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Temporal Profiling of Rat Transcriptomes in Retinol-Replenished Vitamin A-Deficient Testis

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Abstract

At least in mammals, retinoic acid is a pivotal factor in maintaining the functionality of the testis, in particular, for the progression of germ cells from mitosis to meiosis. Removal of dietary vitamin A or a targeted deletion of retinoic acid receptor alpha gene (*Rara*), the receptor for retinoic acid, in mice, led to testicular degeneration by a dramatic loss of germ cells and a loss of control of the spermatogenic cycle. The germ cells that remained in the vitamin A deficient (VAD) rat testis were spermatogonia and a few preleptotene spermatocytes. Spermatogenesis can be reinitiated by injection of VAD rats with retinol, the metabolic precursor of retinoic acid, but to date, the functions of retinoic acid in the testis remain elusive. We have applied DNA microarray technology to investigate the time-dependent transcriptome changes that occur 4 to 24 h after retinol replenishment in the VAD rat testis. The retinol-regulated gene expression occurred both in germ cells and Sertoli cells. Bioinformatic analyses revealed time-dependent clusters of genes and canonical pathways that may have critical functions for proper progression through spermatogenesis. In particular, gene clusters that emerged dealt with: (1) cholesterol and oxysterol homeostasis, (2) the regulation of steroidogenesis, (3) glycerophospholipid metabolism, (4) the regulation of acute inflammation, (5) the regulation of the cell cycle including ubiquitin-mediated degradation of cell cycle proteins and control of centrosome and genome integrity, and (6) the control of membrane scaffolding proteins that can integrate multiple small GTPase signals within a cell. These results provide insights into the potential role of retinoic acid in the testis.

Keywords

retinoic acid receptor; retinoids; spermatogenesis; testis; vitamin A deficiency

Introduction

Transforming a single stem germ cell into a functional sperm capable of fertilization is a complicated process that is controlled by both endocrine and paracrine factors. One of the factors found to be essential for this process is vitamin A (retinoids) provided by nutrition in mammals. The removal of vitamin A from the diet leads to a severe degeneration of the testes [Howell et al. 1963], as observed by a decrease in the size of the testes, due to a dramatic loss of germ cells. The early meiotic prophase spermatocytes undergo apoptosis, while the haploid

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Note 1: Aarray data is available in the GEO database as GSE14962.

Note 2: Gene names are italicized and capitalized in the first letter, followed by lowercase letters. Protein names are all capitalized.

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germ cells slough into the lumen of the seminiferous tubules [Akmal et al. 1998]. The germ cells found in the vitamin A deficient (VAD) rat testes are the type A1 spermatogonia and a few remaining preleptotene spermatocytes, that are typically at stages VII and VIII of the spermatogenic cycle [Morales and Griswold 1987].

Spermatogenesis can be reinitiated in the VAD testes by an injection of a pharmacological dose of retinol (the alcohol form of vitamin A) or multiple injections of retinoic acid (the acid form of vitamin A), followed by feeding animals a vitamin A-sufficient diet [Huang and Hembree 1979; Morales and Griswold 1987; van Pelt and de Rooij 1991]. The re-initiation of spermatogenesis occurs from the type A1 spermatogonia in a synchronized fashion, such that the regenerated seminiferous tubules have only a few stages of the spermatogenic cycle represented, instead of the fourteen stages usually found in the rat testes from animals fed a normal diet [Morales and Griswold 1987]. The few remaining preleptotene spermatocytes undergo apoptosis soon after the re-initiation of spermatogenesis [Ismail et al. 1990].

Retinol is metabolized in the target tissue, including testis, into retinoic acid that can activate retinoid receptors [Gottesman et al. 2001]. These receptors are ligand-dependent transcription factors, belonging to the nuclear hormone receptor superfamily. There are two families of retinoid receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). Each family is composed of three protein subtypes, RARA, RARB, and RARG and RXRA, RXRB, and RXRG, respectively. Typically, a RAR dimerizes with its obligate partner RXR, and as a heterodimeric complex, binds to the retinoic acid response element (RARE) on target genes, in the presence of the retinoic acid ligand, to regulate the transcription of RARE-containing target genes [Evans 1994; Chambon 1996].

Compelling genetic evidence indicate that retinoid receptors play a critical role in spermatogenesis. Spermatid maturation is blocked in the *Rxrb* knockout testes [Kastner et al. 1996]; while the early meiotic prophase spermatocytes undergo apoptosis and the spermatogenic cycle is abnormal in the *Rara* knockout testes [Lufkin et al. 1993; Chung et al. 2004; Chung et al. 2005; Doyle et al. 2008]. Expression studies of RARs and RXRs revealed that both Sertoli cells and germ cells are the target cells of retinoic acid in the testis [Akmal et al. 1997; Dufour and Kim 1999]. Furthermore, the levels of *Rara* mRNA and RARA protein peaked within 2-8 h, and the mRNA level decreased by 24 h in Sertoli cells of retinol-replenished VAD testes [Kim and Griswold 1990; Akmal et al. 1998], indicating a tight regulation.

Analysis using spermatogonial transplantation model revealed that complete germ cell development in the testis requires RARA in both germ cells and Sertoli cells [Doyle et al. 2008]. When wild type germ cells were transplanted into the *Rara* knockout testis, the donor germ cells could not develop efficiently past the meiotic stage of development in a RARA negative microenvironment. In the reciprocal experiment, where *Rara* knockout germ cells were transplanted into a W/W^v mouse testis, it was observed that the majority of donor *Rara* knockout germ cells could not colonize normally [Doyle et al. 2008]. Collectively, these results suggested that the presence of RARA in the germ cells is critical for early spermatogonial proliferation and differentiation, and that RARA in somatic cells is responsible for promoting the survival and progression of the meiotic spermatocytes to the next stage of germ cell development.

The effects of retinoic acid on the expression of transcripts in the testis have been studied for some time, although mostly one gene at a time. In this study, we have taken a microarray technology approach to globally determine transcriptome changes after acute retinol treatment of VAD rats in a time-dependent manner. The VAD rat testis model provides an opportunity to study an *in vivo* system that contains fewer germ cell types, type A spermatogonia and a few

preleptotene spermatocytes, and somatic cells possibly acting as primary responders to retinoic acid during spermatogenesis, when compared to the normal, vitamin A sufficient adult testis. We have identified a number of gene clusters under the control of retinoids that may be important for spermatogonial proliferation and differentiation, and for proper somatic cell functions that are necessary for supporting germ cell development.

Results

Vitamin-A Deficient State and Retinol Replenishment

Male Sprague-Dawley rats on a vitamin-A deficient diet for nine weeks were injected with retinol and their testes collected at 0 (VAD state), 4, 8, and 24 h. To determine if the rats were indeed in a VAD state, a portion of testis from each animal was histologically processed and stained with hematoxylin and eosin. Extensive degeneration of seminiferous tubules in the VAD testis was evident (Fig. 1A), as compared to seminiferous tubules containing a full complement of germ cells in normal, vitamin A-sufficient adult animals (Fig. 1B). No morphological differences were observed for the VAD samples and the 4 to 24 h retinol-treated samples (data not shown). To determine if the VAD animals responded to retinol, the protein expression of RARA, a known retinoic acid-regulated protein [Akmal et al. 1998] was checked by Western blot analysis using anti-RARA antibody. RARA protein expression increased by 8 and 24 h post-retinol treatment of VAD animals (Fig. 1C). In addition, RNA collected from the VAD and retinol-replenished testes were used to perform real time RT-PCR using primer sequences (Table 1) for three known retinoic acid-regulated genes: stimulated by retinoic acid 8 (*Stra8*) [Oulad-Abdelghani et al. 1996]; transferrin [Huggenvik et al. 1987], and cellular retinol binding protein 1 (*cRBP1*) [Faraonio et al. 1993]. The levels of these three retinoic acid-regulated transcripts were increased after retinol injection (Fig. 1E). These pre-microarray experiments verified that VAD rats responded to retinol, and we could proceed with microarray hybridization.

Microarray Hybridization and GeneSpring Analysis

RNA from four separate sets of VAD and retinol-replenished testes were used to generate cRNA targets. These targets were hybridized to the Affymetrix rat U34A microarray chips (Affymetrix Inc., Santa Clara, CA). The raw signals generated from the microarray hybridization were initially acquired and scaled to target intensity by Affymetrix GeneChip® Operating Software (GCOS). Then, they were exported to GeneSpring 7.3 (Agilent Technologies, Santa Clara, CA) and normalized and filtered, as described in Materials and Methods. A total of 431 genes that had at least one sample having a raw signal of 50 or greater and at least one statistically significant change with a *P* value of ≤ 0.05 passed these filters.

Of the 431 significant genes, 309 genes changed by retinol ± 1.5 fold or greater compared to the VAD value (Fig. 2A). The complete list of 309 individual genes, with the highest raw scores to indicate the abundance, are shown in the Supplement available in the electronic version of this manuscript. The 104 up-regulated genes (Supplement Table S1) are separated from 205 down-regulated genes (Supplement Table S2). Analyzing each time point, 90 genes had a fold change of ± 1.5 or greater at 4 h, 249 genes at 8 h, and 266 genes at 24 h (Fig. 2A). An almost equal number of genes were up-regulated (46 genes) and down-regulated (44 genes) after 4 h of retinol treatment. However, at 8 and 24 h post-retinol treatment, more genes were down-regulated than up-regulated. To further understand the relationship of the changes in genes between time points, Venn diagrams were generated (Fig. 2B and C). They showed that the majority of genes regulated at 4 h remained regulated for all time points (41/46 up-regulated and 35/44 down-regulated). In addition, 28/81 genes that were up-regulated at 8 h and 105/168 genes that were down-regulated at 8 h were regulated in a similar manner at 24 h.

Retinol-controlled Genes Regulated by the Classical Retinoic Acid Receptor Mechanism

To determine which genes had a putative retinoic acid response element (RARE) that could be regulated directly by the classical retinoic acid/receptor pathway, the first 2000-bp sequence of the promoter region, upstream from exon 1 of the genes (258 genes) that had Genbank identifiers were analyzed by the Transcription Element Search System (TESS) (www.cbil.upenn.edu/teess/). For controls, the first 2000-bp promoter sequences of genes, known not to have RAREs (*Gata1*, *Pten*, *Dmrt1* and *Sgp1*), were analyzed by TESS and shown not to have recognizable RAREs. Of 258 genes, 121 genes (46.9%) had putative RAREs, while 137 genes did not contain any RARE in the first 2000-bp promoter sequence (Table 2). However, the result does not eliminate the possibility that the other 137 retinol-regulated genes may have RAREs in the region, outside the 5' 2000-bp promoter sequence, or are indirectly regulated by a secondary mechanism, associated with a gene that was earlier regulated by a retinoid receptor-mediated mechanism. In addition, a slightly higher percentage, 56.6% (47/83), of up-regulated genes had putative RAREs compared to 42.3% (74/175) of the down-regulated genes with putative RAREs. There seemed to be no specific clustering of genes with or without RAREs at any specific time point.

Cell-type Expression in the Rat Testis

To determine if there is a propensity for retinoid-regulated genes to be expressed in specific cell types in the rat testis, the database (GSE8978, GEO Accession data set) that contains raw signals from the microarray chip sets (Affymetrix 230 2.0 rat chip) for an enriched, combined population of rat spermatogonia and early spermatocytes, separate populations of pachytene spermatocytes and round spermatids, and an enriched rat Sertoli cell population [Johnston et al. 2008] was searched to identify the 309 retinoid-regulated rat genes. Of 281 genes identified, the number of genes with a signal of 50 or greater that were expressed in the spermatogonial/spermatocyte population and the Sertoli cell population were similar, 245 genes (87.2%) in the former and 247 genes (87.9%) in the latter (Table 3). These results were not unexpected since both Sertoli and early germ cells have been shown to respond to retinoic acid [Kim and Griswold 1990; Zhou et al. 2008] and they both express retinoic acid receptors [Boulogne et al. 1999; Dufour and Kim 1999; Vernet et al. 2006]. Moreover, the ratio of up-regulated genes to down-regulated genes for the spermatogonial and spermatocyte population, and the Sertoli cells were similar, 72 and 77 up-regulated or 173 and 170 down-regulated in spermatogonial/spermatocyte and Sertoli cells, respectively (Table 3).

Retinol Regulation in Retinol-replenished VAD testis and Sertoli Cells by Real time PCR

Nuclear receptor subfamily 0, B1 (*Nr0b1*, alias *Dax1*), growth arrest and DNA-damage-inducible 45 alpha (*Gadd45a*), acyl-CoA synthetase long-chain family member 4 (*Acsl4*), soluble glycerol-3-phosphate dehydrogenase 1 (*Gpd1*), dihydropyrimidinase-like 4 (*Dpysl4*, alias *Crmp3*), cAMP-specific phosphodiesterase 4B (*Pde4b*) were selected as examples with a 4-fold or higher expression level in rat Sertoli cells and with a raw signal level greater than 500 in the GSE8978 GEO Accession data set [Johnston et al. 2008]. In addition, platelets and synovial fluid phospholipase A2, group IIA (*Pla2g2a*) with a 2.5-fold higher expression in Sertoli cells and a raw signal of less than 300 was selected. Lastly, serine protease 35 (*Prss35*) with almost an equal level of expression in Sertoli cells or germ cells was selected. Real time RT-PCR was performed using the RNA samples from the retinol-replenished VAD testes and RNA samples from cultured 20 day-old rat Sertoli cells treated with 1 μ M retinoic acid for 0, 8, and 24 h. Real time RT-PCR assays confirmed the positive regulation by retinol for *Nr0b1* (*Dax-1*), *Gadd45a*, *Acsl4*, *Gpd1*, *Pla2g2a*, and *Prss35* genes, and the negative regulation by retinol for *Pde4b* and *Dpysl4* genes in retinol-replenished VAD testes (Fig. 3), as shown by Affymetrix microarray data (Supplemental Table S1 and S2). In addition, the real time RT-PCR assay showed that *Nr0b1* (*Dax-1*), *Gadd45a*, *Acsl4*, *Gpd1*, *Pde4b* and *Dpysl4*

were regulated by retinoic acid in primary Sertoli cells (Fig. 3). Interestingly, only *Acls4*, *Pde4b*, and *Dpysl4* showed a similar decrease or a greater increase in Sertoli cells, as in the retinol-replenished testes (Fig. 3), indicating that regulation by retinoic acid for these genes primarily occurred in Sertoli cells. In contrast, the fold changes observed for *Pla2g2a* and *Prss35* expression in Sertoli cells were 3 or more fold lower than the fold changes seen for the retinol-replenished VAD testes, indicating that the increasing transcriptome profile seen with *Pla2g2a* and *Prss35* in retinol-replenished testis could not be all from the Sertoli cell. The result for *Pla2g2a* is consistent with a previous report indicating that *Pla2g2a* is found at low levels in Leydig cells [Masuda et al. 2004]. The *Prss35* expression pattern is not available for testicular cells, but it is found in the theca cell layer of developing follicles [Wahlberg et al. 2008].

Functional Clustering and Pathway Analysis

To annotate potential gene functions and canonical pathways over time, the Affymetrix identifiers of 90, 249, and 266 retinol-regulated genes for the 4, 8, and 24 h time points, respectively, were imported into Ingenuity Pathway Analysis software (www.ingenuity.com) (Ingenuity® Systems, Redwood City, CA). This strategy incorporates the fold change as well as the positive and negative regulation of each gene to find potential functions or canonical pathways for a cluster of genes. 59, 120, and 144 genes were annotated for the 4, 8, and 24 h time points, respectively, generating a list of primary functions and canonical pathways, associated with *P* values. The *P* values indicate the overall significance of a functional cluster or a canonical pathway, dependent on the number of retinol-regulated genes found in a functional category and the number of the total genes in a functional category in the software package. The primary cellular functions (Table 4) or the organ-specific diseases (Table 5), with the list cut-off of at least 6 genes per category, were ordered by time and *P* value. There was a time-dependent shuffling of functions, indicating a change in significance for these cellular functions with time, a loss of functions, and a gain of functions (◊ Table 4). However, 15 functional categories (* Tables 4 and 5) were found for all time points, and in general, the number of genes identified increased with time. Interestingly, molecular transports of lipids, fatty acids, arachidonic acid, and amino acids were prominent at 4 h, whereas the cell cycle category emerged as the top significant function at 8 and 24 h. Related to this category, the cell death category also rose to prominence at 8 and 24 h. Equally pertinent, the cellular function concerning embryonic development was the second significant category on the list at 4 h, and remained highly significant at both 8 and 24 h. This could be reflective of the stem germ cells present in the retinol-replenished VAD testes, as represented by doublecortin and CaM kinase-like 1 (*Dclk1*), also known as *Ania4* or *Dcamkl1* that was down-regulated 4.8 fold in the retinol-replenished testis. In neurons and intestinal cells, it is a marker for stem cells [Montgomery and Breault 2008], involved in migration and cell division [Friocourt et al. 2007]. In addition, functional categories related to cellular assembly and organization, cell morphology, and cellular development were revealed for all time points.

Tracking diseases and disorders (Table 5), the reproductive system disease was most significant (1.49E-04) at 24 h. Interestingly, the next category was cancer, in terms of significance and the largest number of genes involved, indicating that retinol may regulate genes involved in cell cycle and associated signaling pathways. Other diseases or disorders that appeared in all time points were immunological, skeletal and muscular, renal and urological, and hematological diseases. These results convey the notion that genes associated with these diseases are germane to immune cells, peritubular myoid cells, and endothelial cells of the testis.

To further dissect and provide a second validation of functions, analysis was conducted in a different way, examining canonical pathways. Again, the strategy was to generate a time-

dependent list to determine which canonical pathways were significant at each time point. To eliminate duplicates, if a canonical pathway was found in both 4 and 8h time points, the canonical pathway was listed with the time point that had the highest *P* value. We also eliminated any canonical pathway with a *P* value of more than 4.0E-01. All genes associated with each canonical pathway were listed (Table 6).

At 4 h after retinol treatment, two canonical pathways for retinoic acid signaling and metabolism were observed. The genes associated with these pathways that were up-regulated were dual specificity phosphatase 1 (*Dusp1*), FBJ murine osteosarcoma viral oncogene homolog (*Fos*), and retinol dehydrogenase 10 (*Rdh10*) and down-regulated was cAMP-dependent protein kinase, regulatory, type II beta (*Prkar2b*). In addition, canonical pathways related to glycerophospholipid metabolism, arachidonic acid metabolism, eicosanoid signaling, fatty acid metabolism, linoleic acid metabolism, and hepatic cholestasis emerged. Furthermore, LPS/IL-1-mediated inhibition of RXR function and IL-10 signaling canonical pathways that indicated some immune function by retinoic acid surfaced. Moreover, G2/M DNA damage signaling, BRCA1 in DNA damage response, and nucleotide excision repair canonical pathways that indicated some genomic stress-related functions were also revealed.

At 8 and 24 h after retinol treatment, a number of signaling pathways emerged, notably, ERK/MAPK signaling, p53 signaling, PTEN signaling, IGF-1 signaling, cAMP-mediated signaling, interferon signaling, and serotonin receptor signaling. In addition, both pathways concerned with the biosynthesis of cholesterol and reverse cholesterol transport, as in bile acid biosynthesis, surfaced. Moreover, retinoic acid appears to have a role in a number of metabolic pathways (amino acid, ketone body, butanoate, citrate cycle, pyruvate, glycosaminoglycan, nitrogen, tryptophan-based serotonin (5-hydroxytryptamine), phenylalanine-based dopamine, purine, aminosugars and glycosaminoglycan). The emergence of some of these metabolic functions may be related to the cells' attempt at bringing metabolic processes back into homeostasis to generate energy for the retinol-replenished testes. Consistent with this notion, Sertoli cells are known to use a combination of glucose metabolism, fatty acid oxidation and amino acid metabolism to generate energy [Griswold 1993]. Sertoli cells also secrete pyruvate and lactate, important for germ cell development.

Most significantly, we noticed that different canonical pathways shared genes, indicative of an even higher order of functional clustering. In this context, we grouped some of the pathways together to form larger "super" functional clusters. These functional clusters include: (1) cholesterol and oxysterol homeostasis, (2) the regulation of steroidogenesis, (3) glycerophospholipid metabolism, (4) the regulation of acute inflammation, (5) the regulation of the cell cycle including ubiquitin-mediated degradation of cell cycle proteins and control of centrosome and genome integrity, and (6) the control of membrane scaffolding proteins that can integrate cell-cell adhesion, cell cycle and cell signaling within a cell. The significance of these clusters of genes is elaborated below.

Discussion

The present study was undertaken to determine the time-dependent transcriptome profiles of VAD rat testes replenished with retinol for 4, 8, and 24 h in order to identify potential functional clusters and canonical pathways, to build insights into the functional significance of the retinoid-regulated transcriptomes in the testis. Many clusters of genes emerged from our functional and pathway analyses, underscoring the profound influence of retinoic acid in the testis.

Retinoic Acid Decreases Cholesterol Synthesis and Increases Cholesterol Efflux

Our microarray results suggest that retinoic acid may have a role in decreasing cholesterol synthesis in the testis. This is similar to previous results that showed cholesterol esters (neutral fat) accumulated in Sertoli cells of mice lacking *Rxrb* function [Vernet et al. 2008]. The expression of seven genes (*Acat1*, *Acat2*, *Hmgcs1*, *Fdps*, *Fdft1*, *Sqle*, and *Cyp51a1*) involved in the synthesis of cholesterol and the precursors of cholesterol, lanosterol, follicular fluid meiosis activating sterol (FF-MAS), testicular meiosis activating sterol (T-MAS), zymosterol, and desmosterol [Rozman et al. 2005] (Fig. 4A), were down-regulated in the retinol-replenished testis. Further, the transcript levels of ATP-binding cassette transporter (*Abcd3*), and adipose differentiation-related protein (*Adfp*) were both reduced in retinol-replenished VAD testes. ABCD3 is implicated in the import of fatty acids and/or fatty acyl-CoAs and ADFP is known to promote cholesterol storage and contribute to lipid accumulation in the arterial wall [Larigauderie et al. 2004]. Collectively, these results strongly suggest that retinoic acids may protect the testis from the cholesterol overload that can lead to apoptosis of cells.

On one hand, retinoic acid seems to increase the cholesterol efflux, generating oxysterol intermediates in the bile acid synthesis pathway. In particular, the expression of cytochrome P450, family 27, A1 (*Cyp27a1*) and hepatocyte nuclear factor 4, alpha (*Hnf4a*), which code for gene products that can convert cholesterol to 27-hydroxycholesterol, an oxysterol, or can up-regulate *Cyp27a1* at the transcriptional level [Chen and Chiang 2003], respectively, were up-regulated. On the other hand, the expression of acetyl coenzyme A acetyltransferase (*Acaa2*), which codes for the penultimate enzyme in the bile acid synthesis pathway, was down-regulated in the retinol-replenished testes, suggesting that the oxysterol intermediates upstream of the ACAA2 enzyme may be important in the testis.

The significance of oxysterols in the testis was demonstrated in mice carrying double mutations of liver X receptor alpha and beta (*Lxra* and *Lxrb*), which displayed a male infertility phenotype [Volle and Lobaccaro 2007]. LXRs are the receptors for oxysterol ligands [Fu et al. 2001] and they have been shown to control the transcription of *Cyp27a1* [Crestani et al. 2004]. In addition, oxysterols have been shown to increase the efflux of lung surfactant, a mixture of disaturated phosphatidylcholine and hydrophobic proteins that prevents lung collapse [Agassandian et al. 2004]. Curiously, the expression of surfactant associated protein C (*Sftpc*) was increased by 2.5 fold after retinol replenishment. The SFTPC is an extremely hydrophobic protein, implicated to play a role in alveolar development, anti-inflammatory response, and phagocytosis [Glasser et al. 2008]. The role of SFTPC in the testis remains unknown. However, retinoic acid has been shown to increase *Sftpc* mRNA and cause alveolar abnormality in transgenic neonatal lungs, over expressing a dominant negative *Rara* [Yang et al. 2003].

Retinoic Acid Up-regulates Progesterone and Down-regulates Testosterone and Estrogen

Our microarray results showed a 2.2-fold increase in the expression of 3 beta- and steroid delta isomerase 1 (*Hsd3b1*), and 3.1-fold and 2.8-fold decrease in the expression of cytochrome P450, family 17, A1 (*Cyp17a1*) and hydroxysteroid (17 beta) dehydrogenase 1 (*Hsd17b1*), respectively. This is interesting because, examining the metabolic pathways for the progesterone, testosterone, and estradiol production from cholesterol (Fig. 4B), it is clear that the patterns of enzyme expression after retinol replenishment support an intriguing notion that progesterone could be preferentially produced, while the testosterone and estradiol levels could be reduced in retinol-replenished testes. Moreover, the increased expression of *Nr0b1* (*Dax1*) by 5.3 fold is congruent with the diminished steroidogenic acute regulatory protein (*StAR*) and aromatase (*Cyp19*) mRNA expression (data not shown) in retinol-replenished testes, because DAX1 is known to inhibit transcription of *StAR* [Jo and Stocco 2004] and *Cyp19* [Wang et al. 2001]. *StAR* transfers cholesterol within the mitochondria, the rate-limiting step in the production of steroid hormones and aromatase converts testosterone to estradiol.

However, the role of progesterone in the mammalian testis remains unknown. In fish, progesterone has been shown to be essential for the DNA replication of spermatogonia before initiation of the meiotic prophase in the testis and, later, for spermiation [Miura et al. 2006]. In mammalian females, evidence is mounting that progesterone, via the classical receptor pathway, is important for controlling the timing of the prostaglandin E2 rise for the release of the oocyte [Stouffer et al. 2007]. Moreover, progesterone increased the expression of protease serine 35 (*Prss35*) at the time of ovulation and maintained it at an elevated level in the developing corpus luteum [Miyakoshi et al. 2006]. Interestingly, *Prss35* expression was stimulated 4.4 fold after retinol replenishment. However, the role of this protease in the testis is not known.

Retinoic Acid May Regulate Phosphatidylcholine Homeostasis

The expression of three genes, lipoprotein lipase (*Lpl*), glycerol-3-phosphate dehydrogenase 1 (*Gpd1*), and choline kinase alpha (*Chka*), was increased by 1.6, 4.3, and 2.5 fold after retinol replenishment, respectively. They code for enzymes that participate in phosphatidylcholine synthesis (Fig. 4C) [Prochazka et al. 1989; Stein and Stein 2003]. This is significant because phosphatidylcholine can be a precursor to many physiologically important membrane molecules including arachidonic acid and docosahexaenoic acid, which have been shown to be critical for male and female fertility [Stoffel et al. 2008]. Similarly, increased expression of phosphatidylcholine transfer protein (*Pctp*) after retinol replenishment also suggests the importance of readily available phosphatidylcholine in retinol-replenished VAD testes. In addition, retinoic acid seems to indirectly regulate its own retinoid metabolism, since phosphatidylcholine can donate the sn-1 acyl-group to retinol by lecithin retinol acyltransferase (LRAT) to produce retinyl ester [Gottesman et al. 2001].

Retinoic Acid May Have a Role in Resolution of Acute Inflammation

A resolution of acute inflammation is known to require activation of a sequential series of phospholipase isoforms, first to produce, from arachidonic acid and docosahexaenoic acid, pro-inflammatory molecules and then anti-inflammatory molecules [Gilroy et al. 2004; Farooqui et al. 2007; Tassoni et al. 2008]. In this light, retinoic acid may participate directly in acute inflammation resolution by activating secretory phospholipase A2, synovial fluid group IIA (PLA2G2A) and calcium-dependent G5 (PLA2G5) that are known to mediate the production of platelet activating factor and lipoxin A4, anti-inflammatory molecules [Gilroy et al. 2004]. This is strikingly interesting since *Pla2g2a* was the top up-regulated gene with a 13-fold increase while *Pla2g5* had a 4-fold increase at 24 h after retinol replenishment. Consistently, retinoic acid increased the expression of *Pla2g2a* in rheumatoid arthritis, mediated by LXR, in smooth muscle cells [Antonio et al. 2003].

Similarly, cytoplasmic epoxide hydrolase 2 (EPHX2) can decrease the level of anti-inflammatory epoxyeicosatrienoic acids (EET) by converting EET to dihydroxyeicosatrienoic acids for excretion through the kidney [Morisseau and Hammock 2005]. In addition, acyl-CoA synthetase long-chain family member 4 (ACSL4) preferentially esterifies arachidonic acid into membrane phospholipids, preventing continuous synthesis of potent inflammatory eicosanoids [Kang et al. 1997]. Thus, a decreased expression of *Ephx2* mRNA and an increased expression of *Acs14* mRNA in retinol-replenished testes are consistent with a role for retinoic acid in the resolution of acute inflammation. Furthermore, calcium binding protein A8 and A9 (S100A8 and S100A9) are transport proteins that bind arachidonic acid and move it to the extracellular space, for transcellular metabolism, in inflamed tissues [Nacken et al. 2003]. An increased expression of *S100a8* and *S100a9* may be necessary for moving arachidonic acid to where it is needed.

Finally, the expression of dual-specificity phosphatase 1 (*Dusp1*) was up-regulated by 3.2 fold after retinol replenishment. This was expected from a previous result that showed retinoic acid facilitates the binding of CREB and USF1 to the *Dusp1* promoter, to increase the transcription of *Dusp1* mRNA [Lu et al. 2008]. Interestingly, DUSP1 is a negative attenuator that can decrease the magnitude and duration of a pro-inflammatory response [Salojin and Oravec 2007; Wang et al. 2008]. It is a dual-specific threonine and tyrosine phosphatase that preferentially dephosphorylates p38 MAPK or c-Jun N-terminal kinase (JNK) [Salojin and Oravec 2007], both critical for activation of multiple transcription factors that control the expression of pro-inflammatory cytokine genes, such as IL-6, IL-10, and TNF α . Thus, retinoic acid may be regulating a master negative attenuator that can decrease many pro-inflammation events at the same time.

Retinoic Acid Controls Cell Cycle Progression

Retinoic acid appears to promote cell cycle progression by up-regulating cyclin D3 (*Ccnd3*) expression by 3.2 fold in retinol-replenished testis. Cyclin D is known to phosphorylate the retinoblastoma (RB) family of proteins, which then release E2F, activating E2F-mediated transcription and cell cycle progression through the G1/S transition. Moreover, a Sertoli cell-only phenotype displayed in E2F1-deficient mice suggest a role for E2F1 in stem cell renewal or spermatogonial proliferation [El-Darwish et al. 2006]. Similarly, *Ccnd3* was found to have a role in hematopoietic stem cell expansion [Cooper et al. 2006]. Thus, altogether, it is reasonable to suggest that retinoic acid may activate CCND3 and E2F in stem germ cells or in spermatogonia.

This is supported by the observation that the expression of retinoblastoma 1 (*Rb1*) and retinoblastoma-like 2 (*Rbl2*), members of the RB family were down-regulated after retinol-replenishment. In addition, the expression of FBJ murine osteosarcoma viral oncogene homolog (*cFos*) was up-regulated by retinol. Consistently, cFOS was shown to increase the expression of *Ccnd3* [Phuchareon and Tokuhisa 1995]. Similarly, protein phosphatase 1 regulatory subunit 9B (*Ppp1r9b*) was up-regulated. PPP1R9B promotes cell cycle progression by recruiting protein phosphatase type 1 (PP1) to the cytoplasm [Terry-Lorenzo et al. 2002], thus making PP1 unavailable to dephosphorylate RB proteins in the nucleus [Rubin et al. 2001]. The down-regulation of A-kinase anchor protein 12 (*Akap12*) and B-cell translocation gene 3 (*Btg3*) expressions are consistent with E2F activation. AKAP12 has been shown to sequester the cyclin D proteins and induce cell-cycle arrest [Lin et al. 2000] while BTG3 has been shown to inhibit E2F1 transactivation [Ou et al. 2007].

Retinoic Acid Regulates Genome Integrity

Equally interesting, retinoic acid may regulate genes that are associated with centrosome maturation and stability and exit from M phase of the cell cycle. Any abnormality in centrosome function or exit from the cell cycle has been shown to cause genomic instability such as aneuploidy and tetraploidy [Liebermann and Hoffman 2008; Man et al. 2008]. Overexpression of inhibitor of DNA binding 1 (*Id1*) that encodes a dominant negative helix-loop-helix protein, was shown to induce multiple mitotic and genomic aberrations, including tetraploidy, centrosome amplification, binucleation, spindle defects, and microtubule perturbation [Man et al. 2008]. Overexpressed ID1 can bind CDH1 abnormally, leading to stabilization of aurora A kinase at the M phase. Normally, aurora A kinase is degraded by the anaphase promoting complex (APC/C), a member of the ubiquitin ligase family, when CDH1, associated with APC/C, recognizes aurora A kinase as a target at the mitosis exit. Down-regulation of *Id1* in retinol-replenished testes should allow a breakdown of aurora A kinase, allowing a proper exit from the M phase. Similarly, GADD45A has a role in centrosome maturation and maintenance by interacting with aurora A kinase, and inhibiting its kinase activity [Shao et al. 2006]. A targeted deletion of *Gadd45a* in knockout mice caused abnormal centrosome amplification and

chromosome segregation, leading to extensive aneuploidy, and aborted cytokinesis [Liebermann and Hoffman 2008]. The expression of *Gadd45A* was up-regulated in the retinol-replenished testes by 3.3 fold. The expression of response gene for complement (*Rgc32*) decreased in retinol-replenished testis. Its gene product has also been shown to be located on centrosomes during mitosis causing a G2/M arrest [Saigusa et al. 2007], but its exact role is not known. In the ovary, it is highly regulated, induced by luteinizing hormone in preovulatory follicles [Park et al. 2008].

Additionally, decreased expression of glypican 1 (*Gpc1*) in retinol-replenished testes may lead to a smooth exit out of the M phase of the cell cycle, promoting genome stability. Previously, overexpression of GPC1, a heparin sulfate proteoglycan attached to the lipid rafts in endothelial cells, resulted in delayed exit from the M phase, accompanied by aneuploidy, polyploidy, and apoptosis [Qiao et al. 2008]. It was correlated with an up-regulated expression of early mitotic inhibitor 1 (*Emi1*) in the M phase, causing a dramatic increase in mitotic cyclin and securin. Normally, EMI1 accumulates in S and G2 phase of the cell cycle to inhibit APC/C and then it decreases in the M phase, when APC/C is re-activated to degrade target proteins such as mitotic cyclins and securin.

Finally, it is interesting that F box only proteins 21 and 22 (*Fbxo21* and *Fbxo22*) were down-regulated in retinol-replenished testes by 2.3 and 1.9 fold, respectively. The functions of FBXO21 and 22 are not known. Although there are many F box proteins, each one is a part of a cullin-based E3 ubiquitin ligase complex called SCF, composed of SKP1, CUL1, and an F box protein. The complex degrades specific cellular regulatory proteins, recognized by the associated F box protein, in a timely manner. A targeted deletion of β -transducin repeat-containing protein (β -*Trcp*), the F box protein 47, caused reduced fertility in male mice [Guardavaccaro et al. 2003]. The M phase was lengthened and centrosomes were overduplicated, leading to accumulation of metaphase I spermatocytes and multinucleated spermatids in these mice. Interestingly, in *C. elegans*, gonads with a mutation in an F box protein gene, *prom1*, completed mitotic proliferation and premeiotic replication, but delayed entry into meiosis. In this case, homologous chromosome pairing was impaired [Jantsch et al. 2007]. These results are particularly notable, since *Stra8* has been shown to be essential for the mitosis-to-meiosis decision in spermatogonia [Baltus et al. 2006; Bowles et al. 2006; Koubova et al. 2006]. Furthermore, it has been shown recently that a targeted deletion of *Stra8* leads to normal pre-meiotic DNA synthesis, but to incomplete meiotic prophase [Anderson et al. 2008] or to premature timing of chromosome condensation [Mark et al. 2008].

Retinoic Acid Controls the Expression of Membrane Scaffold Proteins for Small GTPase Signals

The expression of the connector enhancer of KSR 3 (*Cnkr3* or *Cnk3*), also known as Maguin-like with PDZ and sterile α domains, was down-regulated 2.2 fold in retinol-replenished VAD testes. This is significant because it encodes a multidomain scaffold protein in the family of membrane-associated guanylate kinase homologs (MAGUKs), situated inside the plasma membrane. In mammals, the complex of connector enhancer of KSR (CNK) and kinase suppressor of RAS (KSR) serves as a scaffold platform, where multiple GTPase and phosphatidylinositol triphosphate effectors, namely, RAS, RHO, RAC, RAL, or ARF, can bind simultaneously [Claperon and Therrien 2007]. This way, the CNK/KSR complex can essentially integrate the upstream external stimuli on the plasma membrane with the downstream multiple effectors. Moreover, CNK can dictate which GTPase signals crosstalk to each other and what magnitude of GTPase signaling is present at the plasma membrane. Further control is provided by a SH2 SRC homology domain protein that binds CNK to activate it and the 14-3-3 protein that binds phosphorylated KSR to inactivate it [Kolch 2005]. In rats,

CNK3 is specifically associated with guanine exchange factors (GEF) for ARF6, a small GTPase involved in vesicular trafficking and cytoskeletal changes or phosphatidylinositol triphosphate mediating JNK pathway. Thus, it was remarkable that so many potential effector molecules, such as SH2 domain containing 4A (*Sh2d4a*), Ras association (RalGDS/AF-6) domain family 2 (*Rassf2*), tyrosine 3-monooxygenase/tryptophane 5-monooxygenase activation protein, eta polypeptide (Ywhah or 14-3-3), phosphatidylinositol 4-kinase, beta polypeptide (*Pi4kb*), Rap guanine nucleotide exchange factor (GEF)3 (*Rapgef3*), Cdc42 effector protein (Rho GTPase binding) 3 (*Cdc42ep3*), were all down-regulated in retinol-replenished testes. Altogether, these results strongly suggest that retinoic acid can potentially orchestrate GTPase signals, a subset at a time, directing fundamentally important cellular processes at a precise time.

In conclusion, we were able to group the cellular functions and canonical pathways into several super-functions, concerned with cholesterol and oxysterol metabolism, resolution of acute inflammation, and steroidogenesis. In addition, functions included cell cycle control, such as Ccnd3 and E2F mechanism for cell cycle progression, and the APC/C and SCF ubiquitination mechanisms that are, in effect, surveillance systems for maintaining genomic stability and smooth progression into the next cell cycle, whether it is the mitotic or meiotic cell cycle. Lastly, we observed that retinoic acid could regulate a scaffold protein for RAS/RAF/MAPK, essentially integrating multiple GTPase signals, directing some fundamental cellular processes and functions. Collectively, these diverse roles of retinoic acid underscore the importance of retinoic acid in the testis. It is also apparent that retinoic acid may act to integrate many signals, essentially acting upstream of physiologically important pathways. At the end, as should be expected from all microarray experiments, hypotheses were generated that require further experimentation to sort through the exact cellular localization of gene functions and the precise timing of molecular mechanisms.

Materials and Methods

Animals

Male Sprague-Dawley rats were obtained from Charles River Laboratories (Hollister, CA) or an inhouse vivarium. Rats, between 30–40g body-weight, were placed on a VAD diet (Harland Teklad, Madison, WI) at 20 days of age and testes were collected after 9 weeks on the VAD diet, as previously reported [Akmal et al. 1998]. To obtain retinol-replenished animals, the VAD rats were injected with 7.5mg of all-trans retinol in 50% ethanol, followed by a dietary supplementation of 1mg retinol/rat mixed with normal rodent diet (Harland Teklad). After treatment, testes were collected at 4, 8, and 24 h post-retinol injection. The experiment was carried out at least in quadruplicate to provide RNA samples for Affymetrix® GeneChip® array and real time PCR assays, proteins for Western blot analysis, and tissue for histology. For histology, one testis from each animal was collected and fixed for 6 h in Bouin's solution, embedded in paraffin, cut into 4µm sections on poly-L lysine coated microscope slides, and stained with hematoxylin and eosin. The remaining testis was used to isolate RNA and protein. Procedures using animals followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Washington State University.

Cell Culture

Primary Sertoli cells were isolated from the testes of 20-day-old male Sprague Dawley rats by sequential enzymatic digestion, as described previously [Kim and Griswold 1990]. Briefly, decapsulated testis fragments were digested with 0.25% (w/v) trypsin (Invitrogen, Carlsbad, CA), followed by 0.7 mg/ml of collagenase (Sigma St. Louis, MO). Sertoli cells were plated under serum-free conditions and were maintained in Ham F-12 medium (Invitrogen) at 32°C in a 5% CO₂ atmosphere. After the culture medium was changed twice to reduce endogenous

all-trans retinoic acid, on the third day of culture, the cells were treated for 0, 8, and 24 h with 1 μ M of all-trans retinoic acid (Sigma, St Louis, Mo). After treatment, RNA was collected.

RNA and Protein Extractions

RNA was collected with an RNeasy RNA extraction kit, following the manufacturer's protocol (Ambion, Austin, TX). Protein extracts were collected by centrifugation (at 13,200 rpm, for 30 min) after a 30-min incubation on ice of the homogenized testes in lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 50 mM NaF, and 5 mM EDTA), containing a cocktail of proteinase inhibitors (100 μ M PMSF, 10 μ M aprotinin, and 10 μ M leupeptin). The protein concentration was determined by the Bradford assay, with BSA as the standard.

Western Blot Analysis

Equal amounts of proteins were separated using a 10% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA), blocked with 5% Blotto (Nestle USA, Inc., Solon, Ohio), and incubated with anti-RARA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:300 dilution for 2 h at room temperature. After washing with 0.5% TBS-T (Tris buffered saline and 0.5% Tween-20), membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG antibody at 1:10,000 and the antibody-antigen complexes detected by the Enhanced Chemiluminescence System (Amersham Pharmacia Biotechnology, Piscataway, NJ). Coomassie Blue staining of the Immobilon-P membranes was performed to confirm an equal loading of proteins in each lane.

Real time PCR assays

Real time PCR primers were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table 1. cDNA synthesized from 500 ng of RNA using iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA) was used as template for real time PCR assays with an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA). Typically, a 25 μ l reaction contained 12.5 μ l of 2 $\times$ Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA), 0.15 μ l (final 500nM) of forward and reverse primer, 0.5 μ l ROX (Invitrogen), 5 μ l of cDNA (diluted 1:20 with distilled water), and distilled water. The PCR run was 1 cycle at 50 $^{\circ}$ C 2 min, 1 cycle at 95 $^{\circ}$ C 10 min, and 40 cycles at 95 $^{\circ}$ C 15 s and 60 $^{\circ}$ C 1 min. The real time PCR was conducted on three treatment groups with each individual treatment group in triplicate. Threshold values (Ct) for the gene of interest and a housekeeping gene ribosomal S2 were determined using ABI Prism SDS software version 1.1 (Applied Biosystems, Foster City, CA). The expression level of the gene of interest was evaluated using the $2^{-(\Delta\Delta C_t)}$ method [Livak and Schmittgen 2001]. Specifically, Ct values for the gene of interest were normalized to Ct values for ribosomal S2 in each sample and then the fold change for the gene of interest was calculated relative to the level in the untreated control. One-way ANOVA was performed, followed by pairwise comparisons of the means at P value of ≤ 0.05 , using JMP IN 5.1 (SAS Institute Inc., Cary, NC).

Microarray Array Processing and Data Analysis

Transcription profiling of RNA was performed using RG-U34A GeneChip® microarrays, containing approximately 9,000 genes (Affymetrix, Santa Clara, CA), using one chip per RNA sample. Briefly, the double stranded cDNA, synthesized from 10 μ g total RNA using reverse transcriptase and DNA polymerase, was used as template to prepare the antisense cRNA, using T7 RNA polymerase, incorporating biotinylated ribonucleotides. 15 μ g biotin-labeled target cRNA was fragmented and hybridized with the GeneChip probe array for 16 h, followed by washing steps and incubation with streptavidin phycoerythrin conjugate, and detection with

Affymetrix Genechip® scanner 3000. Resulting image files produced by the scanner were analyzed with Affymetrix GCOS software to determine the raw signal intensity, which is proportional to the amount of bound target RNA.

The raw intensity data sets were first normalized using default parameters of GeneSpring 7.3 (Agilent Technologies, Foster City, CA). In addition, normalization included dividing the raw signal for each gene by the median signal value of control VAD samples. Then, the normalized data for each gene were filtered for at least one sample having a raw signal of 50 or greater, and for at least one statistically significant change with a *P* value of ≤ 0.05 when comparing all experimental samples to each other. ANOVA tests were performed using a *P* value of ≤ 0.05 for each gene, assuming variances not equal, and including calculations made from the Cross-Gene Error Model. No multiple testing correction method was used and the Student-Newman-Keuls Post Hoc test was applied.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

VAD	vitamin A deficient
RAR	retinoic acid receptors
RXR	retinoid X receptors
RARE	retinoic acid response element
TESS	Transcription Element Search System
Sftpc	surfactant associated protein C
StAR	stereoidogenic acute regulatory protein
EET	epoxyeicosatrienoic acids

References

- Agassandian M, Mathur SN, Zhou J, Field FJ, Mallampalli RK. Oxysterols trigger ABCA1-mediated basolateral surfactant efflux. *Am J Respir Cell Mol Biol* 2004;31:227–233. [PubMed: 15039140]
- Akmal KM, Dufour JM, Kim KH. Retinoic acid receptor alpha gene expression in the rat testis: potential role during the prophase of meiosis and in the transition from round to elongating spermatids. *Biol Reprod* 1997;56:549–556. [PubMed: 9116160]
- Akmal KM, Dufour JM, Vo M, Higginson S, Kim KH. Ligand-dependent regulation of retinoic acid receptor alpha in rat testis: in vivo response to depletion and repletion of vitamin A. *Endocrinology* 1998;139:1239–1248. [PubMed: 9492059]
- Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, Page DC. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci U S A* 2008;105:14976–14980. [PubMed: 18799751]

- Antonio V, Janvier B, Brouillet A, Andreani M, Raymondjean M. Oxysterol and 9-cis-retinoic acid stimulate the group IIA secretory phospholipase A2 gene in rat smooth-muscle cells. *Biochem J* 2003;376:351–360. [PubMed: 12882648]
- Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, de Rooij DG, Page DC. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet* 2006;38:1430–1434. [PubMed: 17115059]
- Boulogne B, Levacher C, Durand P, Habert R. Retinoic acid receptors and retinoid X receptors in the rat testis during fetal and postnatal development: immunolocalization and implication in the control of the number of gonocytes. *Biol Reprod* 1999;61:1548–1557. [PubMed: 10570002]
- Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, et al. Retinoid signaling determines germ cell fate in mice. *Science* 2006;312:596–600. [PubMed: 16574820]
- Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB J* 1996;10:940–954. [PubMed: 8801176]
- Chen W, Chiang JY. Regulation of human sterol 27-hydroxylase gene (CYP27A1) by bile acids and hepatocyte nuclear factor 4alpha (HNF4alpha). *Gene* 2003;313:71–82. [PubMed: 12957378]
- Chung SS, Sung W, Wang X, Wolgemuth DJ. Retinoic acid receptor alpha is required for synchronization of spermatogenic cycles and its absence results in progressive breakdown of the spermatogenic process. *Dev Dyn* 2004;230:754–766. [PubMed: 15254909]
- Chung SS, Wang X, Wolgemuth DJ. Male sterility in mice lacking retinoic acid receptor alpha involves specific abnormalities in spermiogenesis. *Differentiation* 2005;73:188–198. [PubMed: 15901285]
- Claperon A, Therrien M. KSR and CNK: two scaffolds regulating RAS-mediated RAF activation. *Oncogene* 2007;26:3143–3158. [PubMed: 17496912]
- Cooper AB, Sawai CM, Sicinska E, Powers SE, Sicinski P, Clark MR, Aifantis I. A unique function for cyclin D3 in early B cell development. *Nat Immunol* 2006;7:489–497. [PubMed: 16582912]
- Crestani M, De Fabiani E, Caruso D, Mitro N, Gilardi F, Vigil Chacon AB, Patelli R, Godio C, Galli G. LXR (liver X receptor) and HNF-4 (hepatocyte nuclear factor-4): key regulators in reverse cholesterol transport. *Biochem Soc Trans* 2004;32:92–96. [PubMed: 14748721]
- Doyle TJ, Braun KW, McLean DJ, Wright RW, Griswold MD, Kim KH. Potential functions of retinoic acid receptor A in Sertoli cells and germ cells during spermatogenesis. *Ann N Y Acad Sci* 2008;1120:114–130. [PubMed: 17905941]
- Dufour JM, Kim KH. Cellular and subcellular localization of six retinoid receptors in rat testis during postnatal development: identification of potential heterodimeric receptors. *Biol Reprod* 1999;61:1300–1308. [PubMed: 10529278]
- El-Darwish KS, Parvinen M, Toppari J. Differential expression of members of the E2F family of transcription factors in rodent testes. *Reprod Biol Endocrinol* 2006;4:63–84. [PubMed: 17147820]
- Evans RM. The molecular basis of signaling by vitamin A and its metabolites. *Harvey Lect* 1994;90:105–117. [PubMed: 8803322]
- Faraonio R, Galdieri M, Colantuoni V. Cellular retinoic-acid-binding-protein and retinol-binding-protein mRNA expression in the cells of the rat seminiferous tubules and their regulation by retinoids. *Eur J Biochem* 1993;211:835–842. [PubMed: 8382159]
- Farooqui AA, Horrocks LA, Farooqui T. Modulation of inflammation in brain: a matter of fat. *J Neurochem* 2007;101:577–599. [PubMed: 17257165]
- Friocourt G, Liu JS, Antypa M, Rakic S, Walsh CA, Parnavelas JG. Both doublecortin and doublecortin-like kinase play a role in cortical interneuron migration. *J Neurosci* 2007;27:3875–3883. [PubMed: 17409252]
- Fu X, Menke JG, Chen Y, Zhou G, MacNaul KL, Wright SD, Sparrow CP, Lund EG. 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. *J Biol Chem* 2001;276:38378–38387. [PubMed: 11504730]
- Gilroy DW, Newson J, Sawmynaden P, Willoughby DA, Croxtall JD. A novel role for phospholipase A2 isoforms in the checkpoint control of acute inflammation. *FASEB J* 2004;18:489–498. [PubMed: 15003994]

- Glasser SW, Senft AP, Whitsett JA, Maxfield MD, Ross GF, Richardson TR, Prows DR, Xu Y, Korfhagen TR. Macrophage dysfunction and susceptibility to pulmonary *Pseudomonas aeruginosa* infection in surfactant protein C-deficient mice. *J Immunol* 2008;181:621–628. [PubMed: 18566429]
- Gottesman ME, Quadro L, Blaner WS. Studies of vitamin A metabolism in mouse model systems. *Bioessays* 2001;23:409–419. [PubMed: 11340622]
- Griswold, MD. Unique aspects of the biochemistry and metabolism of Sertoli cells.. In: Russell, LD.; Griswold, MD., editors. *The Sertoli Cell*. Cache River Press; FL: 1993. p. 485–492.
- Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, Margottin-Goguet F, Jackson PK, Yamasaki L, Pagano M. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. *Dev Cell* 2003;4:799–812. [PubMed: 12791266]
- Howell JM, Thompson JN, Pitt GA. Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid. I. The male rat. *J Reprod Fertil* 1963;5:159–167. [PubMed: 13955162]
- Huang HF, Hembree WC. Spermatogenic response to vitamin A in vitamin A deficient rats. *Biol Reprod* 1979;21:891–904. [PubMed: 526508]
- Huggenvik JI, Idzerda RL, Haywood L, Lee DC, McKnight GS, Griswold MD. Transferrin messenger ribonucleic acid: molecular cloning and hormonal regulation in rat Sertoli cells. *Endocrinology* 1987;120:332–340. [PubMed: 3023031]
- Ismail N, Morales C, Clermont Y. Role of spermatogonia in the stage-synchronization of the seminiferous epithelium in vitamin-A-deficient rats. *Am J Anat* 1990;188:57–63. [PubMed: 2346119]
- Jantsch V, Tang L, Pasierbek P, Penkner A, Nayak S, Baudrimont A, Schedl T, Gartner A, Loidl J. *Caenorhabditis elegans* *prom-1* is required for meiotic prophase progression and homologous chromosome pairing. *Mol Biol Cell* 2007;18:4911–4920. [PubMed: 17914060]
- Jo Y, Stocco DM. Regulation of steroidogenesis and steroidogenic acute regulatory protein in R2C cells by DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1). *Endocrinology* 2004;145:5629–5637. [PubMed: 15358680]
- Johnston DS, Wright WW, Dicaneloro P, Wilson E, Kopf GS, Jelinsky SA. Stage-specific gene expression is a fundamental characteristic of rat spermatogenic cells and Sertoli cells. *Proc Natl Acad Sci U S A* 2008;105:8315–8320. [PubMed: 18544648]
- Kang MJ, Fujino T, Sasano H, Minekura H, Yabuki N, Nagura H, Iijima H, Yamamoto TT. A novel arachidonate-preferring acyl-CoA synthetase is present in steroidogenic cells of the rat adrenal, ovary, and testis. *Proc Natl Acad Sci USA* 1997;94:2880–2884. [PubMed: 9096315]
- Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, Decimo D, Krezel W, Dierich A, Chambon P. Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev* 1996;10:80–92. [PubMed: 8557197]
- Kim KH, Griswold MD. The regulation of retinoic acid receptor mRNA levels during spermatogenesis. *Mol Endocrinol* 1990;4:1679–1688. [PubMed: 2177839]
- Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* 2005;6:827–837. [PubMed: 16227978]
- Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci USA* 2006;103:2474–2479. [PubMed: 16461896]
- Larigauderie G, Furman C, Jaye M, Lasselin C, Copin C, Fruchart JC, Castro G, Rouis M. Adipophilin enhances lipid accumulation and prevents lipid efflux from THP-1 macrophages: potential role in atherogenesis. *Arterioscler Thromb Vasc Biol* 2004;24:504–510. [PubMed: 14707038]
- Liebermann DA, Hoffman B. Gadd45 in Stress Signaling. *J Mol Signal* 2008;3:15–22. [PubMed: 18789159]
- Lin X, Nelson P, Gelman IH. SSeCKS, a major protein kinase C substrate with tumor suppressor activity, regulates G(1)-->S progression by controlling the expression and cellular compartmentalization of cyclin D. *Mol Cell Biol* 2000;20:7259–7272. [PubMed: 10982843]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402–408. [PubMed: 11846609]
- Lu TC, Wang Z, Feng X, Chuang P, Fang W, Chen Y, Neves S, Maayan A, Xiong H, Liu Y, et al. Retinoic acid utilizes CREB and USF1 in a transcriptional feed-forward loop in order to stimulate MKP1

- expression in human immunodeficiency virus-infected podocytes. *Mol Cell Biol* 2008;28:5785–5794. [PubMed: 18625721]
- Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub MP, LeMeur M, Chambon P. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. *Proc Natl Acad Sci USA* 1993;90:7225–7229. [PubMed: 8394014]
- Man C, Rosa J, Yip YL, Cheung AL, Kwong YL, Doxsey SJ, Tsao SW. Id1 overexpression induces tetraploidization and multiple abnormal mitotic phenotypes by modulating aurora A. *Mol Biol Cell* 2008;19:2389–2401. [PubMed: 18353975]
- Mark M, Jacobs H, Oulad-Abdelghani M, Dennefeld C, Feret B, Vernet N, Codreanu CA, Chambon P, Ghyselinck NB. STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation. *J Cell Sci* 2008;121:3233–3242. [PubMed: 18799790]
- Masuda S, Murakami M, Matsumoto S, Eguchi N, Urade Y, Lambeau G, Gelb MH, Ishikawa Y, Ishii T, Kudo I. Localization of various secretory phospholipase A2 enzymes in male reproductive organs. *Biochim Biophys Acta* 2004;1686:61–76. [PubMed: 15522823]
- Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C. Progesterone is an essential factor for the initiation of the meiosis in spermatogenic cells of the eel. *Proc Natl Acad Sci USA* 2006;103:7333–7338. [PubMed: 16651536]
- Miyakoshi K, Murphy MJ, Yeoman RR, Mitra S, Dubay CJ, Hennebold JD. The identification of novel ovarian proteases through the use of genomic and bioinformatic methodologies. *Biol Reprod* 2006;75:823–835. [PubMed: 16870946]
- Montgomery RK, Breault DT. Small intestinal stem cell markers. *J Anat* 2008;213:52–58. [PubMed: 18638070]
- Morales C, Griswold MD. Retinol-induced stage synchronization in seminiferous tubules of the rat. *Endocrinology* 1987;121:432–434. [PubMed: 3595524]
- Morisseau C, Hammock BD. Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. *Annu Rev Pharmacol Toxicol* 2005;45:311–333. [PubMed: 15822179]
- Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. *Microsc Res Tech* 2003;60:569–580. [PubMed: 12645005]
- Ou YH, Chung PH, Hsu FF, Sun TP, Chang WY, Shieh SY. The candidate tumor suppressor BTG3 is a transcriptional target of p53 that inhibits E2F1. *EMBO J* 2007;26:3968–3980. [PubMed: 17690688]
- Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *J Cell Biol* 1996;135:469–477. [PubMed: 8896602]
- Park ES, Choi S, Muse KN, Curry TE Jr. Jo M. Response gene to complement 32 expression is induced by the luteinizing hormone (LH) surge and regulated by LH-induced mediators in the rodent ovary. *Endocrinology* 2008;149:3025–3036. [PubMed: 18308847]
- Phuchareon J, Tokuhisa T. Deregulated c-Fos/AP-1 modulates expression of the cyclin and the cdk gene in splenic B cells stimulated with lipopolysaccharide. *Cancer Lett* 1995;92:203–208. [PubMed: 7541312]
- Prochazka M, Kozak UC, Kozak LP. A glycerol-3-phosphate dehydrogenase null mutant in BALB/cHeA mice. *J Biol Chem* 1989;264:4679–4683. [PubMed: 2466839]
- Qiao D, Yang X, Meyer K, Friedl A. Glypican-1 regulates anaphase promoting complex/cyclosome substrates and cell cycle progression in endothelial cells. *Mol Biol Cell* 2008;19:2789–2801. [PubMed: 18417614]
- Rozman D, Seliskar M, Cotman M, Fink M. Pre-cholesterol precursors in gametogenesis. *Mol Cell Endocrinol* 2005;234:47–56. [PubMed: 15836952]
- Rubin E, Mitnacht S, Villa-Moruzzi E, Ludlow JW. Site-specific and temporally-regulated retinoblastoma protein dephosphorylation by protein phosphatase type 1. *Oncogene* 2001;20:3776–3785. [PubMed: 11439341]
- Saigusa K, Imoto I, Tanikawa C, Aoyagi M, Ohno K, Nakamura Y, Inazawa J. RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G2/M arrest. *Oncogene* 2007;26:1110–1121. [PubMed: 17146433]

- Salojin K, Oravec T. Regulation of innate immunity by MAPK dual-specificity phosphatases: knockout models reveal new tricks of old genes. *J Leukoc Biol* 2007;81:860–869. [PubMed: 17289800]
- Shao S, Wang Y, Jin S, Song Y, Wang X, Fan W, Zhao Z, Fu M, Tong T, Dong L, et al. Gadd45a interacts with aurora-A and inhibits its kinase activity. *J Biol Chem* 2006;281:28943–28950. [PubMed: 16772293]
- Stein Y, Stein O. Lipoprotein lipase and atherosclerosis. *Atherosclerosis* 2003;170:1–9. [PubMed: 12957676]
- Stoffel W, Holz B, Jenke B, Binczek E, Gunter RH, Kiss C, Karakesisoglou I, Thevis M, Weber AA, Arnhold S, et al. Delta6-Desaturase (FADS2) deficiency unveils the role of omega3- and omega6-polyunsaturated fatty acids. *EMBO J* 2008;27:2281–2292. [PubMed: 19172737]
- Stouffer RL, Xu F, Duffy DM. Molecular control of ovulation and luteinization in the primate follicle. *Front Biosci* 2007;12:297–307. [PubMed: 17127300]
- Tassoni D, Kaur G, Weisinger RS, Sinclair AJ. The role of eicosanoids in the brain. *Asia Pac J Clin Nutr* 2008;17(Suppl 1):220–228. [PubMed: 18296342]
- Terry-Lorenzo RT, Carmody LC, Voltz JW, Connor JH, Li S, Smith FD, Milgram SL, Colbran RJ, Shenolikar S. The neuronal actin-binding proteins, neurabin I and neurabin II, recruit specific isoforms of protein phosphatase-1 catalytic subunits. *J Biol Chem* 2002;277:27716–27724. [PubMed: 12016225]
- van Pelt AM, de Rooij DG. Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. *Endocrinology* 1991;128:697–704. [PubMed: 1989855]
- Vernet N, Dennefeld C, Klopfenstein M, Ruiz A, Bok D, Ghyselinck N, Mark M. RXR beta expression in Sertoli cells controls cholesterol homeostasis and spermiogenesis. *Reproduction* 2008;136:619–626. [PubMed: 18713813]
- Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, Mark M. Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology* 2006;147:96–110. [PubMed: 16210368]
- Volle DH, Lobaccaro JM. Role of the nuclear receptors for oxysterols LXRs in steroidogenic tissues: beyond the “foie gras”, the steroids and sex? *Mol Cell Endocrinol* 2007;265–266:183–189. [PubMed: 17208362]
- Wahlberg P, Nylander A, Ahlskog N, Liu K, Ny T. Expression and localization of the serine proteases high-temperature requirement factor A1, serine protease 23, and serine protease 35 in the mouse ovary. *Endocrinology* 2008;149:5070–5077. [PubMed: 18566130]
- Wang Z, Cao N, Nantajit D, Fan M, Liu Y, Li JJ. Mitogen-activated protein kinase phosphatase-1 represses c-Jun NH2-terminal kinase-mediated apoptosis via NF-kappaB regulation. *J Biol Chem* 2008;283:21011–21023. [PubMed: 18508759]
- Wang ZJ, Jeffs B, Ito M, Achermann JC, Yu RN, Hales DB, Jameson JL. Aromatase (Cyp19) expression is up-regulated by targeted disruption of Dax1. *Proc Natl Acad Sci USA* 2001;98:7988–7993. [PubMed: 11427738]
- Yang L, Naltner A, Yan C. Overexpression of dominant negative retinoic acid receptor alpha causes alveolar abnormality in transgenic neonatal lungs. *Endocrinology* 2003;144:3004–3011. [PubMed: 12810556]
- Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, Pouchnik D, Banasik B, McCarrey JR, Small C, et al. Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. *Biol Reprod* 2008;78:537–545. [PubMed: 18032419]

Figure 1. Verification of retinol response in VAD and retinol-replenished testes

Hematoxylin and eosin stained testicular sections from rats on vitamin A deficient (VAD) diet for 9 weeks (A) and normal adult testis (B). VAD seminiferous tubules lack all advanced germ cells and contain vacuoles (arrows), where germ cells once resided. Western blot analysis of testicular extracts from vitamin A deficient (VAD) rats, rats replenished with retinol for 4 h, 8h, and 24h using anti-RARA antibody (C). The Coomassie-stained blot to show equal loading of proteins (D). Real time PCR of *Stra8*, *Transferrin*, and *cRBP1*, known retinoic acid responsive genes (E). Changes are expressed as the fold change ± SEM. Data are representative of 3 separate experiments.

Figure 2. Global analysis of changing transcriptomes in retino-replenished VAD testes

Number of up-regulated and down-regulated transcriptomes significantly ($P < 0.05$) changing by ≥ 1.5 fold (A). Venn diagram for transcriptomes up-regulated (B) and down-regulated (C) in retinol-replenished testes. The circles represent a single time point. Numbers represent the number of genes expressed in a single time point, in two time points, or shared by all time points (innermost).

Figure 3. Expression of selected genes by real time PCR to verify microarray results and their expression in primary Sertoli cells

Real time RT-PCR results for RNA from retinol-replenished testes (left) or RNA from rat Sertoli cells treated with 1 μ M retinoic acid (right). The Y-axis shows the fold change $\pm$ SEM of the transcript level, relative to that of the VAD level for *Nr0b1* (*Dax-1*) (A), *Gadd45a* (B), *Acls4* (C), *Gpd1* (D), *Pde4b* (E), *Dpysl4* (F), *Pla2g2a* (G), and *Prss35* (H). Real time PCR was conducted in triplicate. Letters above the bar indicate statistically significant difference of means, $P < 0.05$.

Figure 4. Potential roles for retinol-regulated genes

Retinol-regulated genes for the pathway of (A) cholesterol synthesis, (B) progesterone, testosterone, and estradiol production, and (C) phosphatidylcholine synthesis.

Table 1

Primers used for real time RT-PCR.

Gene Name	Forward Primer	Reverse Primer
Stra8	TGGTAGGGCTCTTCAACAACCT	TTGTCCTATTCAGTACCTGCCACTT
Transferrin	CAACCACTGCAAGTTCGATGAA	CACAGGTCACAGAGGGTGAAT
cRBP1	CTAGCACAGGGCTGAAACACAT	AAGGAAAAGAGACAAGGCAATGA
Ribosomal S2	CTGACTCCCCGACCTCTGGAA	GAGCCTGGGTCCTCTGAACA
Nr0b1	TCCAGGCCATCAAGAGTTTCTT	CCCTTCAGATAGGCGTACTCTTTG
Pla2g2a	TTGGTGCTGTGTGACTCATGACT	AGGTCAGAACTTTGTGCCACAT
Gadd45a	CTGGCTGCGGATGAAGATG	CACGAATGAGGGTGAAATGGA
Gpd1	CAGCTCCTTCAACCCGTATTCTT	AGCGCTCTGGCTGTTGAAA
Dpysl4	CTCCCACATAGTCCCTTGCAA	CTAAGGGATCATCAAACCTCCCTAGAG
Acsl4	TAGGAGGAAATGTCCGCATGAT	CAGCAGAAGCAGACATTCATGAA
Pde4b	CATCACGGCTTCCCAAATTAG	GGCACTGCCGCAGGTTAA
Prss35	TGGGATCTGAAACGGAACCA	GGCAAGGTGGATCGTCTTCTC

Table 2

Potential canonical RAREs in retinol-replenished VAD testes.

Conditions	Number of Genes	RARE (121 genes)		No RARE (137 genes)	
		Up-regulated	Down-regulated	Up-regulated	Down-regulated
Total	258	47	74	36	101
4 h Retinol	78	21	13	20	24
8 h Retinol	209	32	60	34	83
24 h Retinol	220	41	59	36	84

Table 3

Distribution of retinol-regulated genes in testicular cell types.

Genes found in:	# of Genes	Regulation	
		Up-regulated	Down-regulated
Rat testicular database *	281	95	186
Spermatogonia + early spermatocytes	245	72	173
Sertoli cells	247	77	170

* GEO Accession data set, [GSE8978](#) [Johnston et al. 2008]

Table 4

Time-dependent Primary Functions and the Number of Associated Genes.

4-h Functions	P value	# of Genes
* Molecular transport	1.86E-04	14
* Embryonic development	2.09E-04	12
* Gene expression	2.76E-04	22
* Small molecule biochemistry	6.95E-04	24
* Lipid metabolism	6.95E-04	19
* Cell cycle	6.95E-04	17
* Cellular development	1.38E-03	12
* Cellular assembly and organization	1.54E-03	11
* Cellular compromise	1.54E-03	7
* Cell morphology	2.27E-03	8
* Cell death	3.07E-03	30
† DNA replication, recombination, repair	3.37E-03	9
* Tissue morphology	3.37E-03	8
* Cellular growth and proliferation	4.67E-03	24
* Tissue development	1.54E-02	12
* Cellular function and maintenance	1.54E-02	6

8-h Functions	P value	# of Genes
* Cell cycle	2.84E-04	18
* Cellular development	2.84E-04	14
* Embryonic development	7.55E-04	20
* Small molecule biochemistry	1.76E-03	43
* Cell death	1.76E-03	11
* Cellular compromise	1.76E-03	9
* Gene expression	5.10E-03	37
* Lipid metabolism	5.10E-03	30
* Molecular transport	5.10E-03	19
* Cell morphology	5.13E-03	16
* Cellular function and maintenance	5.13E-03	14
◇ Cell to cell signaling and interaction	5.13E-03	11
◇ Cell movement	5.13E-03	7
* Cellular assembly and organization	9.98E-03	23
* Tissue morphology	9.98E-03	14
* Cellular growth and proliferation	3.21E-02	12
† Nucleic acid metabolism	3.75E-02	7
* Tissue development	4.20E-02	11

24-h Functions	P value	# of Genes
* Cell cycle	3.30E-04	21
* Embryonic development	3.30E-04	21

24-h Functions	P value	# of Genes
* Cellular development	3.30E-04	15
◇ Cell movement	3.30E-04	8
* Small molecule biochemistry	1.39E-03	51
* Lipid metabolism	1.39E-03	43
* Cell death	1.95E-03	67
* Cellular compromise	1.95E-03	9
* Molecular transport	3.69E-03	28
* Gene expression	5.67E-03	49
* Cellular assembly and organization	5.67E-03	23
* Cell morphology	5.67E-03	22
◇ Cell to cell signaling and interaction	5.67E-03	11
* Cellular function and maintenance	5.67E-03	9
* Tissue morphology	1.10E-02	17
* Tissue development	1.10E-02	12
† Post-translational modification	1.78E-02	8
* Cellular growth and proliferation	2.60E-02	52
† Protein synthesis	2.60E-02	7

* functions found in all time points

◇ gain of functions at 8 h

† functions only in one time point

Table 5

Time-dependent Presentation of Diseases and the Number of Associated Genes.

4-h Time point of Diseases	P value	# of Genes
* Cancer	2.61E-04	36
* Reproductive system disease	2.61E-04	23
Inflammatory disease	3.27E-03	14
* Immunological disease	3.37E-03	17
Dermatological diseases	3.37E-03	9
* Skeletal and muscular disorders	9.72E-03	16
Connective tissue disorders	9.72E-03	14
* Renal and urological disease	9.72E-03	10
* Hematological disease	1.54E-02	13
Cardiovascular disease	1.54E-02	9
8-h Time point of Diseases	P value	# of Genes
* Cancer	6.88E-04	65
* Hematological disease	3.15E-03	13
Respiratory disease	3.15E-03	10
* Reproductive system disease	5.13E-03	32
* Renal and urological disease	7.66E-03	12
Endocrine system disorder	9.98E-03	8
Development disorder	9.98E-03	7
Neurological disease	1.62E-02	9
Genetic disorder	1.62E-02	7
* Skeletal and muscular disorders	2.36E-02	8
* Immunological disease	2.36E-02	7
24-h Time point of Diseases	P value	# of Genes
* Reproductive system disease	1.49E-04	38
* Cancer	4.22E-04	80
Cardiovascular disease	1.95E-03	17
* Hematological disease	4.17E-03	19
Respiratory disease	4.17E-03	10
Connective tissue disorder	5.67E-03	15
* Renal and urological disease	9.99E-03	11
Developmental disorder	1.10E-02	14
* Skeletal and muscular disorders	1.10E-02	10
Endocrine system disorder	1.10E-02	8
Neurological disease	1.78E-02	9
Genetic disorder	1.78E-02	7
* Immunological disease	2.60E-02	6

* diseases or disorders found in all time points

Table 6

Time-dependent Canonical Pathways and Associated Genes.

4-h Canonical Pathway	P value	Genes
Estrogen receptor signaling	4.47E-03	↑Nr0b2 ↑Nr0b1 ↓Med4 ↓Polr2i
Glycerophospholipid metabolism	8.51E-03	↑Gpd1 ↑Pla2g5 ↑Chka ↑Pla2g2a
Glucocorticoid receptor signaling	2.19E-02	↑Fos ↑Hspa1b ↑Dusp1 ↑Fkbp4 ↓Polr2i ↑Fgg ↑Ca2, ↓Ywhah
Arachidonic acid metabolism	2.24E-02	↓Ephx2 ↑Pla2g5 ↑Pla2g2a ↓Cyp51a1 ↓Cbr1 ↑Gpx3
p38 MAPK signaling	2.29E-02	↑Dusp1 ↑Pla2g5 ↑Pla2g2a
Linoleic acid metabolism	3.31E-02	↑Pla2g5 ↑Pla2g2a ↓Cyp51a1
BRCA1 in DNA damage response	4.07E-02	↓Rbl2 ↑Gadd45a ↓Rb1
Aminosugars metabolism	4.07E-02	↑Cyb5r3 ↓Pde4b
Aryl hydrocarbon receptor signaling	4.17E-02	↑Fos ↑Cnd3 ↑Nr0b2 ↑Nfia, ↓Rb1 ↓Mgst3
Eicosanoid signaling	8.71E-02	↑Pla2g5 ↑Pla2g2a
LPS/IL-1 inhibition of RXR function	9.12E-02	↑Nr0b2 ↓Mgmt ↑Acs14 ↑Sod3 ↓Maob ↓Fabp512 ↓Hmgcs1 ↓Maoa ↑Acs14 ↑Cd14 ↓Mgst3
Fatty acid metabolism	1.25E-01	↓Acat1 ↑Acs14 ↓Cyp51a1 ↓Acat2 ↓Acaa2
Nucleotide excision repair pathway	1.44E-01	↓Polr2i
RAR activation	1.79E-01	↑Fos ↑Rdh10 ↑Dusp1 ↓Prkar2b
Circadian rhythm signaling	2.32E-01	↓Bhlhb3
G2/M DNA damage checkpoint	2.44E-01	↑Gadd45a
Retinol metabolism	2.90E-01	↑Rdh10
Hepatic cholestasis	3.61E-01	↑Nr0b2 ↑Hnf4a ↓Prkar2b ↑Cyp27a1 ↑Cd14
14-3-3-mediated signaling	3.78E-01	↑Fos ↓Tuba1a ↓Ywhah ↓Tyro3
IL-10 signaling	3.93E-01	↑Fos ↑Cd14
8-h Canonical Pathways	P value	Genes
Ketone body synthesis and degradation	3.24E-04	↓Acat1 ↓Acat2 ↓Bdh1 ↓Hmgcs1
Gly, Ser and Thr metabolism	3.98E-03	↑Chka ↓Maob ↓Gamt ↑Bhmt ↓Phgdh ↓Maoa
Alanine and aspartate metabolism	1.23E-02	↓Pc ↓Abat ↓Dlat ↓Asns
Butanoate/propanoate metabolism	1.62E-02	↓Acat1 ↓Acat2 ↓Bdh1 ↓Abat ↓Hmgcs1
Pyruvate metabolism	2.51E-02	↓Pc ↓Acat1 ↓Acat2 ↓Dlat ↓Mdh2
Val, Leu and Ile degradation	2.51E-02	↓Acat1 ↓Acat2 ↓Abat ↓Hmgcs1 ↓Acaa2
ERK/MAPK signaling	5.62E-02	↑Fos ↑Dusp1 ↑Pla2g5 ↑Pla2g2a ↓Ptk2 ↓Pak1 ↓Prkar2b ↓Ywhah ↓Rapgef3
Mitochondrial dysfunction	6.92E-02	↓Cox8b ↓Prdx3 ↓Maob ↑Cyb5r3 ↓Ndufb8 ↓Maoa
Serotonin receptor signaling	8.51E-02	↓Maob ↓Spr ↓Maoa
Arginine and proline metabolism	9.12E-02	↓Maob ↓Gamt ↓Spr ↓Maoa
Interferon signaling	1.15E-01	↓Stat2 ↑Mx1
Dopamine receptor signaling	1.26E-01	↓Maob ↓Prkar2b ↓Spr ↓Maoa
Biosynthesis of steroids	1.29E-01	↓Fdps ↓Sqle ↓Fdft1
Citrate cycle	1.44E-01	↓Pc ↓Mdh2
Glycosphingolipid biosynthesis	1.93E-01	↑St3gal2
Phenylalanine metabolism	2.05E-01	↓Maob ↓Maoa

8-h Canonical Pathways	P value	Genes
Tryptophan metabolism	2.14E-01	↓Maob ↓Acat2 ↓Acat1 ↓Cyp51a1 ↓Maoa
PTEN signaling	2.17E-01	↓Cnksr3 ↓Ptk2 ↑Ca2 ↓Ywhah
Nitrogen metabolism	2.20E-01	↑Ca2 ↓Asns
Histidine metabolism	2.20E-01	↓Maob ↓Maoa
Nitric oxide signaling	2.26E-01	↓Slc7a1 ↓Prkar2b ↓Flt1 ↑Kng1
O-Glycan biosynthesis	2.28E-01	↑St3gal2
p53 signaling	2.36E-01	↑Ca2 ↓Rb1 ↑Gadd45a
IGF-1 signaling	2.72E-01	↓Ptk2 ↑Fos ↓Prkar2b ↓Ywhah
Bile acid biosynthesis	2.83E-01	↑Cyp27a1 ↓Acaa2
Glycosaminoglycan degradation	2.91E-01	↑Arsb
cAMP-mediated signaling	3.77E-01	↑Dusp1 ↓Pde4b ↓Akap12 ↓Prkar2b ↓Camk2d ↓Rapgef3
24-h Canonical Pathways	P value	Genes
Cell cycle: G1/S checkpoint	2.29E-02	↓Rbl2 ↑Cend3 ↓Rb1 ↓Hdac2
Pyruvate metabolism	3.02E-02	↓Acat2 ↓Pc ↓Dlat ↓Akr1b1 ↓Mdh2
NRF2 on oxidative stress response	7.08E-02	↑Fos ↑Sod3 ↓Ppib ↓Dnaja4 ↑Actc1 ↓Cbr1 ↓Map2k5 ↓Mgst3
C21-Steroid hormone metabolism	1.10E-01	↓Cyp17a1 ↑Hsd3b1
VEGF signaling	1.24E-01	↓Ptk2 ↑Ca2 ↓Flt1 ↓Eif2b1 ↑Actc1
Toll-like receptor signaling	1.56E-01	↑Fos ↑Cd14
Purine metabolism	1.93E-01	↓Atp1b1 ↑Npr1 ↓Prpsap1 ↑Hprt1 ↓Pde4b ↓Guk1 ↓Polr2i ↓Abcd3 ↑Gucy2e ↑Pde1b
Androgen and estrogen metabolism	2.84E-01	↓Hsd17b1 ↑Arsb ↑Hsd3b1
Tight junction signaling	2.88E-01	↑Fos ↓Cnksr3 ↓Prkar2b ↓Cpsf3 ↑Actc1
Fructose and mannose metabolism	3.21E-01	↑Khk ↓Akr1b1
Protein ubiquitination pathway	3.34E-01	↓Psm4 ↑Uchl1 ↓Hspa8 ↓Psm1 ↓Vhl

↑upregulated gene; ↓ down-regulated gene