

Developmental patterns of PPAR and RXR gene expression during spermatogenesis

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1. ABSTRACT

Members of the family of nuclear receptors that include peroxisome proliferator-activated receptors (PPARs) and retinoid X receptors (RXRs) are important mediators of selective gene activation during development and cellular differentiation. In this study, developmentally-specific PPAR and RXR patterns of expression that occur in somatic and germ cell populations in the testis were determined using quantitative real-time PCR (qRT-PCR) studies on RNAs that were isolated from StaPut purified mouse germ cells and primary rat Sertoli cells. These qRT-PCR studies indicate that transcripts encoding the PPAR-Alpha (α), -Beta (β), and -Gamma (γ) and RXR - Alpha (α), -Beta (β), and -Gamma (γ) are developmentally expressed in both differentiating germ and Sertoli cells. In further experiments aimed at deciphering the physiological role that PPAR-Gamma (γ) plays in Sertoli cells, 15-day primary rat Sertoli cells were infected with recombinant adenoviral vectors containing PPAR-Gamma (γ) cDNA and PPAR-Gamma (γ) RNAi constructs. Affymetrix microarray analysis and qRT-PCR validation studies using total RNA isolated from these transfected cells indicated that PPAR-Gamma (γ) regulates the pattern of expression of key lipid metabolic genes in Sertoli cells.

2. INTRODUCTION

The PPAR subfamily of transcription factors consist of three isotypes, PPAR-Alpha (α), PPAR-Beta (β) and PPAR-Gamma (γ), that are each encoded by separate genes (1). Transcripts from these genes encode PPARs proteins that have highly conserved DNA-binding domains that interact with peroxisome proliferator responsive elements (PPREs) present in the promoter regions of target genes. The PPARs form obligate heterodimers with the RXRs to produce functional transcription factors that are involved in transactivation of several key genes involved in energy homeostasis and cellular differentiation. The PPAR and RXR isoforms exhibit distinct patterns of expressions in several tissues (2). Expression of the RXR isoforms in the mouse and rat testis have been extensively characterized (3-5) however, similar studies have not been performed for the PPAR isoforms.

The PPARs are activated by natural ligands such as polyunsaturated fatty acids, prostaglandin metabolites and synthetic ligands such as thiazolidinediones (TZDs) and glitazones (6). These ligands were demonstrated to modify PPAR-mediated transcriptional activation of a number of key genes involved in energy homeostasis (2). Since the PPARs form obligatory heterodimers with RXRs,

these heterodimeric nuclear receptors complexes are involved in mediating integration of the intracellular network of signal transduction pathways that regulate biological function in many cell types including cells that comprise the ovary and testis. PPAR-Gamma (γ) is highly expressed in human testicular cancer cells and the PPAR-Gamma (γ) ligands that include the thiazolidinediones (TZDs) and prostaglandin J metabolite (15d-PGJ2) have antiproliferative effects on these testicular cancer cells (7). Furthermore, Xenobiotic ligands of PPAR-Gamma (γ) have been recently demonstrated to induce translocation of these receptors into the nucleus of Sertoli cells (8). In addition, PPAR-Alpha (α) which is known to regulate both oxidation of lipids and fatty acid composition of phospholipids in germ cells has been shown to be upregulated by FSH in the testis (9).

The molecular mechanisms involved in mediating PPAR activity in the respective cell types present in the seminiferous epithelium of the testis are currently unknown. In order to elucidate the molecular mechanisms involved in PPAR-mediated target gene activation in the testis, the expression profiles of both PPAR-Alpha (α), -Beta (β) and -Gamma (γ) and their heterodimer partners, RXR-Alpha (α), -Beta (β), and -Gamma (γ) in differentiating mouse germ cells, and primary rat Sertoli cells were investigated using real-time PCR analysis. The changing patterns of PPAR and RXR observed in the differentiating germ cells suggest that different combinations of PPAR/RXR heterodimers are involved in mediating stage and cell-type-specific target gene expression in the germ cell lineages present in the mouse seminiferous epithelium. Members of the PPAR nuclear receptor family are also developmentally expressed in primary rat Sertoli cells, however, PPAR-Gamma (γ) is predominantly expressed in mature Sertoli cells. Investigating the functional and physiological roles that the PPARs play in the seminiferous epithelium of the testis is of major importance since these nuclear receptors mediate integration of several signal transduction pathways that are regulated by hormones, growth factors and metabolic substrates.

3. MATERIAL AND METHODS

3.1. Isolation of seminiferous epithelium and spermatogenic cells

Seminiferous cords and tubules were prepared from Swiss Webster mice (Charles River Breeding Laboratories) by collagenase treatment. Monodispersed suspensions of spermatogenic cells were prepared from the seminiferous cords/tubules with collagenase and trypsin digestion (10,11). Primitive type A spermatogonia were isolated from the testes of 6 day-old prepubertal mice and types A and B spermatogonia were isolated from the testes of 8 day prepubertal mice (300 animals). Preleptotene, leptotene/zygotene and early pachytene spermatocytes (P_{17}) were isolated from seminiferous epithelia of day 17 mice (100 animals) (11). Pachytene spermatocytes (P_{60}), round spermatids (steps 1-8), and residual cytoplasmic bodies were isolated from testes of >60-day-old mice (60 animals). After collagenase and trypsin digestion of the seminiferous

tubules, the germ cells were separated by velocity sedimentation at unit gravity on 2%-4% BSA gradients (10). Adult pachytene and round spermatids were > 95% pure. Populations of the other spermatogenic cell types were > 85% pure based on examination of cell morphology under phase contrast optics. Animals used in these studies were maintained and sacrificed according to procedures outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Approval for these studies was received from the Morehouse School of Medicine institutional animal care and use committee.

3.2. Isolation of primary rat Sertoli cells

Primary Sertoli cells were isolated from the testes of 5, 11, and 20 day old Sprague Dawley rats (12). Decapsulated testes were digested with collagenase (0.5 mg/ml) at 37°C for 12 min in enriched Krebs-Ringer bicarbonate buffer (EKRb). The seminiferous tubules were washed 3 times with EKRb and digested with trypsin (0.5 mg/ml at 37°C for 12 min). Cell aggregates were dispersed using repeated pipetting with a plastic Pasteur pipette. An equal volume of DMEM containing 10% FBS was added to the cellular suspension prior to pelleting (40xg for 5 min). The pellets were resuspended in serum-free medium containing 50% DMEM, 50% Ham F12, insulin (5 mg/ml), transferrin (5 mg/ml), retinoic acid (10^{-6} M), epidermal growth factor (10 ng/ml), cytosine -Beta (β)-D arabinofuranosidase, (3 mg/ml), 1 mM sodium pyruvate, penicillin (100U/ml) and streptomycin (100 mg/ml).

3.3. RNA isolation and quantitative real-time PCR (qRT-PCR) assay

Total RNA was isolated from the StaPut purified mouse germ cells and primary rat Sertoli cells using Trizol reagents (Invitrogen, Calsbad, CA) following protocols provided by the manufacturer. The reverse-transcription reactions for cDNA synthesis were performed at 37°C for 1hour using SensiScript Reverse Transcriptase (Qiagen, Valencia, CA) with 1 μ M random hexamers (Invitrogen, Carlsbad, CA), 1 μ M dNTP, 0.5U HotStart Taq polymerase, total RNA (200ng) and Ribonuclease Inhibitor (1unit). Using the cDNA as template, quantitative real-time PCR was performed in iCycler Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Gene-specific primer sequences were designed using Oligo Primer Analysis Software (National Biomedical Systems, San Diego, CA). The RXR and PPAR gene-specific sequences used for real-time PCR are summarized in Table 1.

The PCR reaction mixture consisted of cDNA template from 200ng of total RNA, 0.3mM of each PPAR or RXR gene-specific primer pair and 1X QuantiTect SYBR green PCR mixture (Qiagen) containing dNTPs (1 μ M) and 5U HotStar Taq DNA Polymerase, to final volume of 50 μ l. Samples were amplified with a pre-cycling activation at 95°C for 15min, followed by 50 cycles of denaturation at 95°C for 15s, annealing at 54°C for 30s and extension at 72°C for 30s.

Internal Reference control (18S rRNA) and reagent control minus cDNA were included in each assay. Each

Table 1. Primers

Name	Forward 3'-5'	Reverse 3'-5'
RXR-Alpha	GATTCCGATACGACGACAGT	CATCACCACCTCTCGCCATC
RXR-Beta	CCAAGTCCTCTCGCAGT	ACCTACTCGTGTGCGTATAAC
RXR-Gamma	CACCAGTTGTACAGTCGTC	TGCTCTTGGCTCTCCGTATAG
PPAR-Alpha	GATTCTGATGGACGATGGCTT	ACAGGAGAGCAGGGATTG
PPAR-Beta	GATGGCGACGGTCAGGTG	TGTGGCAGGCAGAGAAGG
PPAR-Gamma	GAGTCCGTCTAGCAGTGT	CGAGGACATCCAAGACAAC

assay was performed at triplicate to verify the results and the mean Threshold Cycle (Ct) number was used for analysis of relative gene expression by comparative $2^{-\Delta\Delta C_t}$ method (13). Finally the amplification of non-specific PCR product was identified by melting curve analysis. The graphical data reported are the mean \pm SEM from triplicate samples from 4 independent studies. Statistical comparisons were made using unpaired t-test or analysis of variants (ANOVA). Significant differences are defined as $P < 0.05$.

3.4. PPAR adenoviral infection of primary Sertoli cells

Primary Sertoli cell cultures isolated from 15 day-old rat testes were plated (2×10^5 cells) in 60 nm² plates and infected with packaged PPAR-Gamma (γ) adenoviral vector or adenoviral vector controls (multiplicity of infection MOI = 10) in DMEM containing 1% FBS and maintained in culture for 72 hr. The cells were harvested by centrifugation (50 xg, 5 min), resuspended in PBS and lysed by addition of 1% Triton X 100. The supernatants were recentrifuged (1,000 x g for 5 min) to pellet any cellular debris.

3.5. Microarray analysis

The microarray data described in the present study complies with the MIAME (Minimum Information About Microarray Experiment) standards. Total RNAs were purified from the adenoviral PPAR-Gamma (γ) cDNA, adenoviral PPAR-Gamma (γ) RNAi, adenoviral -Beta (β)Gal and control adenovirus (empty vector) infected Sertoli cells using Trizol reagents (Invitrogen). Hybridization, washing and scanning of Affymetrix 430 2.0 mouse array Gene Chip were performed using protocols provided by the manufacturer (Affymetrix, Santa Clara, CA). Fluorimetric data were processed by Affymetrix software (Microarray Suite 5.0 MAS) and the gene chips were globally scored to all probe sets with an identical target intensity value. Only genes with average differences value (avg. diff.) above the threshold of 100 and with an avg. diff value of at least two-fold or greater above the control -Beta (β) Gal adenoviral vector vs. PPAR-Gamma (γ) cDNA or PPAR-Gamma (γ) RNAi adenoviral vector infected Sertoli cells were considered as significant. All reactions and microarray hybridizations were performed in the laboratory for Biotechnology and Bioanalysis 1 (LBB1) at Washington State University.

3.6. Microarray statistical analysis

The data analysis from MAS was imported into GeneSpring GX software (Silicon Genetics, Redwood City, CA). The data was normalized using signal values below 0.01 with total chip normalization to the 50th percentile. Transcripts expressed differentially were determined by one-way ANOVA parametric test with a P-value cut-off of

0.05. Benjamin and Hochberg False discovery rate multiple testing corrections were also applied to the data sets.

3.7 Two dimensional SDS-PAGE analysis of lysate from Sertoli cells

Two-Dimensional SDS-PAGE- Samples (100 ug) were loaded onto immobilized pH gradient (IPG) strips (Bio-Rad). Isoelectric focusing was performed on a Protean IEF Cell (Bio-Rad) for IPG gradient pH 3-10. For the second dimension SDS-PAGE using the Bio-Rad Criterion Cell system (Bio-Rad), focused immobilized strips were equilibrated in SDS buffer and transferred onto the surface of 8-16% gradient gel and embedded in 1% agarose containing traces of bromophenol blue (Sigma). All samples were run in duplicates. Gels were stained with SYPRO Ruby (Bio-Rad) protein gel stain and scanned using a Bio-Rad fluorescence laser scanner. The SYPRO Ruby labeled gels were pseudocolored as blue, red and green using PDQuest image analysis software (Bio-Rad). All gels were scanned with identical parameters.

4. RESULTS

The quantitative real-time PCR (qRT-PCR) data presented in Figure 1 shows the expression patterns of PPAR-Alpha (α), -Beta (β) and -Gamma (γ) in StaPut purified mouse germ cells. Although all the PPARs are expressed in spermatogonia, PPAR-Beta (β) is the predominantly expressed transcript in these proliferating germ cells. The expression levels of PPAR-Alpha (α) increase significantly in the preleptotene spermatocytes, while the levels of PPAR-Beta (β) and PPAR-Gamma (γ) remain the same as observed in the spermatogonia. In the leptotene/zygotene spermatocytes, PPAR-Alpha (α) transcripts are expressed at higher levels than PPAR-Beta (β) and PPAR-Gamma (γ) transcripts although PPAR-Gamma (γ) shows a slight increase at this stage of spermatogenesis. The expression levels of all three PPARs decrease in the pachytene spermatocytes. In the round spermatids, PPAR-Alpha (α) transcripts are expressed at significantly higher levels than PPAR-Beta (β) and PPAR-Gamma (γ) transcripts.

Quantitative real-time PCR studies in Figure 2 show the RXR-Alpha (α), Beta (β) and Gamma (γ) transcript expression profiles in the purified mouse germ cell population. RXR-Alpha (α) transcripts are expressed at higher levels than RXR-Beta (β) and RXR-Gamma (γ) in spermatogonia. The expression levels of RXR-Alpha (α), RXR-Beta (β) and RXR-Gamma (γ) transcripts increase in the preleptotene spermatocytes. In the leptotene/zygotene spermatocytes RXR-Gamma (γ) is expressed at significantly higher levels compared to RXR-Alpha (α) and

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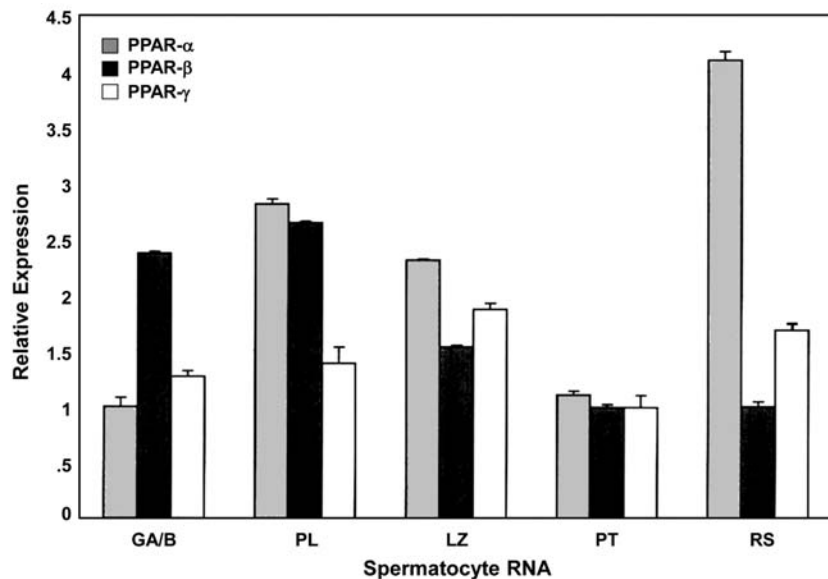


Figure 1. Real-time PCR analysis of expression levels of PPAR transcripts expressed in differentiating mouse germ cells. Total RNAs isolated from StaPut purified mouse germ cells were analyzed in the BioRad iCycler using SYBR green fluorescent real-time PCR with gene-specific primers for PPAR-Alpha (α), -Beta (β) and -Gamma (γ). The mean threshold (Ct) number derived from the BioRad iCycler software program was used to calculate the relative expression of the PPAR transcripts by the comparative $2^{-\Delta\Delta Ct}$ method. Internal reference control (18S rRNA) and (minus cDNA) were included for each assay. The bar graph represents the mean \pm SEM from triplicate samples ($n = 3$) $P < 0.05$. These studies are representative to two independent experiments. GA/B: type A and B spermatogonia; PL: preleptotene spermatocytes; L/Z: leptotene/zygotene spermatocytes; PT: pachytene spermatocytes; RS: round spermatids.

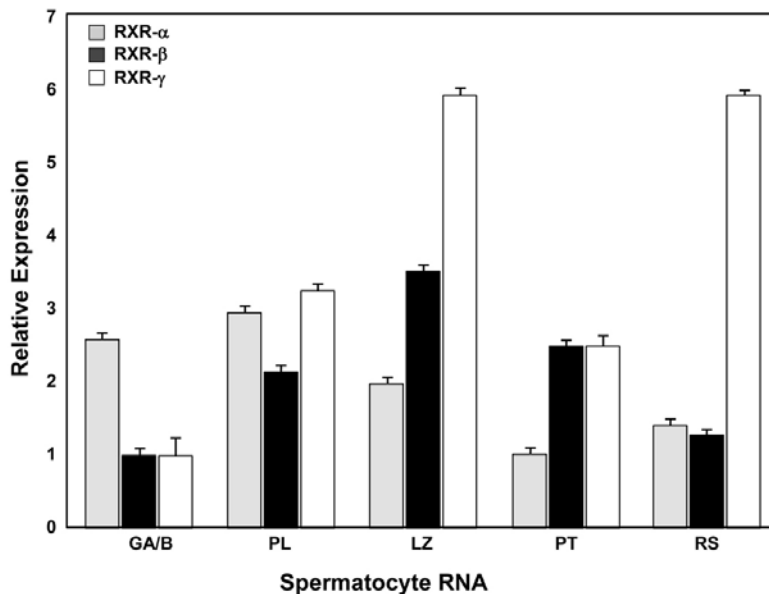


Figure 2. Real-time PCR analysis of the expression levels of RXR transcripts in differentiating mouse germ cells. Total RNAs isolated from StaPut purified mouse germ cells were analyzed in the BioRad iCycler using SYBR green fluorescent real-time PCR with gene-specific RXR-Alpha (α), -Beta (β) and -Gamma (γ) primers. The mean threshold (Ct) number derived from the BioRad iCycler software program was used to calculate the relative expression of the PPAR transcripts by the comparative $2^{-\Delta\Delta Ct}$ method. Internal reference control (18S rRNA) and (minus cDNA) were included for each assay. The bar graph represents the mean \pm SEM from triplicate samples ($n = 3$) $P < 0.05$. These studies are representative to two independent experiments. GA/B: type A and B spermatogonia; PL: preleptotene spermatocytes; L/Z: leptotene/zygotene spermatocytes; PT: pachytene spermatocytes; RS: round spermatids.

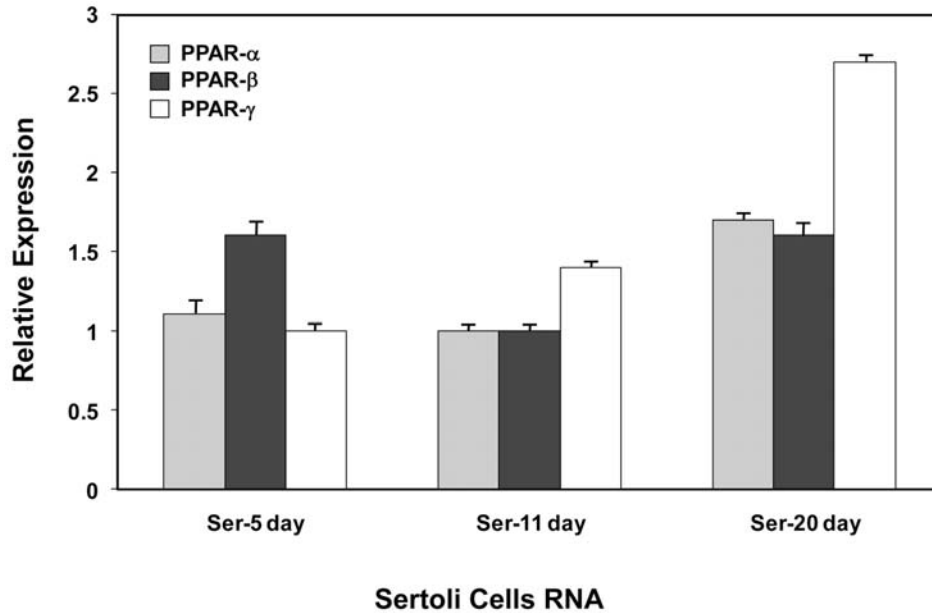


Figure 3. Differential expression of PPAR transcripts in differentiating primary rat Sertoli cells. Total RNAs purified from Sertoli cells isolated from the testis, of 5, 11 and 20 day-old prepubertal rats were analyzed in the BioRad iCycler using SYBR green fluorescent real-time PCR with gene-specific PPAR-Alpha (α), -Beta (β) and -Gamma (γ) primers. The mean threshold (Ct) number derived from the BioRad iCycler software program was used to calculate the relative expression of the PPAR transcripts by the comparative $2^{-\Delta\Delta Ct}$ method. Internal reference controls (18 S RNA) and (minus cDNA) were included for each assay. The bar graphs represent the mean \pm SEM from triplicate samples (n=3) $P < 0.05$. These studies are representative of four independent experiments.

RXR-Beta (β) transcripts. Significantly lower expression levels of all three RXRs-Alpha (α), -Beta (β) and Gamma (γ) transcripts are observed in the pachytene spermatocytes. In the round spermatids, the expression of RXR-Gamma (γ) transcripts are significantly higher than RXR-Alpha (α) and RXR-Beta (β) transcripts.

To examine the expression profiles for PPAR and RXR nuclear receptors in Sertoli cells, quantitative real-time PCR studies were performed on RNAs isolated from the primary rat Sertoli cells from 5, 11 and 20 day-old prepubertal rats as shown in Figure 3. All three of the PPAR-Alpha (α), Beta (β) and Gamma (γ) transcripts are expressed in 5 day-old rat Sertoli cells, with PPAR-Beta (β) expressed at higher levels than PPAR-Alpha (α) and PPAR-Gamma (γ). In the 11 day-old rat Sertoli cells, PPAR-Gamma (γ) transcripts were expressed at higher levels than PPAR-Alpha (α) and PPAR-Beta (β). The mature 20 day-old rat Sertoli cells showed higher levels of expression of all three PPAR transcripts, however, PPAR-Gamma (γ) transcript expression levels were significantly higher than PPAR-Alpha (α) and PPAR-Beta (β) levels. Since RXR isoforms form obligatory heterodimers with the PPAR isoforms, we also examined the corresponding expression profiles for the RXR-Alpha (α), -Beta (β) and -Gamma (γ) transcripts. As shown in Figure 4, real-time PCR studies indicate that although all the RXR-Alpha (α), -Beta (β) and -Gamma (γ) transcript were expressed at low levels, RXR-Beta (β) was the major transcript expressed in the 5 day-old rat Sertoli cells, while RXR-Gamma (γ) was

the predominant RXR transcript expressed in the 11 day old rat Sertoli cells. Significantly higher levels of RXR-Gamma (γ) transcripts were observed compared with RXR-Alpha (α) and RXR-Beta (β) in the mature 20 day-old rat primary Sertoli cells. These studies suggest the encoded PPAR-Gamma (γ)/RXR-Gamma (γ) proteins are the predominant heterodimeric functional transcription factor present in the mature 20 day-old primary rat Sertoli cells.

In the next series of studies we focused on the functional specificity of PPAR-Gamma (γ)/RXR-Gamma (γ) heterodimers in mediating target gene activation in mature Sertoli cells. Affymetrix microarray studies were utilized to analyze the global gene expression patterns in 15 day-old primary rat Sertoli cells. Since the RXRs isoforms can form heterodimeric pairs with other nuclear hormone receptors including thyroid and vitamin D receptors, our experimental strategy was to overexpress PPAR-Gamma (γ) in the primary Sertoli cells (14). Under these experimental conditions it is likely that the majority of available RXR isoforms will pair with overexpressed PPAR-Gamma (γ) to form functional heterodimeric transcription factors that would subsequently interact with PPRE elements in the promoter region of target genes. Complementary RNAi mediated knockdown studies of PPAR-Gamma (γ) were also performed as controls to demonstrate that the majority of target genes up-regulated by PPAR-Gamma (γ) showed corresponding down-regulation after RNAi mediated knockdown of PPAR-Gamma (γ). As indicated by the summary shown in Figure5A, Affymetrix microarray analysis identified several

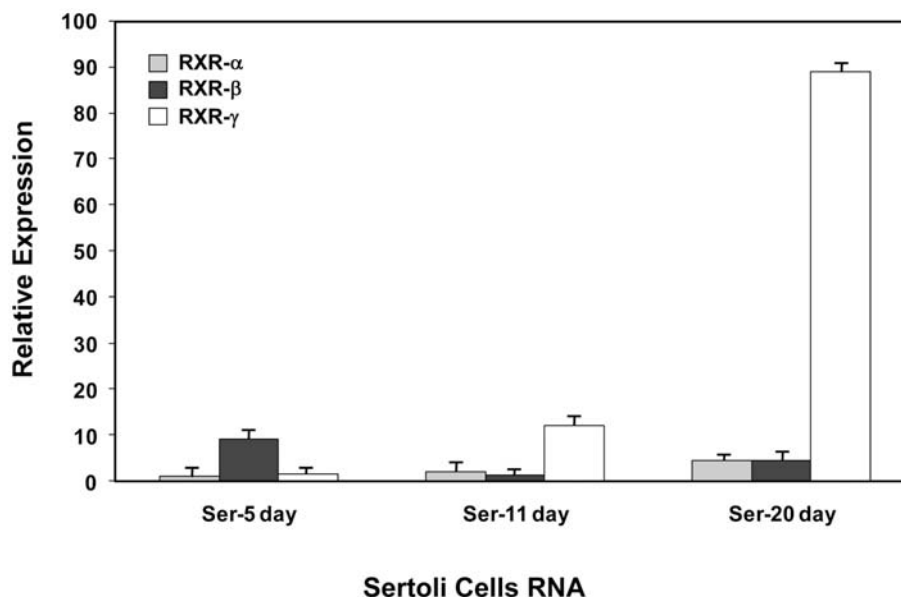


Figure 4. Differential expression of RXR transcripts in differentiating primary rat Sertoli cells. Total RNA purified from Sertoli cells isolated from 5, 11 and 20 day-old prepubertal rats were analyzed in the Bio-Rad iCycler using SYBR green fluorescent real-time PCR with gene-specific RXR-Alpha (α), -Beta (β) and -Gamma (γ) primers. The mean threshold number (Ct) derived from the Bio-Rad icycler software program was used to calculate by the comparative $2^{-\Delta\Delta Ct}$ method. Internal reference control (18S rRNA) and reagent control (minus cDNA) were included in each assay. The bar graphs represent the mean \pm SEM for triplicate samples ($n = 3$) $P < 0.05$. These studies are representative of four independent experiments.

metabolic genes that show 2 fold or greater up-regulation under PPAR-Gamma (γ) overexpression and corresponding 2 fold or greater down-regulation after PPAR-Gamma (γ) RNAi mediated knockdown. These genes play important functional roles in regulating several the lipid and glucose metabolic pathways in Sertoli cells. These studies utilized the Affymetrix mouse Genome 430 2.0 array that contains 39,000 transcripts. After hypothetical genes and expressed sequence tags were removed, the remaining 35,908 probe set were filtered to exclude those genes without a medium significance level of $P < 0.05$ as determined by the Affymetrix software. This filtering process indicated that 3392 highly significant genes were > 2 fold up-regulated after PPAR-Gamma (γ) cDNA transfection and 4844 genes were > 2 fold down-regulated after overexpression of PPAR-Gamma (γ) RNAi transfection into the primary Sertoli cells. Since PPAR-Gamma (γ) is known to regulate lipid metabolism in most cell types, we elected to examine the expression patterns of these lipid genes in the Sertoli cells. Data presented in Table 2 shows that several lipid metabolic genes that were PPAR-Gamma (γ) target genes were up-or down-regulated under these experimental conditions. For the eleven of these selected lipid genes listed in Table 2, quantitative real-time PCR data presented in Figure 5B verified the accuracy of the microarray data set by showing that PPAR-Gamma (γ) mediated up-regulation and down-regulation of these lipid metabolic genes after infection of the primary Sertoli cells with the recombinant PPAR-Gamma (γ) adenoviral vectors.

Since the PPAR-Gamma (γ) gene probes were present on the Affymetrix chips, the microarray data provided an excellent internal control for these expression studies. Infection of the Sertoli cells by the adenoviral PPAR-Gamma (γ) RNA cDNA vectors resulted in x 5 fold overexpression of PPAR-Gamma (γ) while the adenoviral PPAR-Gamma (γ) RNAi vector infection resulted in a significant knockdown of PPAR-Gamma (γ) expression (x 10 fold) in these primary Sertoli cells (See Table 2).

Preliminary studies were performed to demonstrate that changes in expression of mRNAs observed by microarray analysis corresponded to changes at the level of protein expression. Parallel proteomic studies were performed utilizing high-resolution two-dimensional polyacrylamide gel electrophoresis (PAGE) to analyze lysates derived from the adenoviral PPAR-Gamma (γ) cDNA and PPAR-Gamma (γ) RNAi infected primary Sertoli cells. As shown in Figures 6A and 6B significant differences were observed for protein expression profiles in the Sertoli cell lysates after adenoviral PPAR-Gamma (γ) cDNA overexpression compared to adenoviral PPAR-Gamma (γ) RNAi knockdown after infection of the Sertoli cells. Since these gel electrophoretic studies were performed using the BioRad preformed gel platforms, we were able to utilize computer-assisted merging of the imaged gels to verify that there were significant differences in the respective protein expression profiles under these two experimental treatment conditions when compared with the adenoviral GFP vector.

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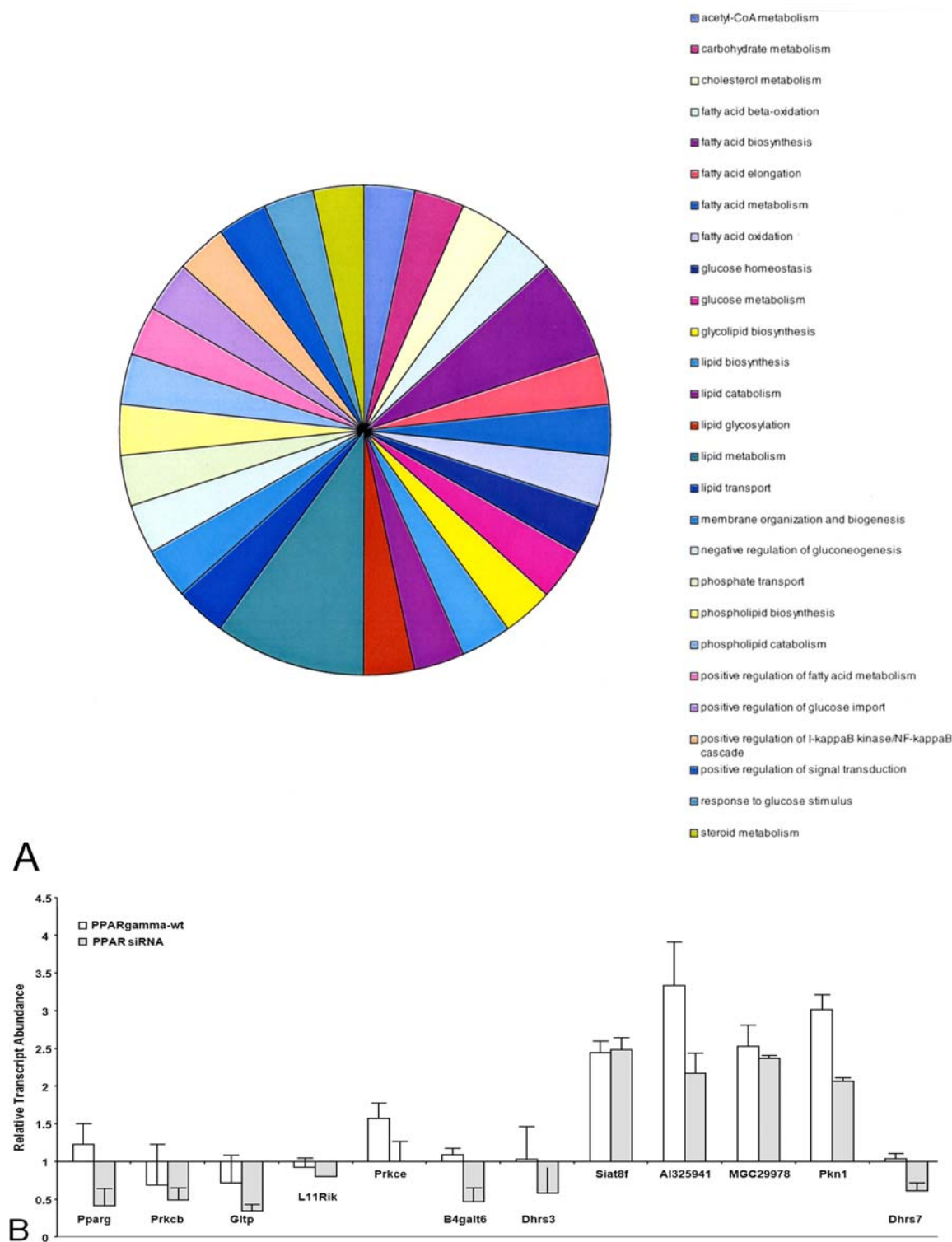


Figure 5.(A). Categorization of genes involved in biological processes regulated by PPAR-Gamma (γ) overexpression or PPAR-Gamma (γ) RNAi knockdown in primary rat Sertoli cells. Data derived from Affymetrix microarray analysis.(B). Quantitative real-time PCR analysis of expression levels of selected lipid metabolic genes showing up-regulation after PPAR-Gamma (γ) overexpression or down-regulation after PPAR-Gamma (γ) RNAi mediated knockdown in primary rat Sertoli cells.

Table 2. Microarray analysis

Gene ID	Common name	Fold Change	GeneBank Name	RefSeq	Product Description
Microarray Analysis of AD PPARγ effect on target Gene expression and fold changes adjusted against AD B-gal control					
1420715_a_at	Pparg	4.847	NM_011146	NM_011146	peroxisome proliferator activated receptor gamma
1443144_at	Prkcb	0.879	BB084315		protein kinase C, beta
1419027_s_at	Gltpt	8.257	BC016584	NM_019821	glycolipid transfer protein
1424312_at	28100031L11Rik	0.761	BC014875	NM_028320	RIKEN cDNA 2810031L11 gene
1437860_at	Prkce	3.351	BB335101	NM_011104	BB335101 RIKEN full-length enriched, 10 days neonate medulla oblongata Mus muscular cDNA clone B830031O06 3', mRNA sequence.
1460329_at	B4galt6	5.405	BG066773	NM_019737	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6
1448390_a_a	Dhrs3	0.462	NM_011303	NM_011303	dehydrogenase/reductase (SDR family) member 3
1456147_at	Siat8f	0.0216	BB486599	NM_145838	sialyltransferase 8 (alpha-2, 8-sialyltransferase) F
143689_x_at	AI325941	2.28	BB204677	NM_178900	expressed sequence AI325941
1424451_at	MGC29978	4.064	BC019882	NM_146230	3-ketoacyl-CoA thiolase B
1445445_s_at	Prkcl1	0.557	BB283614	NM_177262	protein kinase C-like 1
1432892_at	Dhrs7	1.87	AK017856	NM_025522	dehydrogenase/reductase (SDR family) member 7
Microarray Analysis of AD PPARγ SiRNA effect on target Gene expression and fold changes adjusted against AD B-gal control					
1420715_a_at	Pparg	0.0957	NM_011146	NM_011146	peroxisome proliferator activated receptor gamma
1443144_at	Prkcb	0.0358	BB084315		protein kinase C, beta
1419027_s_at	Gltpt	0.339	BC016584	NM_019821	glycolipid transfer protein
1424312_at	28100031L11Rik	0.0605	BC014875	NM_028320	RIKEN cDNA 2810031L11 gene
1437860_at	Prkce	3.351	BB335101	NM_011104	BB335101 RIKEN full-length enriched, 10 days neonate medulla oblongata Mus muscular cDNA clone B830031O06 3', mRNA sequence.
1460329_at	B4galt6	0.256	BG066773	NM_019737	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6
1448390_a_a	Dhrs3	0.155	NM_011303	NM_011303	dehydrogenase/reductase (SDR family) member 3
1456147_at	Siat8f	0.0724	BB486599	NM_145838	sialyltransferase 8 (alpha-2, 8-sialyltransferase) F
143689_x_at	AI325941	1.201	BB204677	NM_178900	expressed sequence AI325941
1424451_at	MGC29978	0.38	BC019882	NM_146230	3-ketoacyl-CoA thiolase B
1445445_s_at	Prkcl1	0.0484	BB283614	NM_177262	protein kinase C-like 1
1432892_at	Dhrs7	0.343	AK017856	NM_025522	dehydrogenase/reductase (SDR family) member 7

5. DISCUSSION

The experimental studies described in this report were designed to investigate the expression patterns of PPAR and RXR transcripts in differentiating mouse germ cells and primary rat Sertoli cells. Although data mining of the Affymetrix microarray gene profiling studies that were performed by Shima *et al.* identified expression patterns of the PPARs and RXRs in germ cells and Sertoli cells (18), these microarray-derived RNA expression patterns were not validated by quantitative real-time PCR (qRT-PCR) studies. Our qRT-PCR studies revealed that PPAR and RXR transcripts encoding members of the PPAR and RXR nuclear receptor family reached maximum levels of expression in the germ cells during the early meiotic stages of spermatogenesis. Interestingly, a significant decrease in both PPAR and RXR transcript expression are observed in the pachytene spermatocytes at the end of meiosis. During germ cell differentiation, PPAR-Alpha (α) and PPAR-Beta (β) expression levels increased from the early spermatogonial stages of spermatogenesis and peaked in the preleptotene spermatocytes, while PPAR-Gamma (γ) levels peaked at a slightly later stage of spermatogenesis in the leptotene/zygotene spermatocytes. Corresponding results were observed for the RXR transcripts, RXR-Alpha

α) transcripts peaked at the early stages of meiosis in the preleptotene spermatocytes while increased levels of RXR-Beta (β) and RXR-Gamma (γ) expression were observed in later stages of meiosis in the leptotene/zygotene spermatocytes as observed for expression of the PPARs. The maximum levels of expression of the RXR and PPAR transcripts occurred during the early meiotic stages of spermatogenesis in the preleptotene and leptotene/zygotene spermatocytes. Interestingly, significantly high levels of PPAR-Gamma (γ) and RXR-Alpha (α) were observed in the round spermatids. This observation verifies the RXR-Alpha (α) expression profile detected previously in the spermatids by *in situ* hybridization studies performed by Kastner *et al.* (4). RNase protection assays studies performed by Gaemers *et al* (5) were able to detect low levels of RXR-Gamma (γ) expression in testes, however, these studies did not determine which of testicular cell types were responsible for this RXR-Gamma (γ) expression. In summary, our studies suggest that the different combinations of PPAR/RXR heterodimeric transcription factors encoded by these transcripts are likely to play a significant functional role in regulating stage and cell-type-specific gene expression successive stages of spermatogenesis in differentiating mouse germ cells.

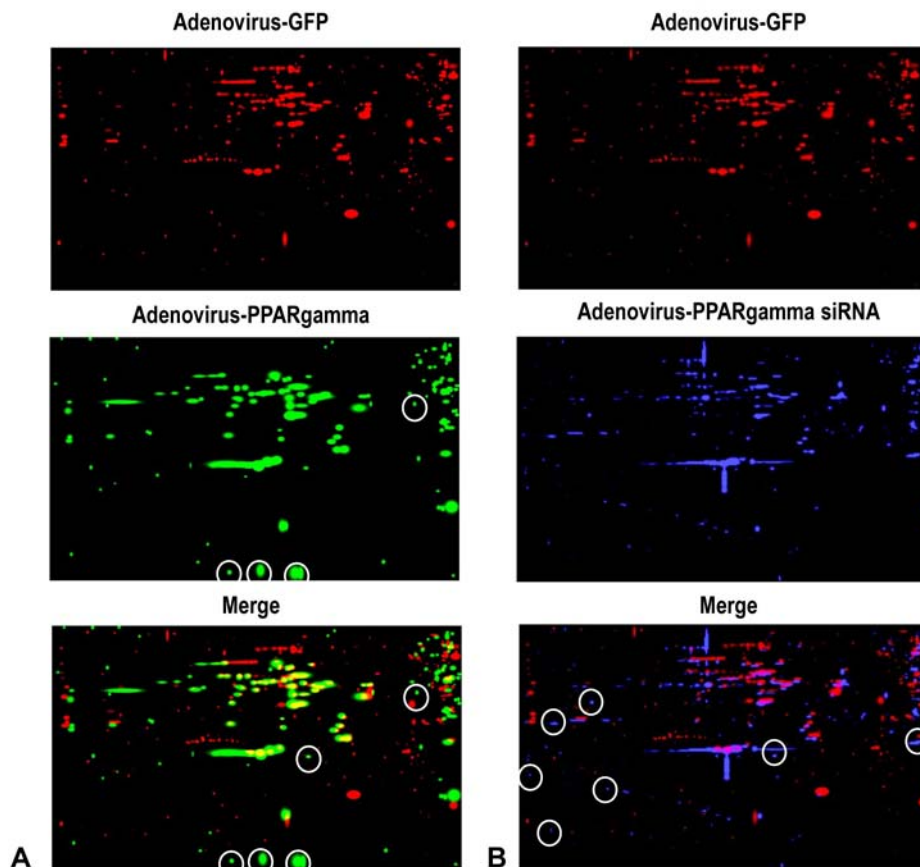


Figure 6.(A). Protein expression patterns derived from lysates isolated from adenoviral (AD) GFP control and PPAR-Gamma (γ) cDNA adenovirus (AD) infected 15 day primary rat Sertoli cells. The lysates were fractionated by IEF followed by SDS PAGE. Proteins expressed in the adenoviral GFP control infected Sertoli cells are shown in red (a) and the PPAR-Gamma (γ) cDNA adenoviral infected Sertoli cells in green (b) Image merging shows that several proteins (white circles) are present only after PPAR-Gamma (γ) overexpression in the Sertoli cells. (B) Protein expression patterns derived from lysates isolated from adenovirus GFP control and PPAR-Gamma (γ) RNAi adenoviral infected 15 day primary rat Sertoli cells. The lysates were fractionated by IEF followed by SDS PAGE. Proteins expressed in the adenoviral control lysates are shown in red (a) and the PPAR-Gamma (γ) RNAi adenoviral infected Sertoli cells are shown in blue (b). Image merging shows that there is a significant change in the profile of proteins expressed in the Sertoli cells after PPAR-Gamma (γ) RNAi mediated knockdown as indicated by the white circles.

Quantitative real-time PCR analysis of the total RNA samples isolated from primary rat Sertoli cells from the testes of 5, 11 and 20 day old prepubertal rats indicated that all three of the PPARs, -Alpha (α), -Beta (β) and -Gamma (γ) transcripts are expressed in the differentiating Sertoli cells. PPAR-Gamma (γ) transcripts were expressed at higher levels than PPAR-Alpha (α) and PPAR-Beta (β) while RXR-Gamma (γ) transcripts was expressed at higher levels than RXR-Alpha (α) and RXR-Beta (β) in the 20 day-old mature Sertoli cells. The pattern of expression of RXR transcripts and their encoded protein isoforms have been extensively studied in the seminiferous epithelium of rat and mouse testis using Northern blot, *in situ* hybridization and immunohistological analysis. Dufour and Kim (3) detected several RXR-Alpha (α), -Beta (β) and -Gamma (γ) protein isoforms in both somatic Sertoli and germ cells isolated from rat testes. These investigators also demonstrated that the RXR isoforms found in rat testis

were similar in size to the mouse testis RXR proteins suggesting that there are no species-specific differences in the respective RXR isoforms. Immunological and Western blot studies performed by these investigators indicated that the RXR receptor isoforms were not always present in the nucleus. In fact, these studies found that detectable RXR-Alpha (α) isoforms were localized in the cytoplasm of germ cells throughout spermatogenesis, while the RXR-Gamma (γ) isoforms were present in the nucleus of early and late spermatocytes and elongating spermatids. Based on these results, the authors suggested that formation of functional PPAR/RXR complexes in germ cells are likely to be regulated by molecular mechanisms that determine which of the nuclear receptor isoforms are translocated from the cytoplasm into the nucleus.

Conflicting published reports exist for expression of the PPARs in testis. Shultz *et al.* (9) reported

differential expression of PPAR-Alpha (α) transcripts in spermatocytes isolated from human and rat testes during spermatogenesis, while studies performed by Braissant *et al.* (15) did not detect any PPAR-Alpha (α) transcript expression in rat germ cells, but detected only low levels of PPAR-Gamma (γ) expression. The quantitative real-time PCR studies performed in our laboratory clearly show that all three PPAR transcripts are developmentally expressed in mouse germ cells. Further proteomics studies are required to investigate whether the observed patterns of PPAR transcript expression reflect corresponding profiles for PPAR proteins isoforms expressed in the respective germ cells.

PPAR-Gamma (γ) and RXR-Gamma (γ) are the predominant transcripts that are expressed in mature Sertoli testicular cells as indicated by quantitative real-time PCR studies performed on RNA isolated from 20 day-old primary Sertoli cells. These transcripts most likely encode functional PPAR-Gamma (γ) and RXR-Gamma (γ) proteins that form heterodimeric transcription factor complexes that subsequently play a major role in mediating target gene activation in the Sertoli cells. Proteomic studies from adipocytes and cardiomyocytes have indicated that PPAR-Gamma (γ) is involved mainly in mediating activation of lipid metabolic target genes in these cell types (16,17). In this study, we have presented experimental evidence to demonstrate that PPAR-Gamma (γ) overexpression results in up-regulation of lipid metabolic target genes in primary Sertoli cells while knockdown of PPAR-Gamma (γ) expression results in down-regulation of these lipid genes. We utilized the Affymetrix mouse 430 2.0 mouse Gene Chip for these studies since 39,000 probe sets are available on these chips rather than the limited 9,000 probe sets available on the Affymetrix rat U34 Gene Chips (18). Since the PPAR genes are included on these chips, analysis of the PPAR-Gamma (γ) expression patterns in the adenoviral transfected Sertoli cells constituted an excellent internal experimental control for our studies (see Table 2). These microarray-based expression studies of RNAs isolated from the adenovirus transfected 15 day-old rat Sertoli cells describe the steady state levels of transcripts expressed in these cells. We are aware that expression of the respective encoded proteins in these cells are likely to be affected by post-transcriptional, translational and post-translational regulatory events.

Preliminary studies to determine whether PPAR-Gamma- γ overexpression and knockdown affected protein expression patterns in the primary Sertoli cells were performed using high-resolution two-dimensional polyacrylamide gel electrophoresis (PACE). These studies confirmed that specific changes occurred in the patterns of protein expression under these experimental conditions. However, we were not able to perform high-throughput quantitative analysis of specific protein expression levels in these Sertoli cell lysates since effective protocols for such as analysis were not available. Published studies by Vogel *et al.* (19) have only recently demonstrated the feasibility of using a novel approach to facilitate high-throughput quantitative analysis of protein expression levels in cellular lysates. These investigators have successfully applied

sophisticated LC/MS/MS mass-spectrometry-based proteomics methods in combination with an extensive bioinformatics statistical analysis program called APEX (20) that was developed in their laboratory for large-scale processing of peptide data. This innovative experimental approach was able to achieve a relatively accurate quantitative measurement of the expression levels of specific proteins present in cellular lysates.

The significance of the observed PPAR-Gamma (γ)-mediated changes in lipid metabolic gene expression in the Sertoli cells is supported by genetic studies performed in both humans and mice which have indicated that male fertility is compromised by inactivation of genes involved in lipid metabolism (21). In the testis, lipids are not only important as source of energy, but they also serve as integral structural components of the membranes forming the tight junctions that comprise the seminiferous epithelium. In addition, several molecular studies have shown that lipids also act as second messengers that are critical components of a number of nuclear receptor-mediated signal transduction pathways. Furthermore, analysis of lipid composition of spermatozoa indicate that their membranes contain extremely high proportions of long-chain fatty acids that are products of the lipid metabolic pathways that mainly occur in the Sertoli cells, and therefore, are important for maintaining male fertility (9, 22,23). In conclusion, the Affymetrix microarray and qRT-PCR molecular studies described in this study have presented new experimental evidence confirming that PPAR-Gamma (γ) play a major physiological role in Sertoli cells by activating key genes involved in the regulation of lipid metabolism.

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Abbreviations: PPAR: peroxisome proliferator-activated receptors; RXR: retinoid X receptors; qRT-PCR: quantitative real-time polymerase chain reaction; PPRES: peroxisome response elements; TZD: thiazolidinediones; EKRB: enriched Krebs-Ringer bicarbonate buffer; DMEM: Dulbecco's Modified Eagle Medium; FBS: fetal bovine serum; PBS: phosphate buffered saline; SDS PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; IEF: isoelectric focusing

Key Words: Spermatogenesis, Gene Expression, Nuclear Receptors, Testis

Nuclear receptor gene expression in the testis

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