

# Gene Expression in the Spermatogenically Inactive “Dark” and Maturing “Light” Testicular Tissues of the Prepubertal Colt

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**ABSTRACT:** In the testis of the 1.5-year-old horse, spermatogenesis initiates locally in grossly light, central areas that contrast with grossly dark, peripheral areas that are as yet inactive in spermatogenesis. Gene expression was compared between “light” and “dark” tissues of 1.5-year-old horse testes to identify mechanisms important to the initiation of spermatogenesis. Microarrays containing human cDNAs were used to assess expression levels of 9132 genes simultaneously in matched pairs of dark and light testis tissues from 3 prepubertal colts. In all 3 analyses, dysferlin (DYS), down-regulated in ovarian cancer 1 (DOC1), and Golgi apparatus protein 1 (GLG1) genes were preferentially expressed in dark tissues, while outer dense fiber of sperm tails (ODF2) and phosphodiesterase 3B (PDE3B) genes were

more highly expressed in light testis tissue ( $>1.7$  balanced difference value, Incyte GEM tools software). Expression levels of 88 additional genes appeared to be different between dark and light tissues in 2 of the 3 microarray analyses. The preferential expression of *DYS*, *DOC1*, *ODF2*, and *PDE3B* genes in dark or light testis tissues was confirmed on Northern blots and localized to cell types by *in situ* hybridization. Future studies to determine the role of genes regulated during the initiation of spermatogenesis may aid in elucidating molecular mechanisms during this critical time as well as in identifying new therapies for enhancing male fertility.

Key words: Gene regulation, testis, spermatogenesis, puberty.  
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The initiation of spermatogenesis begins with a gradual series of events that transform the relatively inactive male gonad into an organ that produces high levels of testosterone and mature spermatozoa. Spermatogenesis in the colt (young stallion, an intact male) initiates in the central region of the testis around the second year of life and spreads peripherally until the entire testis is spermatogenically active. Spermatogenic activity can be noted grossly by the light tan color of the central testicular parenchyma in the 1.5-year-old horse, while peripheral tissues of the testis are dark red-brown (Clemmons et al, 1995). Histologically, dark testis tissues are laden with Leydig cells and large macrophages in the interstitial spaces and have small seminiferous tubules that are not producing sperm and that occupy no more than 50% of the parenchyma (Clemmons et al, 1995). In contrast, light testis tissues have reduced densities of Leydig cells, macrophages, and other interstitial cells as the seminiferous tubules (which are composed primarily of nonpigmented

cells) increase in size and cell density to compose approximately 70% of the testis parenchyma. Thus, the prepubertal colt testis presents an excellent model for functional genomics to identify genes that are turned on or off during the initiation of spermatogenesis. Its adjacent dark and light tissues have similar organizational components, including Leydig cells, seminiferous tubules, and influences of systemic hormones, but they differ in tubule maturity and function; namely, the initiation of spermatogenesis in the light tissue (Clemmons et al, 1995). The long-term goal of this study is to better understand the gene regulation critical to spermatogenesis and to identify new therapeutic approaches to enhance male fertility.

We hypothesize that the induction of spermatogenesis in the light testis tissue of the 1.5-year-old horse is the result of the regulation of 1) hormone receptors that confer abilities for hormone response and/or 2) paracrine growth factors that generate local signals that differ between dark and light testis tissues. Initially, 12 gene products were chosen that were predicted to be differentially expressed in dark and light testis tissues; these gene products included androgen receptor, progesterone receptor, estrogen receptors alpha and beta, steroid hormone receptor coactivator 1, high-density lipoprotein binding protein, transforming growth factor (TGF) beta 1, 2, and 3, oxytocin receptor, glyceraldehyde phosphate dehydrogenase,

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and c-fos. Of the 12, androgen receptor and TGF beta 1 appeared to be differentially expressed, but this was not confirmed on in situ hybridization or Northern blots.

For this reason we turned to microarrays on glass slides in order to simultaneously and efficiently compare levels of expression of 9132 gene products between matched dark and light testis tissues of 3 colts (Rockett et al, 2001b; Schultz et al, 2003). This powerful and unbiased approach identified differential expression of 93 genes in dark and light testis tissues from 2 or more colts. In situ hybridization and Northern blot analysis confirmed and localized the differential regulation of 4 genes chosen for these analyses. These discoveries open investigations into novel pathways that regulate spermatogenesis. Importantly, these data also demonstrate that analysis of equine gene expression can be performed using human cDNA microarrays because of a high level of sequence conservation. This conservation is evident in analyses of 193 horse cDNAs from a testis library that displayed an average of 92% identity between horse and human sequences (Skow, personal communication).

## Materials and Methods

### Animals and Sample Preparations

Seven 1.5-year-old colts were castrated by standard surgical means under appropriate anesthesia. Testes were cut midsagittally, and gross dissection of dark and light tissues from each testis preceded mincing, snap freezing in liquid nitrogen, and storage at  $-80^{\circ}\text{C}$ . Three left testicular samples were used for microarrays, 4 different testes were used for Northern blot analyses, and all 7 horse samples were used for in situ analyses. Samples were consistent across all horses with regard to age and presence of meiosis, as indicated by spermatocytes in light tissues. PolyA<sup>+</sup> RNA was isolated directly from dark and light tissue samples from all 7 horses with the Fasttrack kit (Invitrogen, San Diego, Calif). Testes from the same 7 horses were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2) and embedded in Paraplast (Fisher Scientific, Houston, Tex) for in situ hybridization. Reagents were of molecular biology grade and purchased from Sigma Chemical Company (St Louis, Mo), unless otherwise noted. All animal procedures were approved by the Texas A&M University Animal Care and Use Committee.

### Microarrays

Poly A<sup>+</sup> RNA samples from matched dark and light left testis samples from 3 horses appeared of high quality and purity on an ethidium bromide-stained Northern gel (results not shown). Dark and light testis RNA samples (1.8  $\mu\text{g}$  each) were reverse transcribed and labeled with 5'Cy3 and Cy5 fluoros, respectively; hybridized to immobilized human cDNAs of the UniGEM Human V 2.0 microarray; and analyzed by Incyte (St Louis, Mo). Probe labeling reactions were incubated at  $37^{\circ}\text{C}$  with 200 ng of polyA RNA, 200 units M-MLV reverse transcriptase (Life Tech-

nologies, Gaithersburg, Md), 4 mM DTT, 1 unit RNase Inhibitor (Ambion, Austin, Tex), 0.5 mM dNTPs, and 2  $\mu\text{g}$  of labeled 9-mers for 2 hours. Sample amplification was not used in order to decrease artifacts in expression ratios. The probe samples were then combined, purified, precipitated, and allowed to competitively hybridize to a single microarray. Briefly, the hybridization protocol consisted of the following: resuspension of probe solution with a 5-minute incubation at  $65^{\circ}\text{C}$ , application to array, cover slipping, and sealing in an evaporation chamber for 6.5 hours at  $60^{\circ}\text{C}$ . After hybridization, the glass slides were washed in 3 consecutive washes of decreasing ionic strength. The microarrays consisted of 9132 genes generated from polymerase chain reaction (PCR) products (15% were expressed sequence tags) and included 190 controls. The fluorescence data was converted to "balanced difference" values (balanced to account for differences in fluorescence labeling between the cDNA pools) with GEMTools 2.4 (Incyte Pharmaceuticals Inc, Palo Alto, Calif). Balanced differences with positive values are ratios of signals from the dark testis tissue to light testis tissue (Cy3/Cy5). Negative balanced difference values indicate that the ratio is inverted, with the larger light testis tissue (Cy5) signals divided by the lesser dark tissue signals (Cy3). Normalization was performed per chip to a series of internal controls including yeast cDNAs and fluorescent standards by gridding and region detection algorithms. Gene products with balanced difference values of equal to or more than 1.7 or less than  $-1.7$  were considered differentially expressed between dark and light testis tissues ( $P < .05$ ) (Yue et al, 2001; Moody et al, 2002; Reynolds, 2002). Spots failing to meet minimal criteria (signal strength 2.5 times that of background and covering at least 40% area of the spot) were given balanced difference values of 0 and were excluded from evaluation. Differentially expressed genes in two or more horses were identified with GeneSpring 4.0.2 software (Silicon Genetics, Redwood City, Calif). Complete technical and analytical information, including normalization and ratio determination, is available at (<http://animalscience.tamu.edu/ning/microarraydata/hopprimer.html>).

### Cloning of Down-Regulation of Ovarian Cancer 1 (DOC1), Golgi Apparatus Protein 1 (GLG1), and CDC2 cDNAs From Horse Testis

To make high-specificity probes for androgen receptor (AR), down-regulation of ovarian cancer 1 (DOC1), Golgi apparatus protein 1 (GLG1), and CDC2 mRNAs of the horse, cDNAs were cloned from horse testis, as previously described (Ing et al, 1996). PolyA<sup>+</sup> RNA (200 ng) was reverse transcribed at  $42^{\circ}\text{C}$  with Superscript II (Life Technologies) and random hexamer primers. PCR was performed twice with 2 sets of primers designed from human cDNA sequences (see website), with the set of primers used for the second amplification nested inside the first. cDNAs were cloned into the PCR2.1 vector (Invitrogen), and multiple clones were sequenced. The horse DOC1, GLG1, and CDC2 cDNA sequences are in GenBank (accession numbers AY349169, AF547432, and AF547431, respectively).

*Northern Analyses of Dysferlin (DYS), DOC1, Outer Dense Fiber of Sperm Tails (ODF2), Phosphodiesterase 3B (PDE3B), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs*

PolyA+ RNA samples (5 µg) were analyzed on a denaturing (Northern) gel, as previously described (Ing et al, 1996). RNA Millennium Markers from Ambion (Austin, Tex) were run alongside. The RNA on the gels was transferred to nitrocellulose membranes and the blots were hybridized to radiolabeled antisense cRNA probes produced by in vitro transcription (T3 RNA polymerase) with the Maxiscript kit (Ambion) using [<sup>32</sup>P]-UTP (3000 Ci/mmol; New England Nuclear, Boston, Mass). The cDNA template for DOC1 was a PCR product (described above), while those for dysferlin (DYS), outer dense fiber of sperm tails (ODF2), phosphodiesterase 3B (PDE3B), and GAPDH were constructed from linearized plasmids as follows. Human DYS plasmid (pINCY-4462162 vector; Incyte Genomics, Palo Alto, Calif) was linearized with *EcoRI* and transcribed with T7 RNA polymerase; rat ODF2 plasmid (pBluescript II vector) (GenBank accession number U62821) was linearized with *NcoI* and T7 RNA polymerase; human PDE3B plasmid (pBluescript II vector) (GenBank accession number U38178) with *BamHI* and T3 RNA polymerase; and ovine GAPDH plasmid restricted with *BamHI* and T7 RNA polymerase. After stringent washing, the blots were exposed to x-ray film (XAR, Kodak, Rochester, NY). Densitometry with Intelligent Quantifier software (Bio Image, Ann Arbor, Mich) was used to compare hybridization signals between samples and reported as an average fold change in densitometric units across 4 horses. Individual gene expression units were normalized to GAPDH to account for loading differences.

*In Situ Hybridization*

The mRNA for DYS, ODF2, and DOC1 were localized on serial cross sections from the left testes of 1.5-year-old horses by in situ hybridization analysis, as described previously (Ing et al, 1997). Tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated using in vitro transcription with α[<sup>35</sup>S]-UTP (1250 Ci/mmol, New England Nuclear). The human DYS plasmid (pINCY-4462162 vector, Incyte) linearized with *EcoRI* and transcribed with T7 RNA polymerase to produce antisense cRNA. In addition, negative control sense cRNA from DYS was produced from *NotI* and SP6 RNA polymerase. Rat ODF2 and human PDE3B cDNAs (GenBank accession numbers U62821 and U38178) were restricted within the cDNAs with *NcoI* for ODF2 and with *BamHI* for PDE3B and transcribed with T7 or T3 RNA polymerase for antisense and sense cRNAs. Antisense and sense horse DOC1 cRNAs were produced from PCR products (described above) with T3 and T7 RNA polymerases, respectively. After hybridization, washing, and ribonuclease A digestion, slides were coated with photographic emulsion (Eastman Kodak) and developed 2 or 4 weeks later, depending on hybridization signal strength. Cell nuclei were counterstained with 1% toluidine blue. Micrographs were captured on a Zeiss Axioplan 2 Microscope (Gottingen, Germany) using Adobe Photoshop (Adobe Systems, Seattle, Wash).

## Results

*Microarray Analyses Identify a Wide Variety of Expressed Genes*

The complete data sets from the microarray studies are available online at <http://animalscience.tamu.edu/ning/microarraydata/horse1.html> for “horse1” and adjacent websites for “horse2” and “horse3” (summarized in the Table). Genes are grouped and ranked by average balanced difference values. Incyte microarray gene names are capitalized, while functional descriptions are in lowercase. Two clear results generated from the microarray analysis determined that the majority of genes were not differentially expressed between dark and light tissues of the testes, and individual horses had unique differences in their patterns of testicular gene expression. However, there were a number of genes that appeared to be preferentially expressed in dark or light tissue. DYS, DOC1, and GLG1 genes were preferentially expressed in dark tissue, while ODF2 and PDE3B genes were more highly expressed in light testis tissue of all 3 horses analyzed by microarrays (>1.7 balanced difference value, Incyte GEMTools software). An additional 88 genes appeared to be regulated differently between light and dark testis tissue in at least 2 of the 3 horses analyzed. These genes fall into a number of different functional categories, including 1) signaling molecules; 2) cytoskeletal and adhesion molecules; 3) enzymes and inhibitors; and 4) those of unknown function. These gene products are shown in the Table ranked by their balanced difference values averaged across all 3 microarray analyses (number to the left of the gene name). Larger absolute values of balanced differences related to larger magnitudes of differential gene expression. For example, the largest (−7.8) was for ODF2, indicating that light testis tissue had 7.8-fold more ODF2 mRNA than the dark tissue of the testis. In total, 93 genes appeared to be differentially expressed based on microarray results. These included 58 genes preferentially expressed in dark testis tissue (left panel) and 35 genes preferentially expressed in light testis tissue (right panel). These 93 genes were only 1.3% of the 6940 gene products that had hybridization signals that met minimal criteria on at least 2 microarrays.

The microarray results indicated differential expression of many interesting genes. In the cell-signaling group, the insulin system was implicated, with insulin-like growth factor 2 and insulin-like growth factor binding protein 7 being predominantly expressed in dark tissue, whereas PDE3B was expressed more in light tissue. Gene products that regulate cell cycle progression were also differentially expressed. These included parathymosin and CDC-like kinase in dark tissue and cyclin A1, CDC2, CDC28

## Differentially expressed genes in dark and light testis tissue of prepubertal colts\*

Dark-Specific	
Signaling Molecules	
3.6	Insulin-like growth factor 2
3.6	Parathyrosin–cell cycle regulated
2.1	ATP-binding cassette 3
1.9	CDC-like kinase
1.9	Major histocompatibility complex, Class 1, B
1.8	Insulin-like growth factor binding protein 7
1.8	Protein kinase C, $\mu$
1.8	Phospholipase D2
1.8	CD63 antigen (melanoma I antigen)
1.7	Major histocompatibility complex, Class 1, C
1.5	Methyl-CPG binding domain protein 3
1.4	HLA-G histocompatibility antigen, Class 1, G
Cytoskeleton & Adhesion Molecules	
4.1	Desmoplakin
3.7	Dysferlin, limb girdled muscular dystrophy†
3.0	Laminin, $\beta$ 1
2.8	Keratin 8
2.5	Golgi apparatus protein†
2.3	Down-regulating in ovarian cancer 1†
2.2	Keratin 18
2.1	Absent in melanoma
2.0	Ribosomal proteins L13A & L
1.9	Crystallin, $\mu$
1.9	Ribosomal proteins L3 & L6
1.9	Sarcoglycan
1.8	Calponin 3, acidic
1.8	Cadherin 3, Type 1, placental
1.8	AT-rich sequence binding protein
1.8	Ribosomal proteins L9, L12, S14
1.7	Ribosomal proteins P0 & S3, S7, S10, S23
1.6	Ribosomal protein S15A
Enzymes & Inhibitors	
2.3	O-linked N-acetylglucosamine transferase
2.0	Glutathione-S-transferase $\Omega$
1.9	Carboxypeptidase E
1.9	Argininosuccinate synthetase
1.8	Protein S (Alpha)
1.8	Ribonuclease, RNASE A family
1.8	ATPASE, Na <sup>+</sup> /K <sup>+</sup> transporting, 2 polypeptide
1.8	Vaccinia-related kinase 1
1.8	Serine (cysteine) proteinase inhibitor, clade A 5
1.7	Serine (cysteine) proteinase inhibitor, clade G 1
1.6	Sterol-C4-methyl oxidase-like
1.6	Eukaryotic translation elongation factor 1 $\beta$ 2
1.6	Muscleblind (drosophila)-like
1.5	ESTS, similar to ITF2 Human trans factor 4
Unknown Function	
2.5	KIAA0018 gene product
2.1	Hypothetical protein FLJ20030
2.0	Mesoderm-specific transcript homolog
1.9	KIAA0202 protein
1.7	Homo sapiens MRNA clone 2068071
1.7	KIAA0286 protein

## Table Continued

Light-Specific	
Signaling Molecules	
–5.0	Cyclin A 1
–4.4	Phosphodiesterase 3B, CGMP-inhibited†
–2.5	STE-20-related kinase
–2.3	Cell division cycle 2
–2.2	A kinase (PRKA) protein (Gravin) 12
–2.2	CBP/P300-interacting transactivator 1
–2.0	CDC28 protein kinase 2
–2.0	MAD3L
–2.0	Guanine nucleotide binding protein, $\delta$ 3
–1.9	Phospholipase C, $\delta$ 1
–1.8	Cholecystokinin
–1.8	Sortilin 1
–1.6	Angio-associated, migratory cell protein
–1.6	Purine-rich element binding protein B
–1.6	PRKC—prostate apoptosis response protein 4
Cytoskeleton & Adhesion Molecules	
–7.8	Outer dense fiber of sperm tails 2†
–7.7	Protamine 1
–4.4	Calnegin (Calnexin-t)
–4.0†	Oligodendrocyte transmembrane protein
–22	Kinesin-like 1
–1.7	Coactin 2 (axonal)
–1.7	Leukemia-associated phosphoprotein P18
–1.6	Tubulin, $\alpha$ 1 (testis-specific)
Enzyme & Inhibitors	
–3.3	Glycerol kinase
–2.8	Hydroxyacyl glutathione hydrolase
–2.7†	MUTs ( <i>E. Coli</i> ) homolog 4
–2.3	Coagulation factor C
–2.3	Glucose transporter pseudogene
–2.2	Phosphorylase kinase, $\gamma$ 2 (testis)
–1.8	Serine (cyst) proteinase inhibitor, clade F 1
–1.8	Forkhead box G1B
Unknown Function	
–2.5	Homo sapiens MRNA; DKFZP434P2072
–2.3	Interferon, $\alpha$ -inducible protein (clone IF1616)
–2.0†	KIAA0282 protein
–1.8	HSPC033 protein

\* Differentially expressed genes in dark and light testis tissues from at least 2 of the 3 horses are shown.

† Indicates genes differentially expressed in all 3 horses.

‡ Indicates average of only 2 usable balanced difference values.

protein kinase 2, MAD3L, and PRKC prostate apoptosis response protein 4 in light tissue.

There were many differences in the expression of genes whose products had cytoskeletal or cell adhesion functions. For example, dark testis tissue expressed higher levels of desmoplakin, keratins, and cadherin mRNAs, the products of which are associated with intermediate filaments, as well as myosin-like DOC1, sarcoglycan, and calponin gene products, which are associated with large cytoskeletal filaments. Cell adhesion proteins laminin and GLG1 were also predominantly expressed in dark testis tissue, as were 13 ribosomal proteins and 2 crystallin

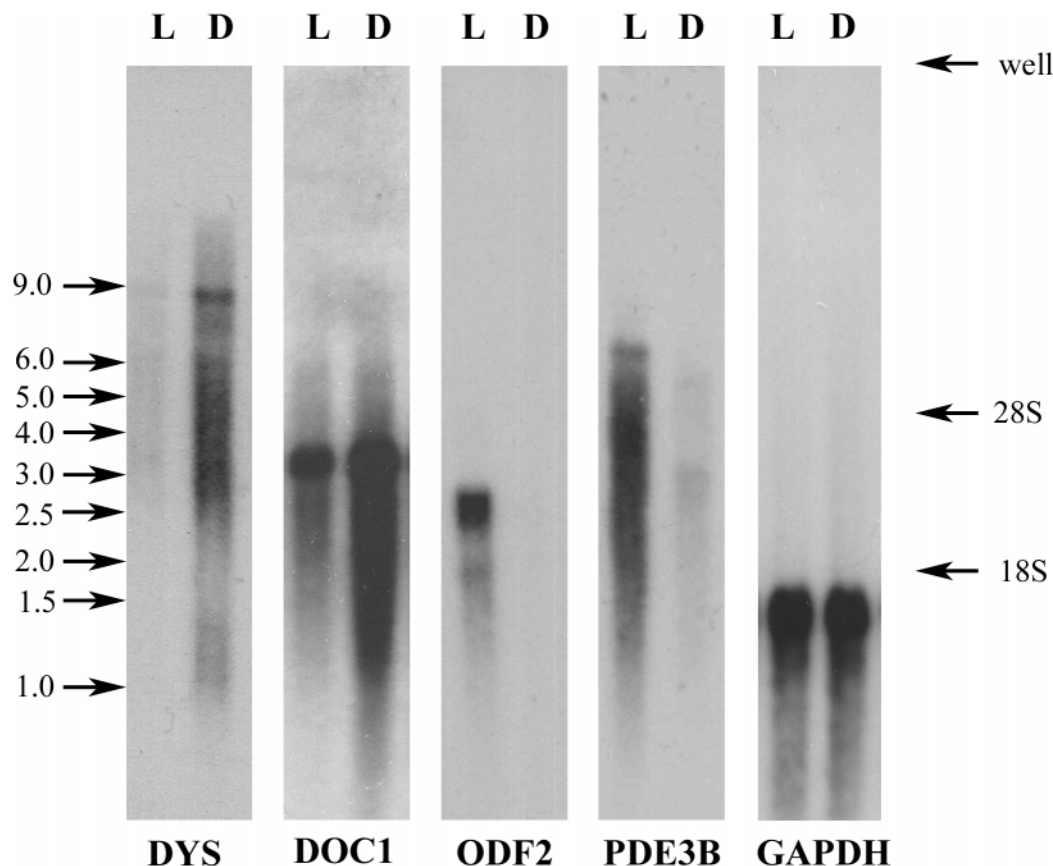


Figure 1. Representative Northern blot analyses of mRNA in dark and light regions of testes from 1.5-year-old horses. PolyA<sup>+</sup> RNA was isolated from light (L) spermatogenically active and dark (D) spermatogenically inactive testicular tissues. Radiolabeled antisense cRNAs specifically hybridized dyslerlin (DYS), down-regulated in ovarian cancer 1, myosin-like (DOC1), outer dense fiber of sperm tails 2 (ODF2), phosphodiesterase 3B (PDE3B), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs on separate blots at 8.0-, 3.8-, 2.5-, 6.5-, and 1.8-kb sizes, respectively. Migration positions of RNA markers are indicated at left and ribosomal rRNAs at right.

genes. Light testis expressed higher levels of gene products associated with sperm-specific structures (ODF2 and protamine 1) and microtubules (kinesin-like 1, stathmin, and the testis-specific tubulin  $\alpha 1$ ). In addition, metabolic enzymes, kinases, protease inhibitors, and transcription factor genes were differentially expressed between dark and light tissues, as were novel gene products of unknown function (such as expressed sequence tags).

**Northern Blots Confirm Differential Expression of *DYS*, *DOC1*, *ODF2*, and *PDE3B* Genes**—Northern blots were used to confirm the differential expression of *DYS*, *DOC1*, *ODF2*, and *PDE3B* genes between light and dark testis tissues, utilizing *GAPDH* mRNAs as a control. Representative Northern blots are depicted in Figure 1. In order to critically analyze and verify these results, horses evaluated were not used in the microarray. Both *DYS* (8.0 kb) and *DOC1* (3.5 kb) mRNA concentrations are greater in dark testis tissue compared to light. Previous Northern blot experiments indicate similar sizes for those mRNAs (Mok et al, 1994; Britton et al, 2000). In contrast, *ODF2* and *PDE3B* mRNAs hybridized weakly in dark tissue but

showed strong expression in the light tissue. Sizes of these transcripts (2.5 and 6.5 kb, respectively) were similar to those observed in other studies (Miki et al, 1996; Schalles et al, 1998). Similar Northern blot analyses of light and dark testis tissue RNA samples of 4 horses were quantitated. Since there were no differences in *GAPDH* mRNA concentrations found between light and dark tissues of other stallions in macroarray, in situ hybridization, or microarray analyses, each lane was normalized to its *GAPDH* mRNA signal and is reported as mean fold change for each mRNA ( $\pm$ SE). Fold differences on Northern blots were comparable to signal intensities for microarrays. *DYS* and *DOC1* were 3.2 ( $\pm$ 0.44) and 2.4 ( $\pm$ 0.27), whereas *ODF2* and *PDE3B* both exhibited fold increases over dark tissue at 4.6 ( $\pm$ 1.6) and 3.9 ( $\pm$ 1.2), respectively.

**In Situ Hybridization Confirms and Localizes Differential Expression of Genes Within Dark and Light Testis Tissues**—*DOC1*, human *DYS* and *PDE3B*, and rat *ODF2* cDNA localization of horse mRNA in light and dark testis from 1.5-year-old horses by in situ hybridization indicate

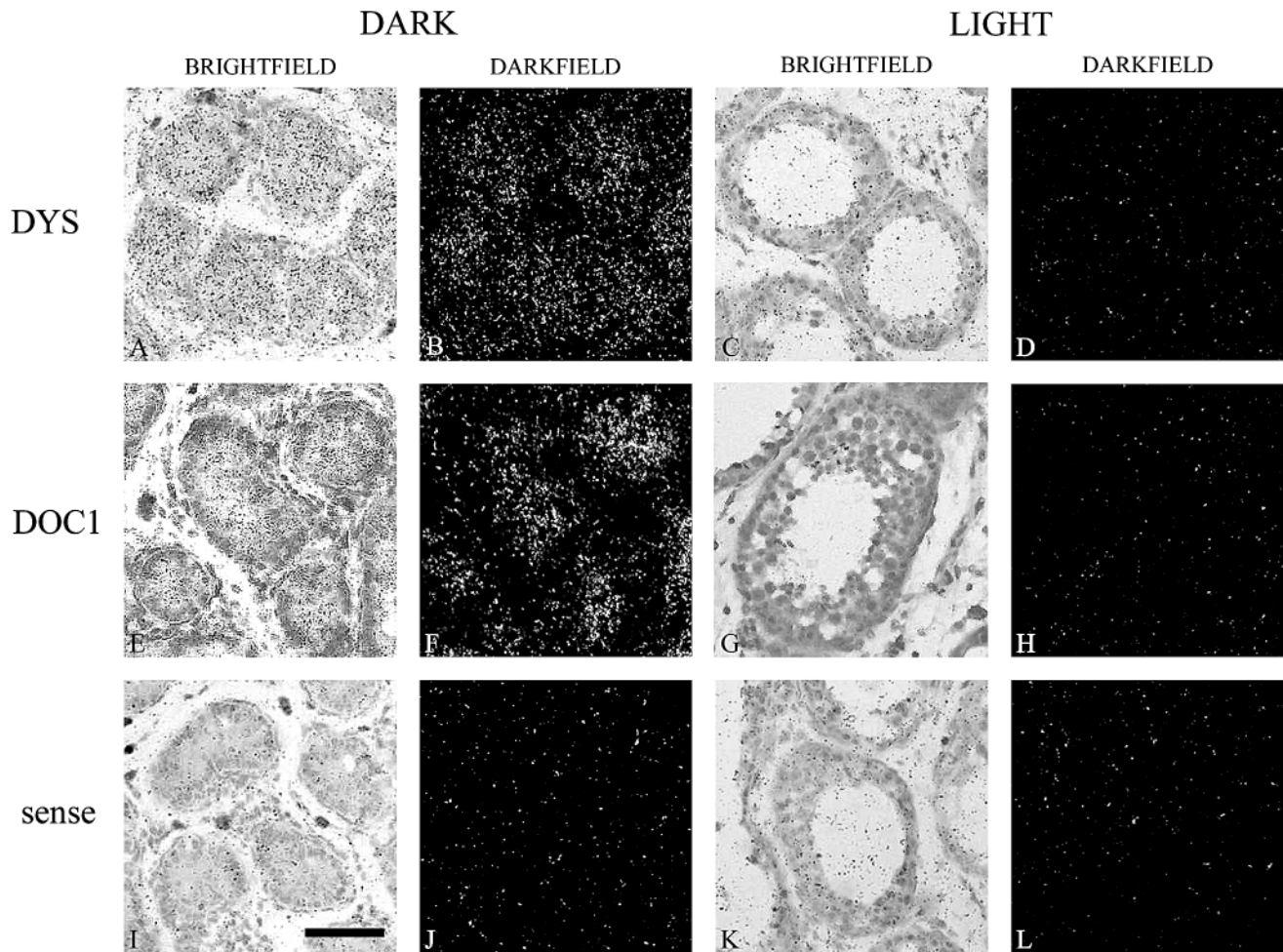


Figure 2. In situ hybridization reveals dysferlin (DYS) and down-regulated in ovarian cancer 1 (DOC1) gene expression predominantly in seminiferous tubules of the dark tissue of a maturing horse testis. Adjacent cross sections from a 1.5-year-old horse testis were hybridized with antisense cRNA probes for DYS and DOC1. Slides were coated with autoradiographic emulsion and developed after 2 or 4 weeks of exposure. Bright-field views (**A, E, and I for dark tissue; C, G, and K for light tissue**) show the nuclei stained lightly with 1% toluidine blue. Small silver grains shine white in dark-field views and indicate hybridization of radioactive probes for DYS (**Panels B and D**) and DOC1 (**Panels F and H**) mRNAs. **Panels I/151/L** show the low level of nonspecific binding to sense DYS cRNA (a negative control). They also clearly demonstrate the smaller diameter and lack of open lumina in the seminiferous tubules of the dark testis Panel compared to the light testis Panel. The bar in **Panel I** indicates 100  $\mu\text{m}$ .

similar patterns to those obtained by microarray and Northern blot analysis. Like that of the horse AR cDNA (GenBank accession number AY032721), the horse cDNA for DOC1 was highly conserved across species, being 92% identical to the human. Representative bright-field and dark-field views of the in situ hybridization of DYS and DOC1 mRNAs are shown in Figure 2. The bright-field views illustrate the histology of the testes; dark testis tissue has small seminiferous tubules with closed lumina and light tissue has larger seminiferous tubules and open lumina. Hybridization signals (small, black silver grains) for DYS and DOC1 mRNAs were markedly stronger in dark testis compared to the light tissue (Figure 2) and were concentrated over the seminiferous tubules. In contrast, an intense accumulation of signal for ODF2 and PDE3B mRNAs (Figure 3) was found

in the light tissue over maturing germ cells (Figure 3). Thus, in situ hybridization confirmed the differential expression between dark and light testis tissue for 4 genes analyzed from the 93 genes listed in the Table and Northern blot hybridization.

## Discussion

A major finding of these studies was that there are few changes in gene expression between light and dark testicular parenchyma of the 9132 genes evaluated. The 93 genes identified by the microarrays as being potentially differentially expressed comprised only 1.3% of those analyzed. The use of matched tissues from the same horse testis provided a "low noise" model system that dem-

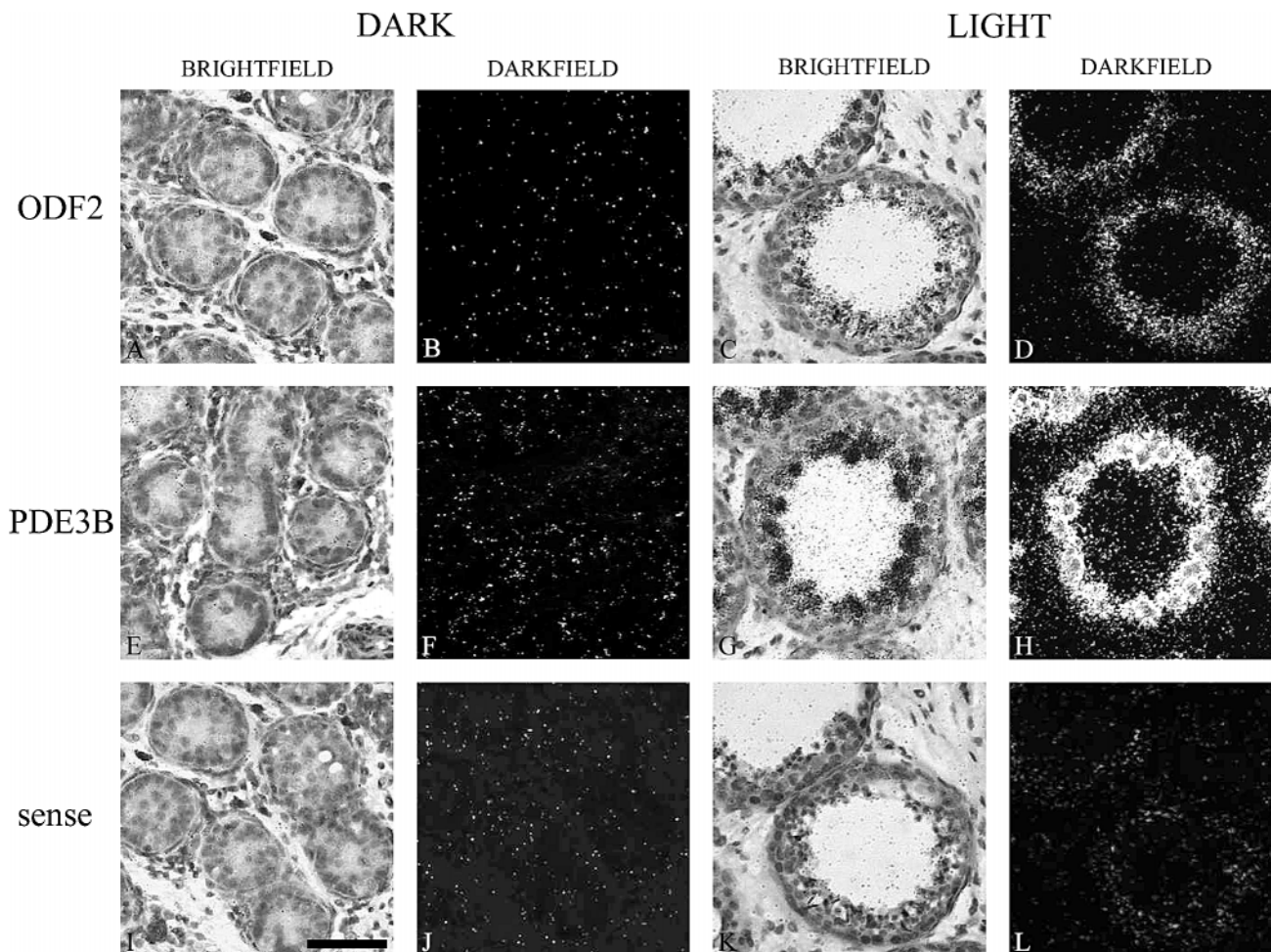


Figure 3. In situ hybridization localizes the expression of outer dense fiber of sperm tails 2 (ODF2) and phosphodiesterase 3B (PDE3B) mRNA in developing male germ cells. Representative views of adjacent cross sections of a testis hybridized with radiolabeled cRNA probes for ODF2 and PDE3B mRNAs. The ODF2 gene is primarily expressed in round and elongated spermatids (**Panel D**), whereas PDE3B mRNA demonstrates very high concentration in mature spermatocytes and round spermatids (**Panel H**). Tissue sections pictured in **Panels I–L** were hybridized to sense ODF2 cRNA (a negative control) and demonstrate the low level of nonspecific hybridization. The bar in **Panel I** indicates 100  $\mu\text{m}$ .

onstrates how few changes in gene expression are concurrent with the initiation of spermatogenesis. Similar results on reproducibility and sensitivity of these types of microarrays have been reported (Yue et al, 2001; Moody et al, 2002; Reynolds, 2002).

The large number of gene products analyzed on the microarrays provided an unbiased and feasible method of identifying genes that are regulated during the initiation of spermatogenesis. The microarray data yielded expected results that validated the analyses. For example, AR and other genes analyzed previously were not differentially expressed between dark and light tissues of the horse testes. However, we were able to identify genes regulated during the initiation of spermatogenesis, including DYS, DOC1, ODF2, and PDE3B.

The genes preferentially expressed in dark testis tissue included DYS and DOC1. DYS is a protein involved in scaffolding and aids in membrane repair between intra-

cellular and extracellular components (Ellis, 2003). Mutations result in the human autosomal recessive limb girdled muscular dystrophy disease. A DYS homolog, the FER-1 gene found in *Caenorhabditis elegans* (Britton et al, 2000), is expressed in spermatid vesicles, and FER-1 mutants produced sperm that were abnormal and were infertile (Achanzar and Ward, 1997). Results demonstrate that DYS gene expression is predominately in spermatogenically inactive tubules that do not have a full complement of germ cells. It is plausible that DYS serves as part of a priming mechanism for spermatogenesis within the seminiferous tubules, enabling them to repair themselves after lumen expansion and compaction during waves of divisions.

The DOC1 gene, a candidate tumor suppressor gene with a myosin-like product, was also found to be more highly expressed in dark tissue. Interestingly, DOC1 is expressed by normal ovarian epithelial cells but not by

ovarian cancer cell lines (Mok et al, 1994). Other than involvement with cytoskeletal and adhesion molecules like that of *DYS*, its role in the initiation of spermatogenesis is still unclear.

Expression of *ODF2* and *PDE3B* genes is predominant in the spermatogenically active horse testis tissue and is well described in other species, including the mouse, rat, and human (Hoyer-Fender et al, 1998; Wiersma et al, 1998; Peterson et al, 1999; Nakagawa et al, 2001). The *ODF2* gene is expressed exclusively in the testis and is part of the cytoskeletal structure of the sperm tail. The function of *ODF2* involves maintaining elastic recoil and providing protection from shear forces during sperm transport in the female reproductive tract. Equine *ODF2* mRNA was localized to mature tubules with round and elongated spermatids, which coincides with the results of other studies in the rat and bull (Schalles et al, 1998). *PDE3B* also showed a definite expression difference in light tissue in all experiments conducted and functions to regulate cAMP levels. In the mammalian ovary, meiotic arrest of oocytes is regulated by cAMP levels. *PDE3B* acts to decrease these levels, and when inhibited with products such as hypoxanthine and milrinone, cAMP levels continue to increase and prohibit maturation (Wiersma et al, 1998). Also, *PDE3B* acts as a mediator in the insulin pathway (Harndahl et al, 2002). *PDE3B* mRNA was expressed only in secondary spermatocytes and round spermatids, but not in elongated spermatids. These results imply that *PDE3B* may play an important role in meiotic division in the testis. Other genes discovered by the microarrays that have greater expression levels in spermatogenically active light testis tissue specific to developing male germ cells include protamine, calmegin, tubulin  $\alpha 1$ , and phosphorylase kinase  $\gamma 2$  (Amat et al, 1990; Prigent et al, 1996; Mochida et al, 1998; Yoshinaga et al, 1999; Steger et al, 2001).

Upon analysis, it is obvious that cross-species hybridization in cDNA microarrays is a strong tool to elucidate important genes involved in spermatogenesis. Although initial predictions of which hormone receptors and paracrine factors would be regulated during the initiation of spermatogenesis did not prove to be true, other genes were identified by the microarray analyses. One example is the *CBP/p300* transactivator that participates in steroid hormone receptor and *c-fos* transactivation pathways and was preferentially expressed in light testis tissue (Fronsdal et al, 1998). Another example is the identification of 3 genes in the insulin-like growth factor–signaling system, which is consistent with the proposed role of this system in testis development and seasonal induction of spermatogenesis (Achanzar and Ward, 1997; Fiszler and Kurpisz, 1998; Liu et al, 1998; Wagoner et al, 2000).

Other groups have indicated that some of the gene products identified here are important to the biology of

the testis (Yu et al, 2003). For example, *ODF2*, cholecystokinin, cyclin A1, calmegin, glucose transporters, and stathmin, predominately expressed in light testis tissues, are involved in meiosis and developing male germ cells (Persson et al, 1989; Weiss et al, 1997; Angulo et al, 1998; Flores et al, 1998; Ohsako et al, 1998; Rouiller-Fabre et al, 1998; Schalles et al, 1998; Bushby, 1999; Ravnik and Wolgemuth, 1999; Yoshinaga et al, 1999; Doege et al, 2000; Muller et al, 2000; Tsuruta et al, 2000; Guillaume et al, 2001). Additionally, placental cadherin and laminin are dark testis tissue–specific gene products that others have localized to peritubular cells and the basement membranes of seminiferous tubules, respectively (Virtanen et al, 1997; Johnson et al, 2000). Importantly, altered expression of *DYS*, kinesin, laminin, and protamine genes has been associated with reductions in male fertility and spermatogenesis in man and other species (Boekelheide et al, 1989; Lee et al, 1995; Virtanen et al, 1997; Mochida et al, 1998; Yoshinaga et al, 1999; Cho et al, 2001; Steger et al, 2001).

In conclusion, this use of human cDNA microarrays to study gene expression in the horse testis was highly successful. These Incyte microarrays have generated data in both human and animal systems that have proven to be reproducible and reliable (Pomp et al, 2001; Yue et al, 2001; Moody et al, 2002). The success of the technique is due to the high level of sequence conservation between horse and human mRNAs. Cross-species hybridization is common in Northern blot and in situ hybridization analyses, as demonstrated here even for species as diverse as rat and horse. Thus, microarrays composed of long human cDNAs (not oligonucleotides) provide a valuable tool for gene expression analyses in domestic animal species. Genetic analysis of equine testis was uniquely powerful in generating a nonbiased list of new gene targets to explore in the developing testis of the prepubertal colt. By understanding the regulation of the identified gene products within testicular cells of immature and mature stallions of high and low sperm, fertility parameters may elucidate the gene networks critical to efficient spermatogenesis as well as novel therapeutic approaches for improving male fertility.

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