A Comparative Analysis of Expression of Selected Genes During Induction of Differentiation in Neonatal Rats by Deprival of FSH or by Hyperthyroidism

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Abstract: The effect of Follicle Stimulating Hormone (FSH) deprivation, or hyperthyroidism, on the expression of selected genes in Sertoli cells of neonatal rats was compared, and significant differences were revealed. While hyperthyroidism resulted in both arrest of proliferation and expression of differentiation markers, FSH deprivation only resulted in arrest of proliferation, as assessed by a decrease in such proliferation markers as connective tissue growth factor (CTGF) and collagen 12A1 and the expression of differentiation markers, such as androgen binding protein, transferrin and clusterin. In Sertoli cells isolated from hyperthyroid rats, a decrease in the expression of FSH receptor was also observed. Microarray analysis of RNA from FSH-deprived or hyperthyroid neonatal rat Sertoli cells suggests that molecular events associated with arrest of proliferation by hyperthyroidism appear to be different from arrest of proliferation by FSH deprivation.

Keywords: FSH, thyroid hormone, proliferation, differentiation, microarray, sertoli cells.

1. INTRODUCTION

In rats, Sertoli cells divide rapidly at birth, but their proliferation declines during the neonatal period and ceases by 15-18 days postnatal [1, 2]. Our previous studies have shown that Sertoli cell proliferation peaks at day 9 after birth in rats [3]. The fact that the primary stimulus for Sertoli cell proliferation is Follicle Stimulating Hormone (FSH) has been established by both *in vitro* and *in vivo* studies [3-10]. After postnatal day 18, Sertoli cell proliferation is arrested despite continued exposure to FSH [11]. Arrest of proliferation of Sertoli cells is accompanied by their differentiation at 14-21 days postnatal, and this is ascertained by increased production of markers characteristic of mature Sertoli cells. These markers are secretory proteins, such as androgen binding protein (ABP) and transferrin, which are important for germ cells, as well as the synthesis of proteins which are involved in the formation of specialized tight junctions between neighboring Sertoli cells to establish the blood-testis-barrier [12, 13]. The importance of FSH in regulating the proliferation of Sertoli cells during the neonatal period is well established, but the differentiation of Sertoli cells, even in the presence of FSH, suggests the possibility that other factors may be involved in the regulation of the transition of Sertoli cells from a proliferative to a differentiative state during this period.

Thyroid hormone plays a crucial role in Sertoli cell development. Neonatal testes express high levels of thyroid hormone receptors, predominantly in Sertoli cells [9, 14-18]. The expression of thyroid hormone receptors in Sertoli cells changes with development. High levels of thyroid hormone receptors are expressed during the fetal and neonatal period, and the increase in the expression of thyroid hormone receptors coincides with the arrest of proliferation of Sertoli cells around day 18 [14]. The regulation of Sertoli cells by thyroid hormone can be studied by using hypothyroid [17, 19-21] animal model systems. Studies have shown that transient neonatal and prepubertal hypothyroidism extends throughout the period of proliferation by delaying Sertoli cell maturation, resulting in an increased number of Sertoli cells in the adult testis [2, 22]. With this in mind, it should be noted that each Sertoli cell supports a fixed number of germ cells [23], suggesting their importance in maintaining testicular functions. The number of Sertoli cells present in the adult testis depends on both the duration of the proliferative phase and the rate of division during that phase. In contrast, transient neonatal hyperthyroidism resulted in an early cessation of Sertoli cell proliferation and had a concomitant stimulatory effect on their maturation, as observed by canalization of seminiferous tubules, decreased testis size and sperm production [20, 21, 24, 25]. Our previous studies have shown that deprivation of endogenous FSH by administration of specific antiserum to FSH (FSH a/s) in neonatal rats resulted in decreased proliferation of Sertoli cells with a concomitant decrease in BrdU incorporation and decrease in proliferation markers such as PCNA and Cyclin A2 [3]. Interestingly, deprivation of FSH only had a marginal effect on differentiation markers, such as ABP and transferrin, during this period [3]. The effects following FSH deprivation and hyperthyroidism are similar insofar as proliferation is concerned; therefore, it was of interest to compare the changes in differentiation markers following these treatments. In the present study, we have compared the effect of FSH deprivation or administration of thyroid hormone on neonatal rat Sertoli cell proliferation

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under *in vitro* and *in vivo* conditions. Our results reveal that hyperthyroidism induces premature differentiation of Sertoli cells. To obtain insights into the molecular events underlying the action of thyroid hormone on the growth and differentiation of Sertoli cells, we have carried out a microarray analysis of RNA from euthyroid and hyperthyroid neonatal rat Sertoli cells using a rat oligo array representing 5600 rat genes and compared the profile obtained with hyperthyroid rats and control immature rats with effects following FSH deprivation and adult rat Sertoli cells.

2. MATERIALS AND METHODS

2.1. Animals and Treatment

Seven-day-old Wistar rats were obtained from the Central Animal Facility, Indian Institute of Science, Bangalore, India, and maintained under standard conditions (12 h of light and 12 h of dark, with water and pelleted food *ad libitum*). The animal procedures employed in the study were approved by the Institutional Ethical Committee.

Hyperthyroidism was achieved in neonatal rats by administration of T3 (100 μg/ kg body weight/ day) in saline, subcutaneously, from birth to 13 days postnatal. The dosage was based on the reported study by Auharek *et al*. [26]. Control or euthyroid rats were administered with equal volume of saline. On day 14, Sertoli cells were isolated and processed for either *in vitro* incubation studies or isolation of RNA.

Antiserum to highly purified ovine FSH (oFSH) was used to neutralize the endogenous FSH in neonatal rats. FSH antiserum (FSH a/s) was raised in the adult male bonnet monkey (*Maccaca radiata*) as previously described [3]. The absence of contaminating antibodies to LH was established by absence of binding to ^{125}I-hCG and the inability of the antiserum to inhibit LH-stimulated testosterone production by Leydig cells [27]. The ability of antiserum to neutralize endogenous FSH has been established in neonatal male rats [3]. Rats administered normal monkey serum (NMS) served as controls.

In order to study the effect of FSH deprivation on the growth and function of Sertoli cells, seven-day-old neonatal rats were administered 100 μl of FSH a/s or NMS by i.p. route twice a day for 6 days. On the fourteenth day, the animals were sacrificed, and the effect of neutralization of endogenous FSH was assessed.

2.2. Isolation of Sertoli Cells and Culture

Sertoli cells were isolated from neonatal (14 days) and adult rats (90 days) as previously described [3, 28]. Briefly, seminiferous tubules were digested in 0.125% of Trypsin (Sigma Chemical Co., St. Louis, MO, USA) and 10 μg/ ml of DNase (Worthington Biochemical Corporation, Lakewood, NJ, USA) in 1× Hanks Balanced Salt Solution (HBSS) for 20 min. After three washes in 1× HBSS, the tubules were subjected to two enzymatic digestions with 1 mg/ml collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and DNase followed by 1 mg/ml collagenase, 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) and DNase for 30 min each. The cells were washed three times with 1× HBSS and pelleted down by centrifugation. The cells were resuspended in 1 ml of DMEM Ham-F12 (Sigma Chemical Co., St. Louis, MO, USA) and counted in a hemocytometer. Sertoli cells from neonatal rats were maintained in DMEM Ham-F12 medium containing 10 μg/ml insulin (Sigma Chemical Co., St. Louis, MO, USA) and 5 μg/ml transferrin (Sigma Chemical Co., St. Louis, MO, USA) at 32°C in 5% CO2. After 24 h of pre-culture, Sertoli cells were subjected to hypotonic shock by replacing the medium diluted with water (1:10) to remove any contaminating germ cells. The characterization of Sertoli cells after hypotonic shock was described earlier [3]. The purity of the Sertoli cells was determined by the absence of germ cells as monitored under the phase contrast microscope. For the absence of Leydig cells, RT-PCR was used to check for LH receptor (LH-R), and for the absence of myoid cells, alkaline phosphatase staining was performed.

2.3. Cell Proliferation Assay

The incorporation of BrdU (5-bromodeoxyuridine) in neonatal rat Sertoli cells was used as a measure of proliferative activity. Forty thousand Sertoli cells were cultured per well in quadruplets in a 96-well culture plate. Each group of Sertoli cells was treated with either oFSH (250 ng/ ml) (a kind gift from Prof. Sairam, Clinical Research Institute of Montreal, Canada) or FSH + T3 (250 ng/ ml oFSH and 100 nM T3). The Sertoli cells were cultured in serum-free medium (DMEM Ham-F12) containing 5 μg/ml transferrin (Sigma Chemical Co., St. Louis, MO, USA) and 5 μg/ml insulin (Sigma Chemical Co., St. Louis, MO, USA) for 16 h at 34°C in 5% CO_2. To maintain uniform conditions, cells from neonatal and adult rats were maintained at 34°C, although Sertoli cells from normal adult rats are generally maintained at 37°C. The Sertoli cells were then incubated in the presence of 10 μM of BrdU for 4 h. Incorporation of BrdU was assessed using the Amersham^{TM} Cell Proliferation ELISA Biotrak system according to the manufacturer's protocol (GE Healthcare UK Ltd). The viability/growth of cells following various treatments was verified using the MTT assay as described by Mosmann, [29]. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl tetrazolium bromide; Thiazolyl blue] is a water-soluble tetrazolium salt. Dissolved MTT can be converted to an insoluble purple formazan by cleavage of the tetrazolium ring by cellular dehydrogenase enzymes. At the end of the incubation period, 50 μl of 5 mg/ml MTT (i.e., one-tenth of the culture volume) was added to the cultures and further incubated for 3-4 hours at 37°C. Following this, the medium was removed and the reaction terminated by the addition of 500 μl of acidified isopropanol (40 mM HCl in absolute isopropanol) to extract the formazan crystals that had formed. Absorbance of the converted dye was measured at 570 nm on a UV Max microplate reader (Molecular Devices, Menlo Park, CA). All tests were performed in triplicate, and data are expressed graphically (OD_{570} as a function of time).

2.4. Semi-Quantitative RT-PCR

Total RNA was extracted from Sertoli cells using TRI reagent (Sigma Chemical Co., St. Louis, MO, USA). To eliminate genomic DNA contamination from the RNA, the RNA samples were treated with RNase-free DNase. Four micrograms of RNA was treated with 1 μL of RQ1 RNase-free DNase in a 1x reaction mixture of RQ1 DNase buffer

plus 1 μL of RNasin® (RNase Inhibitor from GE Healthcare UK, Ltd) for 30 min at 37°C. The reaction was stopped by adding RQ1 DNase stop solution and incubating the mixture at 65°C for 10 min. Following DNase (GE Healthcare UK, Ltd) treatment, 2 μg of total RNA was subjected to reverse transcription using random hexamers (Roche Molecular Biochemicals, Germany) and MMLV-RT (GE Healthcare UK, Ltd) in a 20 μl reaction mixture. PCR amplification was carried out using a 2 μl aliquot of cDNA and specific primers in a 25 μl reaction volume. For amplification, the following reaction parameters were used: dNTPs each: 200 μM; PCR reaction buffer 1x; sense-primer (forward) 50 ng; antisense-primer (reverse) 50 ng; template DNA ~100 ng (except in negative control PCR) DNA polymerase 1-2 U, The final reaction volume was 25 μL. An additive like DMSO was used at a concentration of 5% to 10% for GC-rich templates.

A control reaction without reverse transcriptase was included in every set of RT-PCR reactions to verify the absence of genomic DNA contamination. Preliminary studies were carried out to arrive at the optimum temperature, primer concentrations and template to establish the linear range of RNA amplification. An 18 μl aliquot of the PCR product was electrophoresed on a 1.5% agarose (GE Healthcare UK, Ltd) gel containing Ethidium Bromide (Sigma Chemical Co., St. Louis, MO, USA). Cyclophilin was used as an internal control. The sequences of the primers used in the present study and the RT-PCR details are given in Table **1**. Analysis of images was carried out using a Kodak Electrophoresis and Gel Documentation Analysis System (EDAS-120). The relative ratio of the intensity of the gene of interest was calculated with respect to the internal control, and statistical analysis was carried out.

The authenticity of all the RT-PCR products described in this study was confirmed by purification of transcripts from agarose gels using a gel purification kit (Qiagen, Germany) and sequencing using the ABI Prism 377 automated DNA sequencer.

2.5. Microarray Analysis

2.5.1. RNA Labeling and Hybridization

Total RNA isolated from euthyroid and hyperthyroid neonatal rat Sertoli cells was reverse transcribed into cDNA and labeled using a tyramide signal amplification kit (NEN, Perkin-Elmer) with fluorescein or biotin. Hybridization was carried out by mixing equal quantities of experimental cDNA, control or treated sample labeled with fluorescein or biotin, with the spots on the slides under appropriate conditions. Stringent washes (6XSSPE+0.005% Sarcosine for 1 min at room temperature; 2nd wash with 0.06 X SSPE+0.005% Sarcosine for 1 min at room temperature, followed by treatment with stabilization and drying solution at room temperature for 30 sec.) were carried out to remove excess or unbound labeled sample.

2.5.2. Scanning of Slides

Differential gene expression was assessed by scanning the hybridized arrays with an Axon scanner, using the Genepix Pro program. Hybridized slides were scanned first in the Cy5 channel and then the Cy3 channel, as Cy5 is more susceptible to photo-degradation than Cy3. Data from each

Table 1. **List of Primers and Sequences**

Sl. No.	Name	Primer Sequence (5'- 3')	MER	Annealing Temp (°C)	No. of Cycles	Product Size (bp)
1	ABP FP	ACAATCTCTGGGCTCGGCTT	20	55	24	330
2	ABP RP	TTGCAGGTCCACATCACAGT	20			
3	Col12 A1 FP	GAAGAAAGTGTGCCGGTTATAGG	23	60	26	207
4	Col12 A1 RP	AGGCAGACACAAGAGCAACAATG	23			
5	Clusterin FP	ATGAAGGGCCAGTGTGAGAAGTG	23	60	26	185
6	Clusterin RP	CAGCAGGGATGAGGTGTTGAG	21			
7	CTGF FP	AGTGGAGCGCCTGTTCTAAGAC	22	58	24	205
8	CTGF RP	GCAGCCAGAAAGCTCAAACTTG	22			
9	Cystatin TE 1 FP	GACCTGGAGATGGGCCGTACAC	22	58	24	203
10	Cystatin TE 1 RP	CCTTCAGCTACAGGCACAGAAAGAC	25			
11	Cyclophilin FP	GTGGCAAGTCCATCTACG	18	55	22	381
12	Cyclophilin RP	CAGTGAGAGCAGAGATTACA	20			
13	Egr1 FP	TCCTTCAGCATTCTTATCGCC	21	60	28	150
14	Egr1 RP	GCACGGAGATGGAAAAAATCC	21			
15	FSH-R-3 TM-ICD FP	GCCATCTCTGCCTCCCTCAAGGTG	24	62	28	236
16	FSH-R-3 TM-ICD RP	GCTCTTTCGGGCATGGAAGTTGTG	24			
17	Transferrin FP	CTGGCTGTCCCTGACAAAACG	21	57	24	343
18	Transferrin RP	TGGAGCTGGTTCAGCTGGAAG	21			

fluorescence channel were collected and stored as separate 16-bit TIFF images. These images were analyzed to calculate the relative expression levels of each gene and to identify differentially expressed genes. Each hybridized slide was scanned at different PMT gains. Scanning at different PMT gains, i.e., at very low PMT gain, does not allow the signal intensities of the Cy3 and Cy5 to be saturated. Scanning at medium PMT gains allows the signal intensities of both channels to be balanced, and, finally, scanning at higher PMT gains increases all the weak signal intensities of both channels.

2.5.3. Image Processing and Quality Control

Image processing and data extraction were done using the Genepix Pro program, and quality control of the slides was done using such parameters as background distribution, dye bias or dye specks.

2.5.4. Image Processing

2.5.4.1. Gridding

Depending upon the number of grids and number of rows and columns in each grid of the array, gridding was performed.

2.5.4.2. Aligning of Features

The Genepix Pro program automatically aligns both the blocks and spots on the image of the array. Some of the spots which could not be aligned automatically were manually aligned.

2.5.4.3. Flagging

The Genepix Pro program automatically flags the spots that are bad or absent. Manually flagging for some of the spots was also done for good and bad by circling them, or by putting a cross through them.

2.5.4.4. Raw Data

After gridding on the image, data were extracted for all the features in the image and saved as GPR files (Genepix Results file).

2.5.4.5. Normalization of Raw Data

Raw data were normalized using multiple methods. The best normalization method is one which yields a ratio median close to 1 and duplicate spots variance less. Grid-wise normalization was chosen as the optimum for the slides. Normalization was calculated for each grid, as follows: Calculation of Normalization factor=Sum of CH1-Intensity/CH2-Intensity (excluding control and non-array specific spots); Calculation of Raw Ratio=CH1-Intensity/CH2-Intensity; Calculation of Normalized Ratio=Raw Ratio (for each spot)/Normalization factor (for the grid). Likewise, Ratio was calculated for spots in each grid separately.

2.5.5. Ranking of Differentially Regulated Genes

Differential regulation of genes was ascertained on the basis of genes that were at least one-fold up- or down-regulated in more than 50% of the slides/experiments. Thus, up- and down-regulated genes were ranked on the basis of average fold change, consistency of the fold change across experiments, and intensity of the spots and flags.

2.5.6. Data Analysis

The data obtained after normalization were analyzed using a hierarchical clustering program called "Cluster" that uses standard statistical algorithms to arrange genes according to similarity in pattern of gene expression. The average linkage clustering technique was used to cluster the data. For cluster analysis, a table was constructed in which rows represent all genes for which data have been collected, columns represent individual array experiments, and each cell represents the measured Cy5/Cy3 fluorescence ratio at the corresponding target element on the appropriate array. All ratio values were log transformed (base 2 for simplicity) to treat inductions or repressions of identical magnitude as numerically equal, but with opposite sign. Clusters were made for genes belonging to a common pathway or genes with a similar or common function. The data obtained from Cluster were displayed graphically using a program called "Tree view".

The data were normalized by calculating the ratio between the intensities of the two channels. Genes that were at least two-fold up/down-regulated in more than 50% of the experiment were selected for further analysis. Thus, up/down-regulated genes were ranked on the basis of average fold change, consistency of the fold change across experiments, and the intensity of the spots. For the present study, genes were delimited on the basis of their known biological functions.

2. 6. Statistical Analysis

Data are presented as mean ± S.E.M. of at least three independent experiments performed with the same treatment protocol, and data were analyzed using the unpaired two-tailed '*t*' test.

3. RESULTS

3.1. Thyroid Hormone Regulates Sertoli Cell Proliferation

3.1.1. In Vitro Regulation of Sertoli Cell Proliferation

To understand the role of thyroid hormone on Sertoli cell proliferation, Sertoli cells were isolated from 10-day-old neonatal rats. In our studies, we found that the peak of proliferation was seen on day 9 after birth [30], and an equal number of Sertoli cells (40,000) were cultured under three different conditions. One group of Sertoli cells was cultured in the presence of 250 ng/mL of oFSH. Another group was cultured in the presence of 250 ng/mL oFSH and 100 nM T3, and the last group, which served as a control, received vehicle (medium only). After 16 h of incubation, all three groups of Sertoli cells were pulsed with 10 μM BrdU for four hours. Results revealed increased proliferation of Sertoli cells in the presence of oFSH (Fig. **1A**). In contrast, in the group of Sertoli cells which were treated with both oFSH and T3, a decrease in Sertoli cell proliferation was observed, suggesting that T3 inhibited the FSH-stimulated proliferation of neonatal rat Sertoli cells *in vitro*. MTT assay revealed that the cells were viable at the end of the experiment in all three treatment groups (Fig. **1B**), thus ruling out the possibility that the decrease in proliferation resulted from the death of Sertoli cells in the presence of T3.

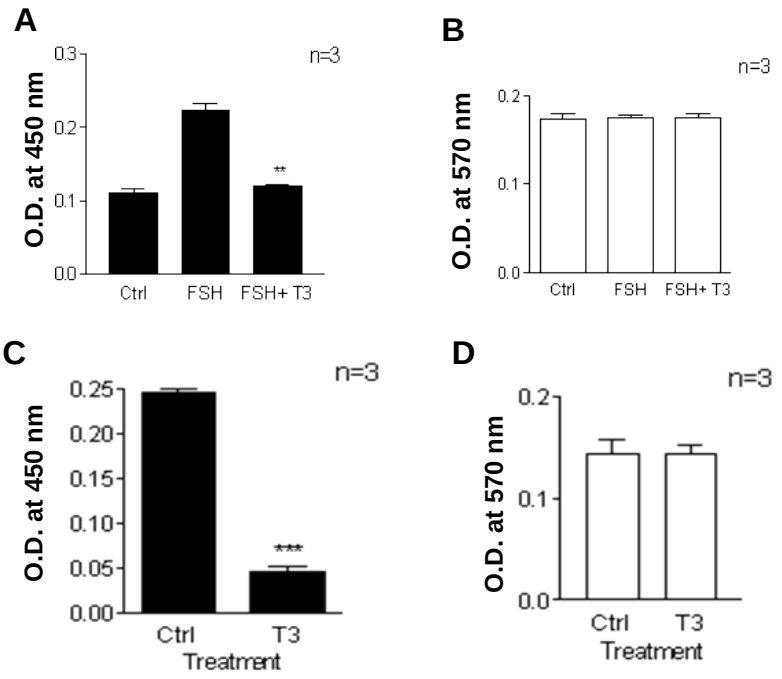

Fig. (1). Regulation of Sertoli cell proliferation by thyroid hormone.

A. BrdU incorporation analysis of neonatal rat Sertoli cells cultured in the presence of oFSH+T3 revealed a significant decrease ($P< 0.01$) compared to proliferation stimulated by FSH. **B**. MTT assay for viability of the cells at the end of the experiment. **C**. BrdU incorporation analysis revealed a significant ($P< 0.001$) decrease in the proliferation of neonatal rat Sertoli cells following administration of T3 to neonatal rats. **D**. MTT assay for viability of the cells used in Figure **1C** at the end of the experiment. The values represent mean ± SEM of three independent experiments.

3.1.2. In Vivo Regulation of Sertoli Cell Proliferation

To validate the observation that thyroid hormone inhibits the FSH-stimulated proliferation of the Sertoli cells, the effect of administration of thyroid hormone to neonatal rats from day one postnatal was evaluated. One group of neonatal rats was subcutaneously administered T3 in saline (100 μg/kg body weight) each day from birth to 13 days postnatal. Another group received only saline (euthyroid). A decrease in proliferation of Sertoli cells in the hyperthyroid neonatal rat was seen compared to Sertoli cells in the euthyroid rat (Fig. **1C**). Thus, as observed in both *in vitro* and *in vivo* experiments, it was established that thyroid hormone inhibits the proliferation of neonatal rat Sertoli cells. The results from the MTT assay established that the cells were viable at the end of the experiment in both treatment groups (Fig. **1D**).

3.2. Effect of Hyperthyroidism on Differentiation of Sertoli Cells

3.2.1. Expression of FSH-R

Our previous studies revealed that the expression of FSH-R decreases with age in the differentiated Sertoli cells isolated from adult rats and that the expression of FSH-R is an important marker to distinguish between the undifferentiated and differentiated rat Sertoli cells [3]. Since arrest of proliferation is a prerequisite to initiate differentiation, we monitored the expression of FSH-R in euthyroid and hyperthyroid rat Sertoli cells. Using the RNA isolated from the euthyroid and hyperthyroid neonatal rat Sertoli cells, RT-PCR revealed that the expression of FSH-R was decreased significantly ($P< 0.01$) in the Sertoli cells isolated from the hyperthyroid rats compared to the euthyroid rats (Fig. **2A** & **B**).

3.2.2. Expression of Sertoli Cell Differentiation Markers

In our previous study, we showed that the mRNA expression of both ABP and transferrin is higher in the adult rat Sertoli cells compared to neonatal rat Sertoli cells [3]. In view of this, we monitored the effect of hyperthyroidism on expression of ABP and transferrin in neonatal rat Sertoli cells. The expression of ABP and transferrin was up-regulated in the hyperthyroid rat Sertoli cells (Fig. **2C** and **D**). The results revealed that the expression of the known Sertoli cell differentiation markers is increased following administration of T3 to neonatal rats, suggesting that hyperthyroidism causes premature differentiation of the Sertoli cells.

3.3. Microarray Analysis of Euthyroid and Hyperthyroid Rat Sertoli Cells

Genes that were at least two-fold up- or down-regulated in more than 50% of the slides used for microarray analysis (three separate sets of slides) were considered to be differentially regulated. Overall, 70 genes were up-regulated, and 62 genes were down-regulated in the neonatal Sertoli cells following hyperthyroidism. Based on their known function, these genes were grouped into different functional classes, such as those related to proliferation, cytoskeleton, metabolism, steroidogenic pathway, proteases, and protease inhibitors. The average fold increase or decrease in the

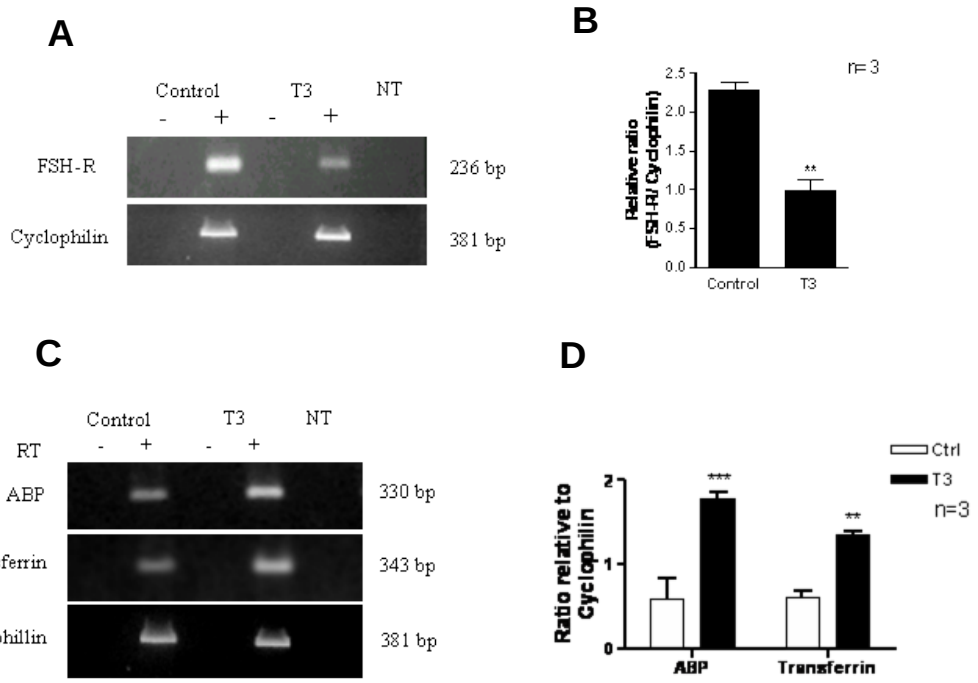

Fig. (2). Effect of T3 administration on Sertoli cell differentiation markers.

A. RT-PCR analysis for FSH receptor (FSH-R) from RNA isolated from the euthyroid and hyperthyroid neonatal rat Sertoli cells. **B**. Graphic representation of data provided in 2A ($P < 0.01$). **C**. RT-PCR analysis for ABP and transferrin in the euthyroid and hyperthyroid neonatal rat Sertoli cells. **D**. Graphic representation of data provided in 2C; the relative ratio is expressed with respect to the internal control cyclophilin. The figure is representative of three independent experiments. The values represent mean ± SEM.

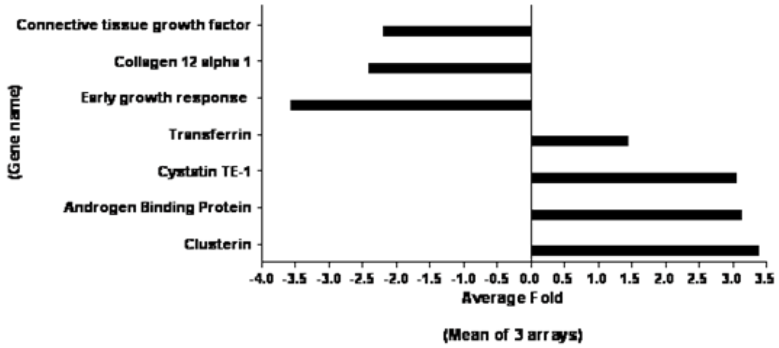

Fig. (3). Regulation of selected genes in neonatal Sertoli cells following induction of hyperthyroidism.

expression of selected genes is presented in Fig. (**3**). Microarray analysis results also revealed the up-regulation of known Sertoli cell differentiation markers, such as ABP, transferrin, protein kinase clusterin, and cystatin TE-1, following hyperthyroidism. However, cell growth promoters like early growth response protein (EGR), connective tissue growth factor (CTGF) and collagen XII alpha 1 (COL12A1) were down-regulated following neonatal hyperthyroidism (Fig. **3**). To establish the role of thyroid hormone in Sertoli cell differentiation, the seven genes were selected on the basis of their known functions.

3.3.1. Validation of the Microarray Results

Semi-quantitative RT-PCR analysis was carried out to validate the expression of genes identified by microarray analysis, in addition to ABP and transferrin, which were up-regulated in hyperthyroid animals (Fig. **2C**). RT-PCR analysis revealed a 3-fold increase in clusterin expression in the 13-day-old hyperthyroid rat Sertoli cells (Fig. **4A** and **B**), and the expression of cystatin TE-1 increased by almost 2.5-fold in Sertoli cells following hyperthyroidism.

RT-PCR analysis for genes involved in cell growth, i.e., COL12A1, EGR and CTGF, revealed a significant ($P < 0.01$) decrease in their expression following hyperthyroidism in neonatal rat Sertoli cells, and mRNA expression of EGR mRNA was almost undetectable. Similarly, the mRNA level of CTGF was also reduced to almost undetectable levels following treatment with thyroid hormone (Fig. **4A** and **B**). Thus, the expression of the genes involved in cell growth was down-regulated following hyperthyroidism in neonatal rat Sertoli cells. The results of the microarray analysis also

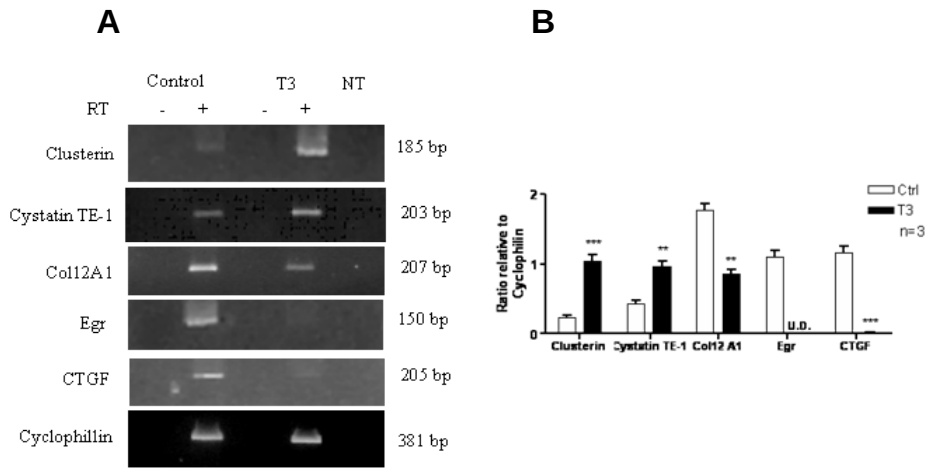

Fig. (4). Validation of the microarray results.

A. RT-PCR analysis for the expression of clusterin, cystatin TE-1, COL12A1, EGR and CTGF in the euthyroid and hyperthyroid neonatal rat Sertoli cells. **B**. The relative ratio is expressed with respect to the internal control cyclophilin. The figures are representative of three independent experiments. The values represent mean ± SEM.

support the hypothesis that thyroid hormone inhibits Sertoli cell proliferation and induces differentiation.

3.3.2. Effect of Deprivation of Endogenous FSH on the Expression of Selected Genes in Sertoli Cells

It has been reported that FSH levels were reduced in T3-injected rats on days 5 and 7, but increased on day 23 after cessation of treatment. It should be noted that serum FSH and LH levels were elevated in both male and female pre-pubertal rats and remained low between day 21 and the immediate pre-pubertal period [31]. Studies by Rao *et al*. [32] have also suggested that thyroid hormone directly regulates FSH receptor gene expression in Sertoli cells. A part of this period corresponds to the period of hyperthyroidism induced in our study, and in spite of the increased FSH levels during hyperthyroidism [21], it should be noted that Sertoli cell proliferation was inhibited, suggesting that changes in the genes involved in inhibition of proliferation or induction of differentiation could be different under both FSH deprivation and hyperthyroidism. This is evident from the fact that RT-PCR analysis following FSH deprivation for clusterin and cystatin TE1 revealed no significant change in the expression level of these markers. These results suggest that arrest of proliferation by FSH deprivation did not result in an increase in differentiation markers. As expected, the level of CTGF expression, which indicates proliferation, was decreased following FSH deprivation, even though COL12A1 and EGR remained unaltered (Fig. **5A** and **B**). As demonstrated earlier [30], following FSH deprivation, there was a decrease in the expression of PCNA Cyclin A2 and Cyclin E2 in the Sertoli cells, as assessed by RT-PCR, as well as a decrease in the incorporation of BrdU by more than 80%. Both of these parameters are indicators of cell proliferation.

3.3.3. Expression of Selected Genes in Differentiated Sertoli Cells of Adult Rat

The results of microarray analysis suggested that thyroid hormone causes premature differentiation of the neonatal Sertoli cells; therefore, we analyzed the expression profile of differentiation marker genes in the neonatal and adult rat Sertoli cells. RT-PCR analysis revealed that the mRNA expression of clusterin and cystatin TE-1 was at least two-fold more in adult rat Sertoli cells, while the mRNA levels of COL12A1, EGR, and CTGF were significantly less in adult rat Sertoli cells (Fig. **6A** and **B**) compared to the neonatal rat. This shows that differentiation markers are more highly expressed in the adult rat Sertoli cells. Under these circumstances, we hypothesized that the prematurely differentiated Sertoli cells, following hyperthyroidism, should have the same expression profile as the adult differentiated Sertoli cells. Compare, for example, the T3 expression profile shown in Fig. (**4**) with the adult expression profile given in Fig. (**6**). It should also be noted that RT-PCR results revealed that the expression pattern of the genes selected from microarray analysis following euthyroidism was similar to neonatal Sertoli cells. In this case, compare the expression profile of control from Fig. (**4**) with the expression profile of the neonatal rat, as shown in Fig. (**6**). This suggests that the Sertoli cells from neonatal rats have prematurely differentiated following exposure to thyroid hormone and behave in a manner similar to normal adult Sertoli cells.

4. DISCUSSION

Although proliferation is generally arrested before differentiation occurs, it is possible that these two processes can occur simultaneously [33]. Previously, we have investigated molecular changes associated with differentiation of cells using BeWo choriocarcinoma cells and Leydig cells [34-36]. In the present study, we have extended this to Sertoli cells, and we analyzed the role of thyroid hormone and FSH in the differentiation of Sertoli cells. Sertoli cells are highly proliferative, but only during the early neonatal period, during which their number increases rapidly [17]. *In vivo* studies by Meachem *et al*. [17] revealed that FSH differentially effects Sertoli and germ cells in an age-dependent manner, promoting Sertoli cell

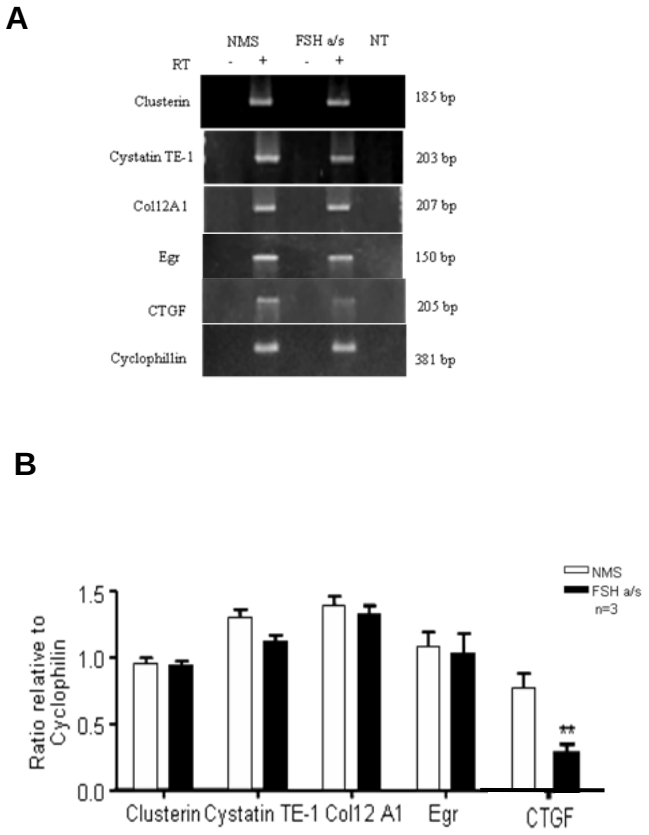

Fig. (5). RT-PCR analyses for selected genes following deprivation of endogenous FSH in neonatal rats.

A. RT-PCR analysis for clusterin, cystatin TE-1, COL12A1, EGR and CTGF in the neonatal rat Sertoli cells following neutralization of endogenous FSH. **B**. The relative ratio is expressed with respect to the internal control cyclophilin. The figures are representative of three independent experiments. The values represent mean ± SEM.

mitosis on day 8 and supporting germ cell viability at day 18. In contrast, our earlier studies revealed that the peak of proliferation with highly purified Sertoli cells is seen on day 9. By day 12, it decreases, and proliferation cannot be observed by day 18 postpartum [30]. Thus, the results obtained in the present study clearly established the dual role of FSH in Sertoli cell proliferation during immature stages and induction of differentiation during adult stages. Although it is possible that both processes may occur simultaneously, a clear demarcation of the time frame of initiation may not be possible. In addition, our earlier results [3] also established that the down-regulation of FSH receptor is indicative of loss of response to FSH by the immature rat Sertoli cells as far as proliferation is concerned. After the cessation of proliferation, Sertoli cells undergo differentiation, and their main function is secretion of growth factors, synthesis and maintenance of the blood-testis-barrier, and secretion of various proteins, such as proteases, protease inhibitors, and transport proteins, essentially to provide a microenvironment for the developing germ cells. Thus, the neonatal and adult rat Sertoli cells can be differentiated at the molecular level by the expression level of differentiation markers, with the adult being functionally more active. As such, these cells can be considered differentiated. Thyroid hormone is known to play an important role in the growth and development of Sertoli cells. Although Bortolussi *et al*. [37] report that the number of FSH receptors, as assessed by binding studies, increases after day 21 as they differentiate, these studies were carried out with crude homogenate and frozen sections. In contrast, we have employed highly purified cells and, as such, we were able to characterize Sertoli cells with the very sensitive RT-PCR approach to assess the expression of FSH receptor. Specifically, following hyperthyroidism, we observed that there is a decrease in the expression of FSH-R in the neonatal rat Sertoli cells, which normally have a high expression. This observation strengthens previous reports suggesting that thyroid hormone induces differentiation of neonatal Sertoli cells [9, 38].

By employing microarray analysis of RNA from euthyroid and hyperthyroid neonatal rat Sertoli cells, we observed an increase in the expression of transferrin cystatin TE-1, ABP, and clusterin. It should be noted that all these molecules are markers of Sertoli cell function. For example, it is known that ABP is necessary for the transport of androgen into the lumen to the epididymis, while transferrin is important for transport of iron to the developing germ cells, which otherwise do not have access to systemic iron by the presence of the blood-testis-barrier. Clusterin is a secretory glycoprotein present in most body fluids and is involved in a variety of physiological processes, including cell adhesion, cellular differentiation and apoptosis [39]. Cystatin TE-1 is a member of the cysteine protease inhibitor family. Cysteine peptidases have an important role in

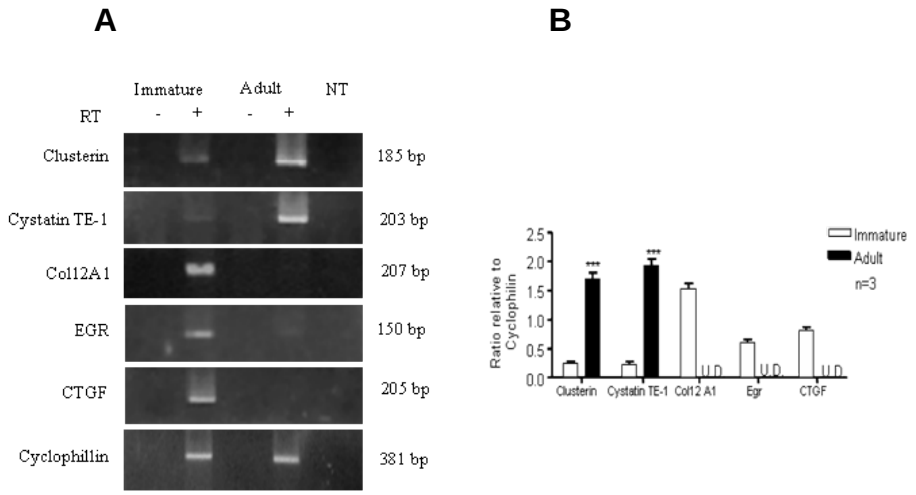

Fig. (6). Comparison of the expression of selected genes in neonatal and adult rat Sertoli cells.

A. RT-PCR analysis for clusterin, cystatin TE-1, COL12A1, EGR and CTGF in the neonatal and adult rat Sertoli cells. **B**. The relative ratio is expressed with respect to the internal control cyclophilin. The figures are representative of three independent experiments. The values represent mean ± SEM.

physiological processes, such as intracellular protein degradation [40]. Thus, cystatin control over these proteases is important. Cystatins may also participate in the defense against microbial infections, which is another important function of Sertoli cells during adult stages [40]. It has been demonstrated that cystatin TE-1 is expressed only in Sertoli cells and epididymis, indicating that it plays a very specialized role in these tissues [41]. Hence, our study provides novel information regarding the role of thyroid hormone in the differentiation of Sertoli cells. In addition, our work supports previous observations [15] following hypothyroidism where the expression of several Sertoli cell factors, such as ABP, clusterin, and inhibin-beta B, were delayed at the mRNA level, including hemiferrin, a spermatid-specific mRNA. Furthermore, thyroid hormone replacement in PTU-treated animals decreased MIS and c-erbA alpha mRNA expression to control levels [15]. It was also observed that the level of expression for clusterin and cystatin TE-1 increased in the adult Sertoli cells.

During our microarray studies, it was observed that the expression COL12A1 was down-regulated in Sertoli cells isolated from hyperthyroid rats. COL12A1 belongs to the collagen family of extracellular matrix (ECM) proteins which are involved in extra- cellular matrix remodeling, growth and proliferation of cells. The ECM proteins are also known to promote cellular proliferation through the regulation of immediate early genes, which are involved in proliferation. Earlier studies have also reported the modulation of basement membrane (BM) components, including type IV collagen, laminin and entactin expression, in prepubertal rat Sertoli cells by the thyroid hormone, as observed by immuno-cytochemical studies [39]. Western blot analysis of Sertoli cell-conditioned media indicated that thyroid hormone treatment of Sertoli cells significantly reduced type IV collagen secretion by 62%, and even the RNA levels for type IV collagen alpha 1 chain were reduced by 50% after thyroid hormone treatment [42]. These observations demonstrated the ability of T3 to differentially regulate the expression of BM components and, hence, could be regarded as a part of the integrated mechanism by which thyroid hormone affects testicular development and differentiation [42].

In the present study, it was observed that the expression of EGR gene is down-regulated following treatment with thyroid hormone. The EGR gene is a transcription factor induced by stress or injury, mitogens, and differentiation factors [43, 44]. Its expression is high in proliferating cells, and antisense to the EGR gene inhibits prostate tumor development in TRAMP mice [45]. The reduced expression of the EGR gene in adult rats supports its importance in the regulation of Sertoli cell proliferation. However, EGR gene expression was not significantly affected following the decrease in Sertoli cell proliferation by FSH deprivation, which points to an important distinction between arrest of proliferation by FSH deprivation and T3 treatment.

CTGF is an important matricellular regulatory factor involved in internal and external cell signaling, growth, and proliferation or differentiation of cells, depending on the cell type [42, 43]. *In vitro* CTGF directly regulates the proliferation and differentiation of chondrocytes [46, 47]. The expression of this gene was observed to be down-regulated by thyroid hormone treatment. In addition, the expression of COL12A1, EGR gene and CTGF was observed to be highly reduced or absent in the adult Sertoli cells. The arrest of proliferation is a prerequisite for initiation of differentiation [29]. However, while deprivation of FSH resulted in arrest of proliferation, we found that it did not result in the expression of markers characteristic of Sertoli cell differentiation. It should be noted that while the results of Ruwanpura *et al*. [48] suggest a direct action of FSH on germ cells, most of the studies [12, 49] reporting the role of FSH in spermatogenesis suggest that it acts indirectly by regulating the function of Sertoli cells. Our results also support such a conclusion and thus our results differ from those reported by Ruwanpura *et al*. [48]. However, induction of hyperthyroidism in neonatal rats results not only in the

arrest of proliferation but also a decrease in the level of expression of differentiation markers in Sertoli cells. Thus, the results of the present study suggest that the molecular changes associated with FSH deprivation and hyperthyroidism appear to affect different pathways.

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