB expression. Although the expression levels of inhibinβB mRNA were similar in the control and FSH and T treated wells of pseudoadult Sc, the inhibinβB expression in the FSH and T treated well was higher than that in the Sc treated with T alone (Fig. 17 lanes 5 to 8). Treatment with FSH or a combination of FSH
Fig. 13  Lactate production by Sc cultured from the pseudoadult monkey in response to increasing doses of rmFSH. F5= 5ng/ml of rmFSH; F10= 10ng/ml of rmFSH; F20= 20ng/ml of rmFSH
Fig 14 Lactate production by Sc cultured from the pseudoadult monkey in response to cAMP. FSH = 5ng/ml of rmFSH; cAMP = 150μM of 8-Br-cAMP.
Fig. 15 Lactate production by Sc cultured from the infant monkey in response to cAMP. FSH = 5ng/ml of rmFSH; cAMP= 150μM of 8-Br- cAMP
Fig. 16  Bioactivity of the rmFSH preparation as reflected from lactate production by Sc from the 18 days old rat in response to treatment with rmFSH. oFSH= 50ng/ml of oFSH; rmFSH= 5ng/ml of rmFSH.  * p<0.05
Fig. 17  RT-PCR analyses of mRNA expression in the Sc from the infant and pseudoadult monkey. A Expression of the inhibin$\beta_B$ mRNA in Sc the from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. B mRNA expression of the housekeeping gene, cyclophilin, in Sc the from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. For both panels, Lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 5ng/ml of rmFSH; 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 5ng/ml of rmFSH and $10^{-7}$M of T. Numbers on the right represent the size of the PCR product (in base pairs) of the respective genes. 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).
and T did not alter the expression levels of the inhibinβB mRNA in the Sc from the infant monkeys (Fig. 17 lanes 1 to 4).

**FSHR Expression in Sc Cultured from the Infant and Pseudoadult Monkeys**

Presence of FSHR mRNA could be detected by RT-PCR in the Sc from infant as well as pseudoadult monkey. The levels of FSHR mRNA were higher in the Sc control wells of the pseudoadult monkey as compared to that of the infant. Treatment with FSH or a combination of FSH and T resulted in a decline in the FSHR mRNA in the pseudoadult monkey. No decline in FSHR mRNA due to treatment with FSH, T or a combination of FSH and T could be observed in the Sc from the infant monkey (Fig 18).

**Quantification of the FSHR mRNA Expression in the Sc Cultured from the Infant and Pseudoadult Monkeys by Real Time PCR Analyses**

Real Time PCR analysis of the level of FSHR mRNA transcripts revealed that the expression of FSHR mRNA was approximately four-fold higher in the Sc from the pseudoadult monkey as compared to the FSHR mRNA expression in Sc from the infant monkey. Treatment with a combination of FSH and T results in the decline of the FSHR mRNA expression in the Sc from the pseudoadult monkey. In contrast, similar treatment does not alter the expression of FSHR mRNA in the Sc from the infant monkey (Fig. 19, 20).

**Cyclic AMP Production by Sc Cultured from the Infant and Pseudoadult Monkeys in Response to Cholera Toxin**

In a manner similar to the action of rmFSH, treatment with cholera toxin, a stimulator of G-protein coupled receptor pathway, resulted in a significant (p <0.05) rise in basal cAMP production by Sc cultured from the pseudoadult monkeys. This rise in cAMP production was equivalent to that observed due to treatment of the pseudoadult Sc with 5ng/ml of FSH (Fig. 21).

Similar to our observations in the pseudoadult monkey, cholera toxin treatment also induced a significant (p<0.05) rise in the amount of cAMP produced by the infant monkey Sc. It is important to note that treatment of infant Sc with rmFSH (5ng/ml) did not augment cAMP production by these cells (Fig. 22).
Fig. 18 RT-PCR analyses of mRNA expression in the Sc from the infant and pseudoadult monkey. A Expression of the FSHR mRNA in Sc from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. B mRNA expression of the housekeeping gene, cyclophilin, in Sc from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. For both panels lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 5ng/ml of rmFSH; 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 5ng/ml of rmFSH and $10^{-7}$M of T. Numbers on the right represent the size of the PCR product (in base pairs) of the respective genes. 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 Real Time PCR analysis of FSHR mRNA expression in the control wells of Sc from the infant and pseudoadult monkeys. Expression was quantified relative to the expression of the housekeeping gene β-Gus. Graph represents the mean expression levels in three monkeys. * p< 0.05

Fig. 20 Real Time PCR analysis of FSHR mRNA expression in the rmFSH (5ng/ml) and T (10^{-7} M) treated wells of Sc from the infant and pseudoadult monkeys. Expression was quantified relative to the expression of the housekeeping gene β-Gus. Graph represents the mean expression levels in three monkeys.
Fig. 21 Cyclic AMP production by Sc cultured from the infant monkey in response to FSH and cholera toxin. rmFSH = 5ng/ml of rmFSH; CT = 1U/ml of cholera toxin. * p< 0.05
Cyclic AMP production by Sc cultured from the pseudoadult monkeys in response to FSH and cholera toxin. rmFSH = 5ng/ml of rmFSH; CT = 1U/ml of cholera toxin. * p< 0.05
**AR Expression in Sc Cultured from the Infant and Pseudoadult Monkeys**

Presence of AR mRNA could be detected by RT PCR in the Sc from the infant and pseudoadult monkey. Treatment with FSH T, or a combination of FSH and T did not exhibit any variation in the mRNA expression of AR in the Sc from the infant as well as the pseudoadult monkey. (Fig. 23).

**Quantification of the Expression of the AR mRNA in the Sc Cultured from the Infant and Pseudoadult Monkeys by Real Time PCR Analyses**

No differences in the expression levels of the AR mRNA were observed in the Sc from the infant and pseudoadult monkey. Treatment of the Sc from both the developmental stages with a combination of FSH and T too did not alter the expression of AR (Fig. 24, 25)

**Androgen Binding Ability of the Sc Cultured from the Infant and Pseudoadult Monkeys**

To compare the number of AR present in the Sc from the infant and pseudoadult monkeys, androgen binding assay was carried out in the Sc cultured from both the age groups. The specific androgen binding ability of the pseudoadult Sc was significantly (p< 0.05) higher than that of the Sc from the infant monkey (Fig. 26).

**Quantification of the mRNA Expression of the AR Co-activator, SRC-1, in the Sc Cultured from the Infant and Pseudoadult Monkeys by Real Time PCR Analyses**

Real Time PCR analyses to quantitate the expression of the AR co-activator, SRC-1, in the Sc from the infant and pseudoadult monkey revealed a two-fold higher expression of SRC-1 mRNA in the pseudoadult Sc in response to treatment with a combination of FSH and T as compared to that observed in the infant Sc subjected to similar treatment *in vitro* (Fig. 27).
Fig. 23 RT-PCR analyses of mRNA expression in the Sc from the infant and pseudoadult monkey. A Expression of the AR mRNA in Sc the from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. B mRNA expression of the housekeeping gene, cyclophilin, in Sc the from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. For both panels lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 5ng/ml of rmFSH; 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 5ng/ml of rmFSH and 10^{-7}M of T. Numbers on the right represent the size of the PCR product (in base pairs) of the respective genes. 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. 24 Real Time PCR analysis of AR mRNA expression in the control wells of Sc from the infant and pseudoadult monkeys. Expression was quantified relative to the expression of the housekeeping gene β-Gus. Graph represents the mean expression levels in three monkeys.

Fig. 25 Real Time PCR analysis of AR mRNA expression in the rmFSH (5ng/ml) and T (10⁻⁷M) treated wells of Sc from the infant and pseudoadult monkeys. Expression was quantified relative to the expression of the housekeeping gene β-Gus. Graph represents the mean expression levels in three monkeys.
Fig. 26  Androgen binding ability of Sc from the infant and pseudoadult monkeys. Each bar denotes mean ±SEM of one monkey. a is significantly different from b. p< 0.05. n=4 infants and 4 pseudoadults
Fig. 27 Real Time PCR analysis of AR co activator SRC-1 mRNA expression in the rmFSH (5ng/ml) and T (10^{-7} M) treated wells of Sc from the infant and pseudoadult monkeys (n=3 each). Expression was quantified relative to the expression of the housekeeping gene β-Gus. Each bar represents the mean ± SE from three monkeys. * p<0.05
Ornithine decarboxylase Expression in Sc Cultured from the Infant and Pseudoadult Monkeys in Response to FSH and T

Expression of the ODC mRNA could be detected in the Sc from both the developmental stages (Fig. 28 lanes 1 to 8). The expression of ODC mRNA in the Sc from the pseudoadult monkey declined due to treatment with T, a further decline in the expression in these cells was noticeable in response to treatment with a combination of FSH and T (Fig. 28 lanes 7 and 8). No change in ODC expression was discerned in the Sc from the pseudoadult monkey due to treatment with FSH alone (Fig. 28 lane 6). In the Sc from the infant monkey no alteration in the expression of ODC could be detected due to similar hormone treatments (Fig. 28 lanes 1 to 4).

DISCUSSION

In primates, the hypothalamic-pituitary-testicular hormonal axis is transiently activated during the first few postnatal months (infancy) as a result circulating levels of gonadotropins (LH and FSH) and gonadal hormones (T and inhibin) are elevated (Bergada et al., 1999a; Burger et al., 1991). However, spermatogonial development is restricted at this age. Histological analysis of the testicular sections of the infant monkey showed presence of Sc and only spermatogonial population in the seminiferous tubules. This observation confirmed that the testis of this age group is spermatogenetically inactive, despite exposure to an adult-like hormonal milieu. Therefore, Sc isolated from the testis of the infant monkeys were considered to be “immature” and the status of the hormone receptor signaling pathway in these cells was compared with that in the Sc from the spermatogenetically active (pubertal/adult) testis. The procedure of Sc isolation and culture followed by us gave high yield of Sc; also PTc contamination of the infant monkey Sc culture was less (2%) than previously reported (4%, Majumdar et al., 1998). We did not attempt the culture of Sc from adult testis because a) the yield of the cells are known low due to the large amount of Gc associated with Sc in the seminiferous tubules and b) the cultured cells do not divide even after 8 days of culture, unlike infant and juvenile Sc (Majumdar et al., 1998). Hence, we chose to culture Sc from pubertal animals. Since, it is difficult to detect the exact timing of the onset of puberty and there are individual variations in this phenomenon, we induced precocious puberty in 18-24 months old juvenile monkeys, with dormant hypothalamo-pituitary-testicular axis, by intermittent pulsatile GnRH infusion. In previous reports, testes of animals subjected to
Fig. 28 RT-PCR analyses of mRNA expression in the Sc from the infant and pseudoadult monkey. **A** Expression of the **ODC** mRNA in Sc from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. **B** mRNA expression of the housekeeping gene, **cyclophilin**, in Sc from infant (lanes 1-4) and pseudoadult (lanes 5-8) monkey. For both panels lanes 1 and 5 represent control Sc wells; lanes 2 and 6 represent Sc well treated with 5ng/ml of rmFSH; 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 5ng/ml of rmFSH and $10^{-7}$M of T. Numbers on the right represent the size of the PCR product (in base pairs) of the respective genes. 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).
this treatment have shown to elicit a pattern of circulating T and immunoactive inhibin concentrations similar to those produced by spontaneous testicular secretion in adults (Abeyawardene et al., 1989). The juvenile animals undergoing such intermittent pulsatile GnRH infusion, in our study, exhibited an adult-like pattern of circulating T concentration, by the end of 4th week of treatment, which was similar to that reported earlier (Abeyawardene et al., 1989). This treatment also resulted in a significant increase in the testicular weight of these animals. The histological analysis of testicular sections of these GnRH treated animals showed presence of primary spermatocytes indicating initiation of Gc development and differentiation. Similar to the observations of Abeyawardene et al (1989) Sc, stem (type Ad and Ap) and differentiated (type B) spermatogonia were discernable in the testicular sections. A few prophase I spermatocytes were also encountered during the histological examination. Such animals were termed as “Pseudoadult”. Sertoli cells, isolated from the testes of such monkeys were considered to be “mature”, since they displayed all signs of spermatogenic initiation in response to gonadotropins. The isolation of Sc was less cumbersome in the pseudoadults as compared to adults, as these testes possessed relatively less numbers of advanced Gc. The Sc at this stage proliferated rapidly in culture and confluent Sc monolayers could be obtained by day 5 of culture. The ability of the pseudoadult Sc to support spermatogenesis and to proliferate efficiently, *in vitro*, rendered the pseudoadult monkey as a model, better than adult Sc, to study the functions of mature Sc. Presence of less than 1.5% of PTC contamination in these Sc cultures was also advantageous. Having established the ‘immaturity’ of the Sc from the infant monkey *vis-a-vis* the “maturity” of the Sc from the pseudoadult monkey we proceeded to compare the status of FSH and T mediated signal transduction pathways in these two developmental stages.

**FSH Mediated Signaling in the Infant and Pseudoadult Sc**

In response to treatment with FSH, the Sc from the pseudoadult monkey produced significantly high levels of cAMP. Production of cAMP in response to FSH is an event consequent to the interaction of the hormone with its receptor (Simoni et al., 1997). Hence, our observations established the presence as well as functional status of the FSHR in the Sc from the pseudoadult monkeys. In the infant Sc, the response to FSH, in terms of cAMP production, was remarkably lower than that found in the pseudoadult Sc. This suggests that, as compared to the pseudoadult Sc, the FSHR in the infant Sc are either less in number or activity. The reports of Huhtaneimi et al (1987), demonstrating a specific
high affinity binding of radiolabeled FSH in homogenates of testes from 19-22 week of gestation up to birth in the rhesus monkey supports our observation of the presence of FSHR during infancy. However, no cAMP response was detected in fetal tissues exposed to FSH (Huhtaniemi et al., 1987). Absence of a cAMP response in the face of the reported hormone-receptor interaction in the fetal testes indicates a defective FSHR signaling in the fetal period. For the first time, we report that the FSHR signaling pathway is compromised in Sc from the infant monkey. Proliferation of primate Sc, in utero, is a gonadotropin-dependant phenomenon as fetal anencephaly in man and fetal hypophysectomy in the rhesus monkey leads to a marked reduction in testicular size (Baker and Scrimgeour, 1980a; Gulyas et al., 1977b). That FSH may be the primary stimulus for the Sc proliferation in the primate testis is suggested by the report of FSH mediated regulation of Sc proliferation in the rat (Orth, 1984b). The report of gonadotropin requirement for primate Sc proliferation (an FSH-dependant phenomenon in the rat) along with the absence of cAMP (produced as a result of FSH stimulation) response in the fetal Sc brings to light the controversy regarding the role of FSH in primate spermatogenesis (Huhtaniemi et al., 1987; Plant and Marshall, 2001). Till now the situation in the fetal testis as regards the FSH signaling and Sc proliferation could be attributed to the ontogenic differences in the fetal and postnatal development as reflected by the studies in rat and mice (Baker and O'Shaughnessy, 2001; Migrenne et al., 2003). Our report of limited FSH signaling in the Sc from the infant monkey coupled with reports of proliferation of Sc in the postnatal period (Cortes et al., 1987; Kluin et al., 1983), highlights the necessity for a more detailed examination of the role of FSH for Sc proliferation in primates. However, requirement of FSH for Sc proliferation may be less than that for other spermatogenically important phenomenon in the infant testis. This also raises a question on the validity of extrapolating the observations in the rodents to the primate system keeping in mind the total dissimilarity in the patterns of hypothalamic-pituitary-testicular axes in these two species. Until the action of FSH on the neonatal/infant Sc is more elucidated in the primates; one may presume that the slight rise in the cAMP upon FSH stimulation is sufficient for the Sc proliferation in this developmental (infant) stage. This would indicate the ability of the infant Sc to transmit the FSH signal to its functional end point. Acting on this presumption we scored for FSH signaling events occurring at the post-cAMP stage. To this end we checked the ability of FSH to regulate the production of E₂ and lactate, the two Sc metabolites essential for Gc metabolism and survival (Jutte et al., 1983a; Newton et al., 1992; Steinberger et al., 1978).
The necessity of E2 for male fertility is well established. Mice with KO of the P450 aromatase gene 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). In men it has been shown that aromatase deficiency consecutive to a P450 aromatase gene mutation leads to sterility (Carani et al., 1997; Morishima et al., 1995). Estradiol is now considered as a survival factor for Gc in human testis (Carreau et al., 2003; Pentikainen et al., 2000a). Presence of ERα and ERβ in testicular Gc and spermatozoa suggests the involvement of E2 in the Gc development (Aquila et al., 2004). A blockade in the spermatid maturation has been observed in male monkeys treated with aromatase inhibitor (Shetty et al., 1997). The importance of E2 in primate spermatogenesis is highlighted by the fact that lack of E2 or ER activity is associated with infertility in men (Carani et al., 1997; Morishima et al., 1995). Very recently, it has been shown that, in irradiated rats, E2 treatment induces Gc differentiation in 88% of seminiferous tubules (Shetty et al., 2004). In the 18-20 days old rat, the aromatization of T to E2 is reported to be under the control of FSH (Newton et al., 1992). The failure of FSH to regulate the aromatization of T to E2 in the infant Sc led us to believe that the FSH signaling may not be optimally functional in these cells. This failure of FSH to augment the aromatization of T to E2 in the pseudoadult Sc, questioned the role of FSH in regulating the activity and/or the expression of aromatase in the primate Sc. Our observation of the FSH-independent aromatization of T to E2 in the Sc from the 9 and 40 days old rat (Chapter I, Fig. 13) support the notion of a constitutive activity and/or the expression of aromatase enzyme in the primate Sc from both the age groups.

Another Sc metabolite important for Gc survival is lactate, the principle source of energy for Gc (Grootegoed et al., 1984; Jutte et al., 1983a) where it is used as a substrate for ATP production in mitochondrial oxidative phosphorylation (Grootegoed et al., 1984). Deprivation of lactate reduces the viability of male Gc in rats (Trejo et al., 1995). A mechanism for the transport of lactate into the Gc has also been reported (Boussouar et al., 2003). Erkkila K et al (2002) demonstrated a prevention of Gc apoptosis in 70% of human seminiferous tubules upon treatment with lactate, in vitro. Treatment with a higher concentration of lactate effectively blocked the Gc apoptosis in their study (Erkkila et al., 2002). Injection of lactate in cryptorchid testis of adult rats was also shown to result into a significant rise in sperm production (Cortes et al., 1987). Past literature convincingly establishes the effect of FSH on the lactate production by the Sc (Jutte et al.,
The FSH-independent production of lactate by the Sc from the pseudoadult monkeys (where FSH response in terms of cAMP production is evident) raised doubts of the efficacy of the amount of FSH (5ng/ml) used in our study. Although FSH at a concentration of 5ng/ml does lead to a significant rise in the cAMP levels of the Sc from the pseudoadult monkey, this rise in cAMP may still be under the threshold required to trigger a full-scale sequence of signal transduction events occurring in response to FSH. Failure of the increased concentrations (10 and 20ng/ml) of FSH to augment lactate production by the Sc from the infant as well as pseudoadult monkeys raised doubts regarding the bioactivity of the FSH preparation used by us. The ability of the rmFSH preparation to significantly augment lactate production by the Sc from the 18 days old rat (used as a test system) confirmed the bioactivity of FSH. Thus the absence of an increase in lactate production by the infant and pseudoadult Sc suggests a FSH-independent production of lactate in the Sc from both the developmental stages. This FSH-independency was convincingly confirmed by the failure of exogenous cAMP to induce a rise in lactate production by the Sc from the infant as well as pseudoadult monkeys. The failure of FSH and/or exogenous cAMP to regulate production of lactate by the infant Sc might be attributed to a deficit lying in these cells; however, absence of modulation of lactate production in the pseudoadult Sc due to the same treatments, suggested a constitutive production of lactate in the primate Sc. Such an absence in modulation of lactate production by FSH has been reported in Sc from the adult rat, which has been attributed to the high activity of PDEs in the adult Sc (McLachlan et al., 1996b; Means et al., 1980). In the previous chapter, FSH-mediated regulation of lactate production by the Sc from the 40 days old rat in the presence of the PDE inhibitor, IBMX was reported (Chapter I, Fig. 16). However, even in the presence of IBMX, no such FSH-mediated regulation of lactate production could be observed in the primate Sc. Since we could not detect a FSH action in the primate Sc at the level of the Sc metabolite production, we attempted to evaluate the FSH response at the level of transcription. This approach to study the FSH response is better suited to primate studies due to the limitations in obtaining Sc in large numbers; as little as 1μg of RNA is sufficient to study the expression of a number of genes. Interaction of FSH with its receptor is known to trigger a sequence of signal transduction events resulting in the phosphorylation of a number transcriptional activators which ultimately leads to the transcription of the FSH-inducible genes (Simoni et al., 1997). One of the highly characterized Sc molecules,
produced in response to FSH is inhibin (Welt et al., 2002). Serum measurements in males suggest that inhibinB of the two forms of inhibin (inhibin A and B), secretion of inhibinB (a dimer of the inhibin α and βB) is regulated by FSH stimulation (Anawalt et al., 1996). The observation of higher expression of inhibinβB in the infant Sc as compared to that in the pseudoadult was surprising. The decrease in the expression (as revealed by RT-PCR analyses) of inhibinβB, below the control levels, in the response to treatment with T in the Sc from the pseudoadult monkey was also unexpected. It is important to note that expression of the inhibinβB mRNA (which was higher than that in the pseudoadults) was not regulated by hormone treatments in the infant Sc; whereas similar treatments to the pseudoadult Sc altered the level of inhibinβB transcripts indicating increased FSHR activity in the pseudoadults. This also proves that expression of inhibinβB mRNA is independent of FSH in infants, this does not necessarily mean that the post-transcriptional events are same in the infants and pseudoadults. Previous studies in the rat suggested the FSH-mediated stimulation of inhibinB secretion, however, expression of βB subunit of inhibin, in vitro or in vivo, was shown to be independent of FSH action (Bicsak et al., 1987; Krummen et al., 1989; Morris et al., 1988). For the first time we report the FSH-mediated regulation of inhibinβB mRNA expression in primate Sc. Exposure to FSH may lead to a down regulation of the FSHR, a feature common to all G-protein coupled receptors termed as receptor desensitization (Simoni et al., 1997). Analyses of the FSHR mRNA expression in the infant and pseudoadult Sc revealed the down regulation of the FSHR in the pseudoadult Sc due to treatment with FSH, whereas no such effect is observed in the case of the infant Sc, again reiterating that FSHR in the pseudoadult Sc is more active than that in the infant Sc. Although treatment with FSH resulted in a rise in inhibinβB expression in the pseudoadult Sc, it led to a decline in the expression of the FSHR upon a 24hr period of exposure. The increase in inhibinβB expression in the face of decline FSHR expression, at first seems to be in contrast to the previously reported increase in inhibinB levels arising due to an increase in circulating FSH (Arslan et al., 1993; Fingscheidt et al., 1990). Looking into the dynamics of inhibinB secretion, Majumdar et al (1997) have reported an immediate elevation in circulating FSH levels due to its infusion in the juvenile (a period where levels of endogenous LH and FSH are undetectable) monkey; however, a significant rise in immunoactive inhibin was observed only after 9-21hrs of the FSH infusion. Withdrawal of FSH infusion led to an immediate decline in the circulating FSH levels but the elevated levels of inhibinB in their study
permitted in these animals for up to 24hrs after the withdrawal of FSH. A similar latency in correlation between an increase and a decrease in the FSH stimulus and the respective response of testicular inhibin secretion, as reflected by circulating inhibinB concentrations, has been observed in normal men (Anawalt et al., 1996). Down regulation of the expression of FSHR due to prolonged FSH exposure to the Sc results in limiting the responses of these cells to the FSH stimulus (Knecht et al., 1983; Simoni et al., 1997).

In the pseudoadult Sc, although FSHR were decreased, the rise in inhibinB expression after 24hrs is probably due to stimulation of these Sc with FSH occurring before the induction of FSHR down regulation. Hence, increased inhibinB expression in the face of a reduced FSHR expression at 24hr of FSH treatment might be attributed to the previous exposure of Sc to the hormone (FSH) before the down regulation of FSH has occurred. Evaluation of inhibinB expression at 24-48hrs after the withdrawal of FSH might represent the true regulation of this molecule by FSH. However, a rise in inhibinB upon FSH treatment clearly suggests that in the pseudoadult Sc FSHR is active and treatment of FSH may regulate inhibinB production. Further long-term studies evaluating the time-course of inhibinB mRNA expression following the addition and withdrawal of FSH from the Sc in culture will help in correlating the kinetics of expression of inhibinB mRNA and the secretion of inhibinB in response to FSH in primates. The T induced rapid decline in inhibinB expression in the pseudoadult monkey suggests a negative regulation inhibinB production by T, in the absence of FSH. This may be a mechanism of limiting the persistence of inhibinB after the decline of circulating FSH. Our observation of a rescue of inhibinB expression in the pseudoadult Sc due to a combined FSH and T treatment, as opposed to its decline due to treatment with T, lends weight to the above argument. Ramaswamy et al (2003), using adult male rhesus monkeys which were rendered hypogonadotrophic-hypogonadal by administration of a GnRH receptor antagonist (acycline), demonstrated a marked T induced suppression of inhibinB secretion. Similarly, infusion of FSH for 11 days in juvenile monkeys induced an increase in the circulating levels of inhibinB, whereas infusion of single-chain recombinant human LH (resulting into elevated T levels) to the juvenile monkeys resulted in the suppression of inhibinB levels (Ramaswamy et al., 2003). Infusion of FSH along with the single-chain recombinant human LH to these animals failed to completely reverse the LH-induced suppression (Ramaswamy et al., 2003). Though the authors were able to correlate the decreased circulating inhibinB levels due to T treatment with a corresponding decline in
the levels of inhibin\(\beta_B\) expression in the testicular homogenates, an increase in the expression of inhibin\(\beta_B\) corresponding to the rise in circulating inhibinB levels due to FSH treatment was not discernible (Ramaswamy et al., 2003). Our observation in the Sc, in vitro, agree with report of Ramaswamy et al (2003), in vivo, and suggests that inhibinB secretion by the monkey testis is governed by the inhibitory and stimulatory action of T and FSH. In their study, Ramaswamy et al (2003) reported the absence of FSH-mediated increase in the mRNA expression of inhibin\(\beta_B\); however, in our study this rise could be observed. This might be due to the fact that the source of RNA, for RT-PCR analyses carried out in our study, was from pure Sc cultures rather than the testicular homogenates, hence might be a more sensitive and pure system to assay the Sc specific responses.

Our study indicated a low FSHR mRNA expression in the infant as compared to the pseudoadults, which might be the primary reason of the absence of a FSH response in these cells. Hence, we proceeded to quantify the level of FSHR transcripts in the Sc from both the developmental stages. Real Time PCR analysis revealed 8-10 fold lower expression of the FSHR in the control wells of infant Sc, as compared to that in the pseudoadult Sc, confirming our RT PCR observations. The Real Time PCR analysis also confirmed our observation of down regulation of FSHR mRNA in the pseudoadult Sc due to treatment with a combination of FSH and T. However, no change in the expression levels of FSHR mRNA in the FSH and T treated infant Sc further emphasizes the lack of FSH action in these cells. In all, our observations suggest that, in the infant Sc, the low expression of FSHR is responsible for the limited cAMP response, rather than the inactivity of the receptors and/or the molecules lying between the receptor and adenylyl cyclase in the FSH signal transduction cascade. This hypothesis was considerably substantiated by our observation of a significant rise in cAMP production by the infant Sc due to addition of cholera toxin, an irreversible stimulator of the intracellular G\(_{as}\) subunit of the G protein coupled FSHR. Taken together, the low level of FSHR expression in the Sc of infant primates may primarily be responsible for the lack of FSH-mediated initiation of spermatogenesis and quiescence of the seminiferous epithelium at this stage of development when the endocrine activity of the hypothalamic-pituitary-testicular axis is robust.
**Androgen Mediated Signaling in the Infant and Pseudoadult Sc**

The similar levels of expression of AR mRNA, as shown by RT PCR analysis, in the infant and pseudoadult Sc indicated that the androgen signaling pathway in the Sc from both the developmental stages may be equally functional. Quantitative measurements of AR mRNA levels by Real Time PCR analysis confirmed the RT PCR findings. The RT-PCR as well as the Real Time PCR results also revealed the absence of FSH and T mediated regulation of the AR mRNA levels in the primate Sc. This finding was surprising, since FSH and T are reported to regulate the AR expression in the rat Sc (O'Shaughnessy et al., 1992; Sanborn et al., 1991; Vihko et al., 1991). The absence of FSH-mediated regulation of the AR mRNA expression, specially in the pseudoadult monkeys, further questions the role of FSH in primates. Suire et al (1997) have demonstrated that in the adult rat, even though the expression of transferrin mRNA was lower than that observed in the infant, level of transferrin protein was more in the adults. The authors attributed the higher levels of transferrin protein to the increased rate of translation of the mRNA in the adult (Suire et al., 1997). Similarly, action of T to regulate the AR in the primate Sc may occur at the post-transcriptonal or translational level rather than at the level of transcription. There is little information available pertaining to the expression of AR protein during primate development. The only study addressing this issue in primates reported a low immuno-expression of AR in the neonatal Sc from the marmoset monkeys, which increased substantially in infant animals, reaching adult levels by the end of infancy (McKinnell et al., 2001). However, the authors have not shown any T-mediated direct regulation of AR immuno-expression in the infant and neonatal marmoset monkeys (McKinnell et al., 2001). The report of McKinnell et al (2001) combined with the observation of similar AR mRNA expression in the Sc from the infant and pseudoadult rhesus monkeys suggest that although AR mRNA expression may be similar during infancy and adulthood, but it may not be translated into AR (protein) like those in adult primates. However, this study design was unable to determine a) the correlation between the immuno-detection of steroid receptors on tissue sections and their biological activity and b) whether or not those receptors are activated by ligands binding. The low androgen binding ability of the infant Sc as compared to that the pseudoadult Sc in the face of similar levels of AR mRNA expression, for the first time, demonstrated the deficit in AR action in the infant Sc, which may explain the lack of spermatogenic activity in the phase of adult-like hormonal milieu during this stage of development. This
is the first study reporting the limited androgen binding ability of the Sc in the infant primate. The assay of the androgen binding ability of the Sc also quantifies the number of androgen binding sites, i.e. AR, in these cells. Thus, this observation suggests that either the AR mRNA fails to get translated or, if translated, the AR protein does not acquire its functional conformation. The absolute necessity of T for spermatogenesis has been reported by a number of studies (McLachlan et al., 2002; Sharpe, 1994b). The inability of the AR of the infant monkey to efficiently bind T may account for the absence of spermatogenesis in this age group. Androgen receptor function is required for the male embryonic sexual differentiation, pubertal development and the regulation of spermatogenesis (Holdcraft and Braun, 2004b). The necessity of AR to potentiate the action of T in the testis is highlighted by the report of Yeh et al (2002) demonstrating the development of tfm syndrome in mice lacking the functional AR. The variable action of T on the testicular cells is demonstrated by the studies of Yeh et al (2002) and De Gendt et al (2004), who report the a complete androgen insensitivity phenotype of the ARKO mice whereas the mice with a Sc-selective KO of AR display normal development of the urinogenital tract and testis descent, however spermatogenesis is extremely compromised in the Sc-ARKO testis. The Sc-ARKO mice are infertile, with spermatogenic arrest predominately at the diplotene premeiotic stage and almost no sperm detected in the epididymides (Chang et al., 2004). As in the case of the rat, we attempted to analyze the functional status of AR by transfection of the MMTV-Luc construct (See Chapter I Discussion) in the infant and pseudoadult Sc (not described in materials and methods section). But we were unable to deliver the construct in our cultures due to the difficulties in transfecting primary cell cultures. Reports exist demonstrating the difficulties in delivering genes to the primary cells (Hamm et al., 2002). Though a variety of methods (adenovirus mediated transfection, Calcium phoshate precipitation and packaging in viral envelopes) of transfection were tried by us, we failed to transfect the MMTV-Luc construct in the Sc. For a steroid hormone receptor, binding of the receptor to its ligand is an essential prerequisite for the initiation of its signaling cascade (Keller et al., 1996). A limited androgen binding ability of AR in the infant Sc is by itself indicative of a limited AR function in this developmental stage, hence, aforementioned experiments using MMTV-Luc might not have provided us any new information about androgen action. Androgen receptor dimerization, nuclear localization, binding of AR to its cognate response element and recruitment co-regulators by the AR to promote the expression of target genes are the events occurring subsequent to the binding of T to its receptor (He et
The first reported AR co-activator is SRC-1 (Onate et al., 1995). This coactivator (SRC-1) functions in transcriptional activation through its histone acetyltransferase activity (HAT) and multiple interactions with the hormone-bound receptors (Xu et al., 1998). The role of SRC-1 in facilitating the action of T is demonstrated by the reduced tissue responses to T in mice lacking SRC-1 (Xu et al., 1998). The importance of coactivators in bridging receptor activation functions with the basal transcription machinery to stimulate trans-activation has been demonstrated (Glass and Rosenfeld, 2000). The observation of a significant difference in the expression levels of the AR co-activator, SRC-1, in the infant and pseudoadult monkeys, as revealed by Real Time PCR analysis, further demonstrates the limited AR signaling in the infant Sc. Since co-activators are reused in the transcription machinery, a marginal change in their expression may also cause a significant difference in the end point. An intact AR signaling results in the regulation at the level of gene expression and protein synthesis in the Sc (McPhaul, 2002). Till date only a limited number of androgen-regulated genes in Sc have been studied (Benbrahim-Tallaa et al., 2002a; Sadate-Ngatchou et al., 2004; Sutton et al., 1998c; Zhou et al., 2005). One of these T regulated genes is the one coding for the enzyme ODC (Weiner et al., 1990). Ornithine decarboxylase is the initial, rate-limiting enzyme in the biosynthetic pathway for polyamines. In general, polyamines play an important role in the structure and function of DNA (Fillingame et al., 1975; Mamont et al., 1976; Sunkara et al., 1977; Sunkara et al., 1979) and in RNA (Kuehn and Atmar, 1982; Tsang and Singhal, 1978) as well as protein synthesis (Bachrach et al., 1973; Russell et al., 1978; Williams-Ashman and Canellakis, 1979). Elevation of ODC activity is closely associated with cell growth, proliferation, and differentiation (Slotkin, 1979; Russell, 1980), while reduction in ODC activity is an early biochemical signal of cessation of tissue growth and organ maturation (Janne et al., 1978). Weiner et al. (1990) have reported the decrease in expression of the ODC mRNA in the rat Sc due to treatment with T. The observation of decreased expression of the ODC mRNA in the Sc from the pseudoadult monkeys due treatment with T indicates the ability of these cells to respond to the T stimulus. Failure of T to modulate the ODC mRNA expression in the infant Sc as compared to that in the pseudoadult Sc, further confirms the inactive status of the AR in these cells. Lack of information regarding a suitable end point of T action has been a handicap in studying the effects of T in vitro. This landmark information about a T regulated factor in primate Sc would essentially provide an measurable end point to study the action of T in primates. The study thus reveals for the
first time that the androgen-mediated signaling is compromised in the infant Sc and the primary deficit in the lies in the limited activity of AR in Sc of the infant testis.

**CONCLUSIONS AND FUTURE DIRECTIONS**

The observations regarding the status of molecules involved in the androgen and FSH-mediated signal transduction cascades in the spermatogenically inactive and active Sc reveal that the primary deficit of the spermatogenically inactive Sc lies at the level of the respective hormone receptors. Though this study addresses the controversy regarding the significance of FSH in spermatogenesis, the reports of compensation by hormones and intratesticular regulatory factors, at least in part, for the absence of some hormones or factors, including FSH (Abel et al., 2000; Dierich et al., 1998a; Kumar et al., 1997) and androgen (Chang et al., 2004; De Gendt et al., 2004a; Holdcraft and Braun, 2004a; Yeh et al., 2002), or luteinizing hormone (Zhang et al., 2004) receptors may not be ruled out. Thus, it is likely that synergism and/or redundancy between regulatory molecules is a characteristic of the spermatogenic process, on which depends species survival. Recent report of Marshall et al. (in press) demonstrates the gonadotropin independent proliferation of type Ap spermatogonia in the adult rhesus monkey; whereas the differentiation of type Ap spermatogonia to B spermatogonia was shown to be an absolutely gonadotropin dependent by these authors (Marshall et al., 2005). However, either of the hormones, FSH or T, may provide the hormonal drive for the differentiation of spermatogonia Ap to type B spermatogonia (Marshall et al., 2005). This study thus demonstrates the ability of FSH and T to compensate for each other even in primates. The observation of a lack of FSH response in the infant Sc contradicts the reports of Sc proliferation, a process reported to be dependant on FSH, during this period of primate development. However, discernible rise in cAMP due to FSH treatment to the Sc from the infant monkeys may indicate that a low level of FSHR activity is sufficient to trigger Sc proliferation. The recent reports of T-mediated proliferation of the rodent and primate Sc may also account for Sc proliferation in infant monkeys (Arslan et al., 1993; Haywood et al., 2003; Johnston et al., 2004a; Ramaswamy et al., 2000). The study reveals a compromised AR activity in the infant Sc; however, the recent reports of the non-genomic actions of androgens might provide an explanation for the infant Sc, possessing inactive AR, to exhibit a T-dependant response with respect to Sc proliferation (Heinlein and Chang, 2002b). These non-genomic actions of AR lead to the activation of mitogen-activated protein kinase and CREB protein, a phenomenon classically occurring in cells
due to the activation of the G-protein coupled receptor (Fix et al., 2004; Simoni et al., 1997). Thus it is possible for T to dually activate a cell a) by triggering the steroid receptor signal cascade though its classical genomic action and b) by triggering the G-protein-cAMP pathway through its non-genomic action. These findings add to the controversy regarding the role of FSH in spermatogenesis. The observations of the FSH-independent aromatization (conversion of T to E₂) and production of lactate by the primate Sc contribute to furthering this controversy. However, the reports of absolute necessity of FSH at certain steps of spermatogenesis as well as contrasting results from studies using the variety KO mouse models precludes the claim of a total redundancy of FSH in spermatogenesis (Dierich et al., 1998a; Krishnamurthy et al., 2000a; Kumar et al., 1997). The failure of the KO studies to yield comparable results might be due to other developmental disorders in the different mouse models. Culture systems are not affected by such drawbacks, although indeed they remain in vitro models. Hence putting together results from KO experiments and in vitro data should help in approaching again the physiological role of these hormones in spermatogenesis. It is important to note that FSH expression and activity is high in the pseudoadult monkeys as compared to the infants. The observation of similar levels AR mRNA expression in the infant and pseudoadult monkeys, but low AR activity in the infant Sc as compared to that of the pseudoadult might indicate a role of FSH in the regulation of AR activity at the post-transcriptional or post-translational level. Thus FSH, by regulating the AR, becomes central to the process of spermatogenesis. This hypothesis can be tested by transfection of the FSHR gene in the Sc of infant testis, in vivo, or seminiferous tubules, in vitro, and evaluating the ability of the transfected infant Sc, possessing increased FSHR transcripts, to support spermatogenic progression during infancy. Although we failed in transfecting the Sc in culture, however studies of Scobey et al. (2001) undertaking transfection of CREB gene in the Sc, in vivo, demonstrate the possibility of delivering the FSHR construct (Scobey et al., 2001). Also the transfection of genes by electroporation to generate transgenic animals indicates that such attempts in the Sc might lead to successfully delivering the relevant genes to these cells (Ike et al., 2004; Widlak et al., 2003; Yamazaki et al., 2000). With the advent of new technologies aimed at delivering genes to hard-to-transfect cell lines as well as primary cells, future attempts to transflect AR and FSHR genes in the Sc might get easier (Arts et al., 2003; Gresch et al., 2004). Such an attempt, if successful, might pave the way for the treatment of certain forms of male idiopathic infertility (of which infant monkey was a surrogate in this study), occurring due to primary testicular
failure, through the \textit{in vitro} induction of Gc differentiation by FSHR and/or AR transfection in the testicular tissue retrieved by fine needle aspiration biopsy of the infertile male.