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## INVITED ORIGINAL ARTICLE

Germ Cell Biology

# Dynamic changes in the expression of apoptosis-related genes in differentiating gonocytes and in seminomas

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Apoptosis is an integral part of the spermatogenic process, necessary to maintain a proper ratio of Sertoli to germ cell numbers and provide an adequate microenvironment to germ cells. Apoptosis may also represent a protective mechanism mediating the elimination of abnormal germ cells. Extensive apoptosis occurs between the first and second postnatal weeks, at the point when gonocytes, precursors of spermatogonial stem cells, should have migrated toward the basement membrane of the tubules and differentiated into spermatogonia. The mechanisms regulating this process are not well-understood. Gonocytes undergo phases of proliferation, migration, and differentiation which occur in a timely and closely regulated manner. Gonocytes failing to migrate and differentiate properly undergo apoptosis. Inadequate gonocyte differentiation has been suggested to lead to testicular germ cell tumor (TGCT) formation. Here, we examined the expression levels of apoptosis-related genes during gonocyte differentiation by quantitative real-time polymerase chain reaction, identifying 48 pro- and anti-apoptotic genes increased by at least two-fold in rat gonocytes induced to differentiate by retinoic acid, when compared to untreated gonocytes. Further analysis of the most highly expressed genes identified the pro-apoptotic genes *Gadd45a* and *Cycs* as upregulated in differentiating gonocytes and in spermatogonia compared with gonocytes. These genes were also significantly downregulated in seminomas, the most common type of TGCT, compared with normal human testicular tissues. These results indicate that apoptosis-related genes are actively regulated during gonocyte differentiation. Moreover, the down-regulation of pro-apoptotic genes in seminomas suggests that they could represent new therapeutic targets in the treatment of TGCTs.
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## INTRODUCTION

Spermatogenesis is a dynamic process regulating the formation of millions of spermatozoa daily throughout the adult lifetime of males. At the base of spermatogenesis are spermatogonial stem cells (SSCs), which constitute a reservoir of cells among which some cells self-renew to maintain an adequate stem cell pool, while others enter the spermatogenic cycle to produce sperm.[1] SSCs are formed from neonatal precursor cells, the transitional gonocytes (also known as pre- and pro-spermatogonia).[1,2] We have previously shown that rat postnatal day 3 (PND3) gonocytes undergo proliferation in response to platelet-derived growth factor (PDGF)-BB and 17β-estradiol via activation of the PDGF receptor (PDGFR), the estrogen receptor (ER), and the MEK/ERK pathway.[3,4] Furthermore, we have shown that gonocytes undergo differentiation when stimulated with all-*trans*-retinoic acid (RA).[5,6] Because gonocyte differentiation requires their migration and relocation to the basement membrane of the seminiferous tubules[7–9] and possibly Sertoli cell-secreted factors other than RA, *in vitro* differentiation is not complete. Nonetheless, it is a convenient tool to study aspects of gonocyte differentiation, such as the upregulation of spermatogonial markers and activation of

signaling pathways. Using this model, we have shown that the activation of PDGFR, SRC, JAK2, and STAT5 pathways is required for gonocyte differentiation.[10] Because germ cells are the repository and vessel by which genetic material gets transferred throughout generations, it is critical that abnormal germ cells get eliminated to prevent intergenerational transfer of defective genome sequences. Apoptosis represents an important means of controlling germ cell quality by allowing the removal of defective germ cells.[11] Indeed, gonocytes that fail to migrate to the basement membrane of the seminiferous cords, where they can complete differentiation, by PND 5–8, undergo apoptosis and are eliminated.[1,12] While the failure of eliminating abnormal gonocytes could jeopardize the quality of the SSC pool, it could also lead to the retention of defective gonocytes implicated in the formation of human testicular germ cell tumors (TGCTs).[13] In this context, it is important to understand not only the mechanisms supporting the gonocyte development, but also those controlling the apoptosis of deficient cells.

Apoptosis is also necessary to maintain adequate ratios of germ to Sertoli cell numbers because Sertoli cells can only support a limited number of germ cells, and thus, any excess of germ cells must be

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eliminated.[14,15] Few studies have examined the mechanisms regulating gonocyte apoptosis, looking at several typical pro- and anti- apoptotic factors. Transforming growth factor-β was shown to induce apoptosis in fetal gonocytes and prepubertal germ cells, whereas its effect on PND3 rat gonocyte apoptosis was minimal.[16–18] Furthermore, FAS, a transmembrane receptor of the tumor necrosis factor receptor family (also called APO-1) known to play a role in germ cell apoptosis,[11,19] is present in gonocytes and its ligand, FASL, is secreted by Sertoli cells.[11,18] Overexpression of anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) in mice was found to inhibit germ cell apoptosis in young mice, whereas in older mice, Bcl-2 overexpression leads to an increase in germ cell apoptosis and sterility.[1,12,20] Finally, the deficiency of the pro-apoptotic factor BAX in mice led to decreased germ cell apoptosis at PND5 and PND15 and disrupted spermatogenesis, further stressing the importance of apoptosis in this process.[21,22]

Interestingly, RA, which induces neonatal gonocyte differentiation, was shown to regulate other processes in fetal (but not neonatal) gonocytes, including a positive effect on fetal gonocyte proliferation and the induction of fetal rat and human gonocyte apoptosis.[23,24]

The goal of the present study was to identify novel pro- and anti-apoptotic genes differentially expressed during the transition from gonocytes to spermatogonia, and to determine if any of these genes were dysregulated in germ cell tumors, as a way to broaden our understanding of germ cell apoptosis and to find novel targets in the treatment of TGCTs.

## MATERIALS AND METHODS

### Animals
PND3 and PND8 male Sprague Dawley rats were purchased from Charles Rivers Laboratories (Saint-Constant, QC, CA). Pups were euthanized and handled according to protocols approved by the McGill University Health Centre Animal Care Committee and the Canadian Council on Animal Care.

### Germ cell isolation/treatment
Germ cells were isolated from 30 to 40 PND3 (and 10 PND8) rat testes per preparation as previously described.[5,6,25,26] In summary, germ cells were isolated using sequential enzymatic tissue dissociation. The resulting mixture of germ cells and somatic Sertoli and myoid cells was then plated overnight for further separation, followed by a 2%–4% bovine serum albumin (BSA) (Roche Diagnostics, Indianapolis, IN, USA) gradient. A final purity of at least 95% was used for gene array analysis and of 85% for other types of analyses. In some of the experiments, isolated PND3 gonocytes were treated with or without $10^{-6}$ M all-*trans*-RA (Sigma-Aldrich, Oakville, ON, CA) and 2.5% fetal bovine serum (FBS) (Invitrogen, Burlington, ON, CA) for 24 h for mRNA analysis, and 72 h for protein analysis. Samples were frozen or fixed in paraformaldehyde for later analyses. All experiments were performed using a minimum of three independent germ cell preparations.

### Human testicular tissue/ethical approval
Human testicular tissues (both normal and tumoral specimen) for quantitative polymerase chain reaction (qPCR) analysis was generously provided by Dr. Peter Chan (Department of Surgery, Division of Urology, McGill University, Montreal, QC, CA) or extracted RNA was purchased from Oncomatrix (San Marcos, CA, USA). Samples were obtained under supervision of the institutional ethics review boards, and informed consent was obtained from all sample providers.

### RNA extraction and cDNA synthesis
Total RNA was extracted using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) and digested with DNase I (Qiagen, Valencia, CA, USA). For human samples, the RNeasy Protect Mini Kit (Qiagen) was used. cDNA was synthesized using the single strand cDNA transcriptor synthesis kit (Roche Diagnostics).

### Apoptosis genes profiling arrays
As a first screening for genes of interest, the mRNA expression levels of genes related to apoptosis were determined in control and RA-treated gonocyte suspensions, using Rat Apoptosis RT² Profiler PCR Array (Qiagen) analysis. Each array plate included probes for 84 genes known to be involved positively or negatively in the process of apoptosis, various housekeeping genes, and internal standards. The reactions were run using the following protocol: an initial step at 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 60 s. The data obtained were normalized using the built-in reference gene that was the least altered between RA-treated and control cells. Genes that were significantly changed between C and RA-treated gonocytes by at least two-fold were further analyzed using the DAVID Bioinformatics Resources version 6.7 software (NIH, Bethesda, MD, USA) and its built-in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Gene array data were also analyzed using the 'search tool for the retrieval of interacting genes/proteins (STRING 9.05; Software developed, in part, by the Novo Nordisk Foundation Center for Protein Research (NNF CPR), the European Molecular Biology Laboratory (EMBL), and the Swiss Institute of BioInformatics (SIB)) for functional partners.[27] Candidate genes were further analyzed using BioGPS, a gene annotation portal system available at biogps.org.[28] Only genes that presented Cp above 35 in at least one of the conditions were considered in further experiments.

### Gene expression array analysis
Rat Illumina and human Affymetrix microarray analyses were performed by the McGill University's Genome Quebec facility as previously described.[6] Briefly, RNA extracts from rat PND3 gonocytes and PND8 spermatogonia (three independent preparations for each age) were analyzed using the RatRef-12 Expression BeadChip for genome-wide expression analysis (Illumina, San Diego, CA, USA). Gene expression in the corresponding somatic cell fractions (containing 70% Sertoli cells and 30% myoid cells) were also analyzed as a comparison (two independent preparations per age). The array chip contains 22,523 probes selected primarily from the NCBI RefSeq database. Human samples (normal testicular tissue $N = 3$, seminoma $N = 3$, embryonal carcinoma $N = 2$, unknown pathology = 1, and Tcam−2 cells $N=2$) were analyzed using the HuGene-1_0-st-V1 array chip (Affymetrix, Santa Clara, CA, USA). This chip contains 33,297 probes selected mainly from the NCBI RefSeq database. Data normalization was performed by Novak *et al.*[29] Apoptosis-related genes were found by comparing already existing lists provided by Qiagen.

### Quantitative real-time polymerase chain reaction
Quantitative polymerase chain reaction was performed using a SYBR Green PCR Master Mix kit (Roche Diagnostics) on a LightCycler 480 (Roche Diagnostics). Specific primers were designed using the Roche primer design software (Roche Diagnostics) (**Table 1**). qPCR cycling conditions were: an initial step at 95°C, followed by 45 cycles at 95°C for 10 s, 61°C for 10 s, and 72°C for 10 s. 18S rRNA was used as a housekeeping gene. Assays were performed in triplicate. All experiments were performed using a minimum of three independent sample preparations.

### Immunohistochemistry
Protein expression of cleaved caspase 3 (*Casp3*) and cleaved *Casp9* was determined using formalhyde-fixed, paraffin-embedded sections of

**Table 1: List of primer sets used for quantitative real-time PCR analysis**

| Genes | Accession number | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|---|---|---|---|
| Rat | | | |
| Anxa5 | NM_013132.1 | GAAGGCCATGAAAGGCTTG | CGGGCTGTCAACAGGTTC |
| Api5 | NM_001127379.1 | CCCAGTGGAAGGTCTGGATA | CCATGTCACCACAAAATGAACT |
| Birc2 | NM_021752 | GAAAATGCTGACCCTCCAGT | TCATCACTGCATCTTCCCAAT |
| Casp2 | AF136231 | CACAGGAAGGGGCTGATG | TCAGGATGCATTCCACACAC |
| Casp8ap2 | NM_001107921.1 | TGGAGAGCTCATGTACAATTCC | GAGTTGAATTGGATGGAAACTCA |
| Cycs | NM_012839.2 | GATGCCAACAAGAACAAAGGT | TGGGATTTTCCAAATACTCCAT |
| Dad1 | NM_138910.2 | TCCTTACCGTGGAGTTGGAG | TCTGATCCAGGAGACGTCAA |
| Gadd45a | NM_024127.2 | CAGAGCAGAAGATCGAAAGGA | CTCGTACACGCCGACAGTTA |
| Mapk1 | NM_053842.1 | GGGTTCTTGACAGAGTATGTAGCC | ACCAAATATCAATGGACTTGGTATAA |
| Mcl1 | NM_021846.2 | AGAAATGTGCTGCTGGCTT | GTTGGTGGCTGGAGGTTTT |
| Prd×2 | NM_017169.1 | GACTCTCAGTTCACCCACCTG | TATTCAGTGGGCCCAAGC |
| STRA8 | XM_575429.2 | TGCTTTTGATGTGGCGAGCT | GCGCTGATGTTAGACAGACGCT |
| 18S | X01117.1 | cgggTGCTCTTAGCTGAGTGTCCcG | CTGGGCCTGCTTTGAACAC |
| Human | | | |
| Anxa5 | NM_001154.3 | GCCACCTCTCTTTATTCCATGA | TGACACGTTAGTCATCTTCTCCA |
| Api5 | NM_001142930 | TACCCCAGTGGAAGGTCTTG | GCCCAACTGGTGAAAACTGT |
| Birc2 | NM_001166.3 | AAAATGCTGACCCACCAATTA | GGTGTATTCATCATGACAGCATCT |
| Casp2 | NM_032982.3 | GGGGTCTTGGTCCACCTT | GCCACACACTCCCAATATCC |
| Casp8ap2 | NM_012115.3 | CCAGAGAGTCGGAGGGAGT | TTCCTTTTGAGAGTCACCACCT |
| Cycs | NM_018947.5 | TGTGCCAGCGACTAAAAAGA | CCTCCCTTTTCAACGGTGT |
| Dad1 | NM_001344.2 | GCGTCTGAAGTTGCTGGAC | ACGAGGAGACAGTAACCGAACT |
| Gadd45a | NM_001924.3 | TTTGCAATATGACTTTGGAGGA | CATCCCCCACCTTATCCAT |
| Mapk1 | NM_002745.4 | CAAAGAACTAATTTTTGAAGAGACTGC | TCCTCTGAGCCCTTGTCCT |
| Mcl1 | NM_021960.4 | AAGCCAATGGGCAGGTCT | TGTCCAGTTTCCGAAGCAT |
| Prdx2 | NM_005809 | CAGACGAGCATGGGGAAG | ACGTTGGGCTTAATCGTGTC |
| STRA8 | NM_182489.1 | cgctgGGACCTTCTGACTGGCAGcG | CTCCGAGAGGTTCTGCCACAG |
| 18S | NR_003286.2 | cggacTGTGATGCCCTTAGATGTCcG | GTAGGTAGGCACACGCTGAG |

PCR: polymerase chain reaction

PND3 and PND8 testes. As previously described, slides were dewaxed and rehydrated using Citrosolv (Fisher Scientific, Toronto, ON, CA) and Trilogy solution (Cell Marque IVD, Rocklin, CA, USA).[6,30] Following treatment with Dako Target Retrieval solution (DAKO, Burlington, ON, CA), the sections were incubated with PBS (Invitrogen) containing 10% goat serum (Vector Laboratories, Burlington, ON, CA), 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega, Madison, WI, USA) for 1 h to block nonspecific protein interactions. Sections were then incubated with cleaved *Casp*3 antibody (Cell Signaling, Whitby, ON, CA) or cleaved *Casp*9 antibody (Cell Signaling) diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) overnight at 4°C. The sections were then incubated with biotin-conjugated goat anti-rabbit secondary antibody (BD Pharmingen, Mississauga, ON, CA) diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) for 60 min at room temperature. Immunoreactivity was detected using streptavidin-peroxidase (Invitrogen) and AEC single use solution (Invitrogen). Sections were counter-stained with hematoxylin (Sigma-Aldrich) and processed for cover-slipping. Slides were examined under bright-field microscopy with a BX 40 Olympus microscope (Olympus, Center Valley, PA, USA) coupled to a DP70 Olympus digital camera (Olympus). Negative controls were done by incubating some sections with Rabbit IgG (preimmune rabbit serum) (Invitrogen) (data not shown).

### Immunocytochemistry
Microscopic slides of isolated gonocytes and spermatogonia were prepared by cytospin centrifugation of germ cells at lower purity, using tail fractions of the BSA gradient that contained Sertoli and myoid cells, since high purity was not required for this type of end-point, and the presence of somatic cells could potentially provide internal controls by showing the immunostainings of other cell types. The expression of cleaved *Casp*3 and *9* was examined by immunocytochemical analysis as previously described.[3,26] In brief, fixed cells were treated with Dako Target Retrieval solution and then blocked with PBS containing 10% goat serum, 1% BSA, and 0.02% Triton X100 for 1 h. The cells were then incubated with either cleaved *Casp3* or cleaved *Casp*9 antibody diluted in PBS containing 1% BSA and 0.02% Triton X100 overnight at 4°C, followed by a biotin-conjugated goat anti-rabbit secondary antibody and immunoreactivity detection as described above in the immunohistochemistry protocol.

### Statistical analysis
For qPCR results, statistical analysis was performed using an unpaired two-tail Student's *t*-test using statistical analysis functions in the GraphPad Prism 5.0 program (GraphPad Inc., San Diego, CA, USA). For all experiments, *N* was equal to a minimum of three independent experiments. A $P < 0.05$ was considered statistically significant.

## RESULTS

### Caspases 3 and 9-dependent apoptosis takes place in postnatal day 8 but not postnatal day 3 germ cells
As mentioned above, apoptosis is an integral part of the regulatory systems insuring proper germ cell development and spermatogenesis. Since germ cell apoptosis has been reported to take place mainly

between PND7 and 14 in rodents, we first examined the expression of activated *Casp3* and *9* in PND3 gonocytes and PND8 spermatogonia either at day 0, or in the case of gonocytes, after 3 days treatment with or without RA. The expression of cleaved *Casp3* and *9* was also examined in PND3 and PND8 testes sections to determine the *in vivo* levels of apoptosis at both developmental phases (**Figure 1a** and **b**). Using trypan blue uptake assays, we had previously shown that PND3 gonocytes viability was well maintained from days 0 to 3 in culture and that RA treatment did not affect cell viability.[5,6] The present data showed that there was no cleaved *Casp3* and *9* expression in isolated PND3 gonocytes in these conditions, in contrast with a large proportion of the somatic cells that were positive for both activated caspases (**Figure 1a**). Indeed, we had previously observed that most somatic cells would die after a few days in the low percent FBS (2.5%) used for germ cell cultures. The present data showed that activated *Casp3* and *9* were driving somatic cell death in these conditions. The immunohistological analysis of PND3 testis sections confirmed that there was no obvious gonocyte apoptosis at PND3, whereas there were strong cleaved *Casp3* and *9* signals in the interstitium, likely corresponding to the apoptosis of fetal-type Leydig cells, a population gradually disappearing from the testis after birth (**Figure 1a**, bottom panel). These data strongly contrasted with intense cleaved *Casp3* and *9* signals in germ cells of PND8 testes mainly positioned in the center of the seminiferous tubules, suggesting that these cells had failed to migrate to the basement membrane and to differentiate (**Figure 1b**, bottom panel). At this age, most fetal Leydig cells had already died out, and there were little-cleaved caspase immunoreactivities in the interstitium.
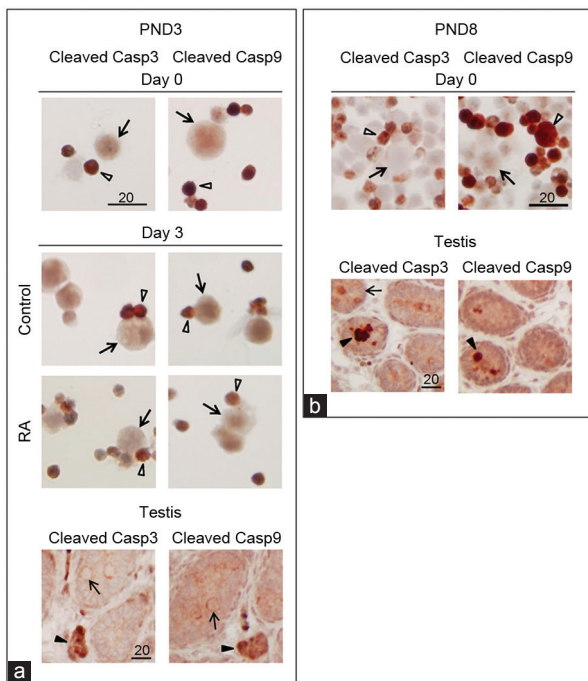
A small proportion of germ cells strongly positive for cleaved *Casp9* were observed in PND8 spermatogonial preparations, as well as a few germ cells weakly positive for cleaved *Casp3* (**Figure 1b**, top panel).

### Changes in the mRNA expression of apoptosis-related genes between control and retinoic acid -treated postnatal day 3 gonocytes

Considering that germ cell apoptosis occurred after gonocyte differentiation in cells that did not migrate and/or differentiate properly, and that only a few apoptosis-related genes had been shown to be involved in these processes, we then performed a comparative gene expression analysis between control and RA-induced differentiating gonocytes to identify new apoptosis-related genes that would be altered during gonocyte differentiation. We first performed a Qiagen RT[2] profiler PCR array analysis on cDNAs from gonocytes treated with or without RA as a screening tool (**Supplemental Table 1**). The on-going differentiation process in the RA-treated gonocyte pool was confirmed by a four-fold increase in the mRNA expression of the spermatogonial marker *Stra8* as expected from previous studies.[5,6] In the arrays, 84 genes most commonly associated with pro- or anti-apoptosis responses were analyzed, alongside housekeeping genes and internal array standards. The data showed that 48 genes presented ≥ two-fold gene expression changes in RA-treated gonocytes compared to controls (**Figure 2a**). Interestingly, the majority of the 84 genes analyzed were more highly expressed in RA-treated gonocytes than in untreated cells, as seen by the heat map (**Figure 2b**) and scatter plot (**Figure 2c**).

To better understand the functional relationships between these genes, we performed KEGG pathway analysis (**Figure 3**), which showed that the altered genes were part of the core network of genes driving apoptosis. The genes upregulated during RA-induced gonocyte differentiation included a majority of pro-apoptotic genes corresponding mainly to the extrinsic apoptotic pathway, but also
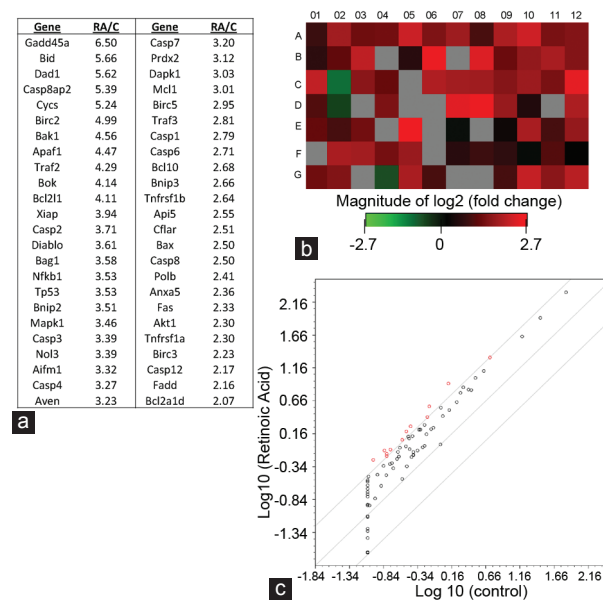


**Figure 1:** Expression of activated caspases (*Casp*) *3* and *9* in postnatal day 3 (PND3) gonocytes and PND8 spermatogonia. Activated caspases were visualized by immunocytochemical and immunohistochemical analyses. (**a**) PND3 gonocytes were either fixed after cell isolation (day 0) or cultured for 3 days with or without retinoic acid ($10^{-6}$ M) and collected on cytospin slides after fixation. Bottom panel: immunohistochemical analyses of *Casp3* and *9* in PND3 testes. (**b**) Immunocytochemical analysis of PND8 spermatogonia at day 0. Bottom panel: immunohistochemical analyses of *Casp3* and *9* in PND8 testes. Representative pictures are shown. Scale = 20 μm. Arrows: germ cells. Arrowheads: apoptotic (cleaved-caspase positive) cells.



| Gene | RA/C | Gene | RA/C |
|------|------|------|------|
| Gadd45a | 6.50 | Casp7 | 3.20 |
| Bid | 5.66 | Prdx2 | 3.12 |
| Dad1 | 5.62 | Dapk1 | 3.03 |
| Casp8ap2 | 5.39 | Mcl1 | 3.01 |
| Cycs | 5.24 | Birc5 | 2.95 |
| Birc2 | 4.99 | Traf3 | 2.81 |
| Bak1 | 4.56 | Casp1 | 2.79 |
| Apaf1 | 4.47 | Casp6 | 2.71 |
| Traf2 | 4.29 | Bcl10 | 2.68 |
| Bok | 4.14 | Bnip3 | 2.66 |
| Bcl2l1 | 4.11 | Tnfrsf1b | 2.64 |
| Xiap | 3.94 | Api5 | 2.55 |
| Casp2 | 3.71 | Cflar | 2.51 |
| Diablo | 3.61 | Bax | 2.50 |
| Bag1 | 3.58 | Casp8 | 2.50 |
| Nfkb1 | 3.53 | Polb | 2.41 |
| Tp53 | 3.53 | Anxa5 | 2.36 |
| Bnip2 | 3.51 | Fas | 2.33 |
| Mapk1 | 3.46 | Akt1 | 2.30 |
| Casp3 | 3.39 | Tnfrsf1a | 2.30 |
| Nol3 | 3.39 | Birc3 | 2.23 |
| Aifm1 | 3.32 | Casp12 | 2.17 |
| Casp4 | 3.27 | Fadd | 2.16 |
| Aven | 3.23 | Bcl2a1d | 2.07 |

**Figure 2:** Genes overexpressed in differentiating gonocytes. (**a**) List of genes overexpressed in retinoic acid (RA)-treated gonocytes compared to untreated cells by at least two-fold. (**b**) Heat map showing the range of fold changes when analyzing RA-treated gonocytes compared to untreated cells. (**c**) Scatter plot showing genes over- and under-expressed in RA-treated gonocytes compared to untreated cells. Red circles indicate genes overexpressed by at least four-fold whereas black circles indicate a fold change of over two-fold.
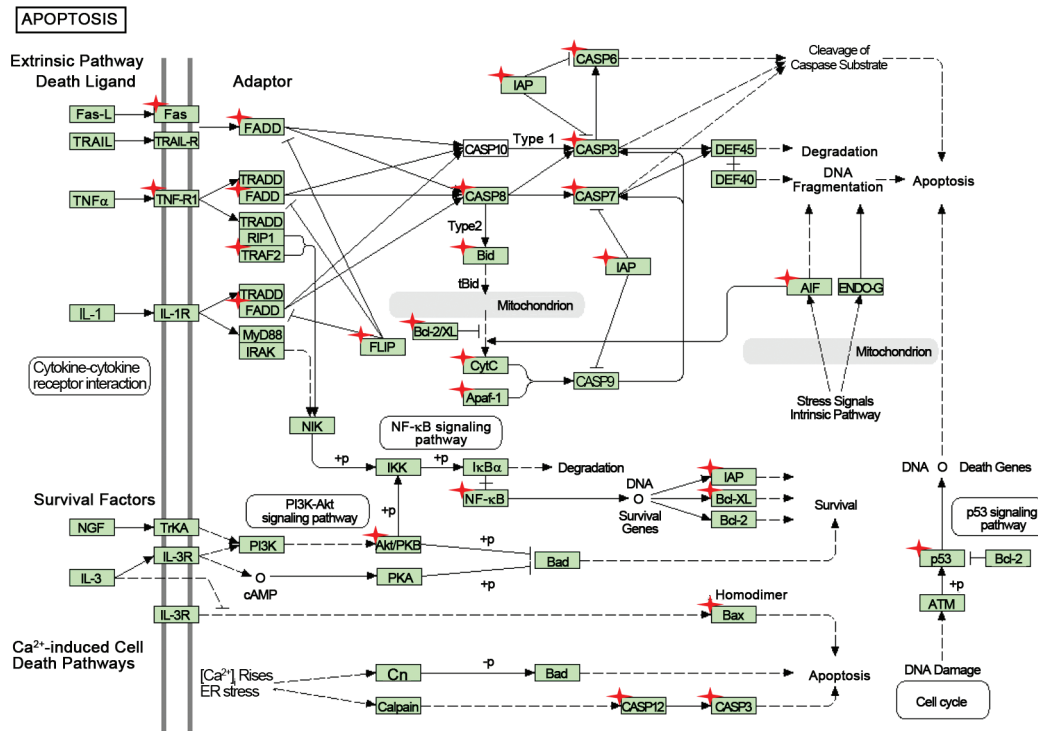
**Figure 3:** Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the apoptosis pathway. The KEGG pathway analysis of genes identified by gene profiling arrays is shown. Highlighted by the red stars are genes that belong to this known apoptosis pathway that were also a part of the 48 genes overexpressed in retinoic acid-treated gonocytes (compared to untreated cells). Adapted from DAVID Bioinformatics Resources version 6.7 software.

**Table 2: Expression of apoptosis-related genes in germ cells measured with gene arrays**

| Symbol | Accession number | Gene expression per cell type (mean±SEM) | | | |
|---|---|---|---|---|---|
| | | G3 | G8 | S3 | S8 |
| Gadd45a | NM_024127.1 | 542.4±34.8 | 939.0±108.0 | 162.8±4.0 | 243.8±38.6 |
| Dad1 | NM_138910.2 | 484.0±32.7 | 531.4±17.3 | 471.3±6.3 | 467.2±18.8 |
| Casp8ap2_pred | XM_232860.3 | 86.1±12.9 | 135.6±7.5 | 116.6±12.0 | 121.4±8.7 |
| Cycs | NM_012839.2 | 1032.1±61.4 | 1274.4±92.4 | 1622.6±262.8 | 1591.7±2.3 |
| Birc2 | NM_021752.1 | 359.3±55.4 | 403.3±14.1 | 410.4±52.6 | 275.6±3.9 |
| Bak1 | NM_053812.1 | 162.3±35.0 | 135.8±20.2 | 314.8±6.1 | 378.5±3.1 |
| Bok | NM_017312.2 | 356.4±35.7 | 339.2±36.8 | 298.4±53.0 | 329.3±27.8 |
| *Casp2* | NM_022522.2 | 480.5±50.9 | 637.7±35.1 | 379.7±5.6 | 382.0±38.5 |
| Bag1_pred | XM_216377.3 | 354.5±53.7 | 353.3±9.5 | 479.8±28.9 | 483.4±8.3 |
| Bnip2_pred | XM_217191.3 | 476.9±17.2 | 411.6±18.8 | 450.0±17.5 | 507.3±44.4 |
| Mapk1 | NM_053842.1 | 118.3±12.7 | 167.6±17.6 | 122.3±14.4 | 148.0±7.5 |
| *Casp7* | NM_022260.2 | 356.8±26.9 | 294.0±29.8 | 253.4±1.6 | 265.9±38.7 |
| Prdx2 | NM_017169.1 | 1177.8±192.1 | 1186.2±88.8 | 1690.8±10.2 | 1646.5±125.9 |
| Dapk1_pred | XM_225138.3 | 499.9±205.2 | 318.3±57.8 | 530.4±86.6 | 305.2±75.8 |
| Mcl1 | NM_021846.2 | 677.6±88.7 | 777.9±16.8 | 473.3±62.1 | 593.5±86.6 |
| Bcl10 | NM_031328.1 | 428.9±52.3 | 384.6±13.8 | 440.7±13.5 | 380.0±18.4 |
| Api5_pred | XM_342470.2 | 315.4±39.6 | 441.5±30.7 | 366.8±58.5 | 387.8±56.2 |
| Anxa5 | NM_013132.1 | 138.7±15.9 | 138.9±16.8 | 147.6±16.9 | 145.0±4.7 |
| Akt1 | NM_033230.1 | 114.6±12.3 | 88.9±4.2 | 156.8±4.9 | 158.3±3.0 |
| Tnfrsf1a | NM_013091.1 | 237.8±85.1 | 143.5±33.1 | 311.0±27.0 | 340.7±30.7 |

Rat gene array analysis was conducted using triplicate samples of isolated PND3 gonocytes (G3), PND8 spermatogonia (G8), and duplicates of the corresponding Sertoli/myoid cell extracts (S3, S8). 43 of the 48 genes upregulated in RA-treated gonocytes (compared to control cells) were represented in the array. Of those, 20 were present with abundance values>100. Pred: predicted; SEM: standard error of mean

several pro-survival genes. Similarly, STRING analysis showed that most of these genes clustered around *Casp3*, including several caspases, death receptors, and adaptors, but also the data highlighted the existence of a cluster of pro-survival genes around Akt, and few minor clusters in rats (**Figure 4**). *Bnip5*, *Birc2* (*XIAP*, *Hipk4*), and *Api5* were the only genes not previously reported to interact with any of the other genes
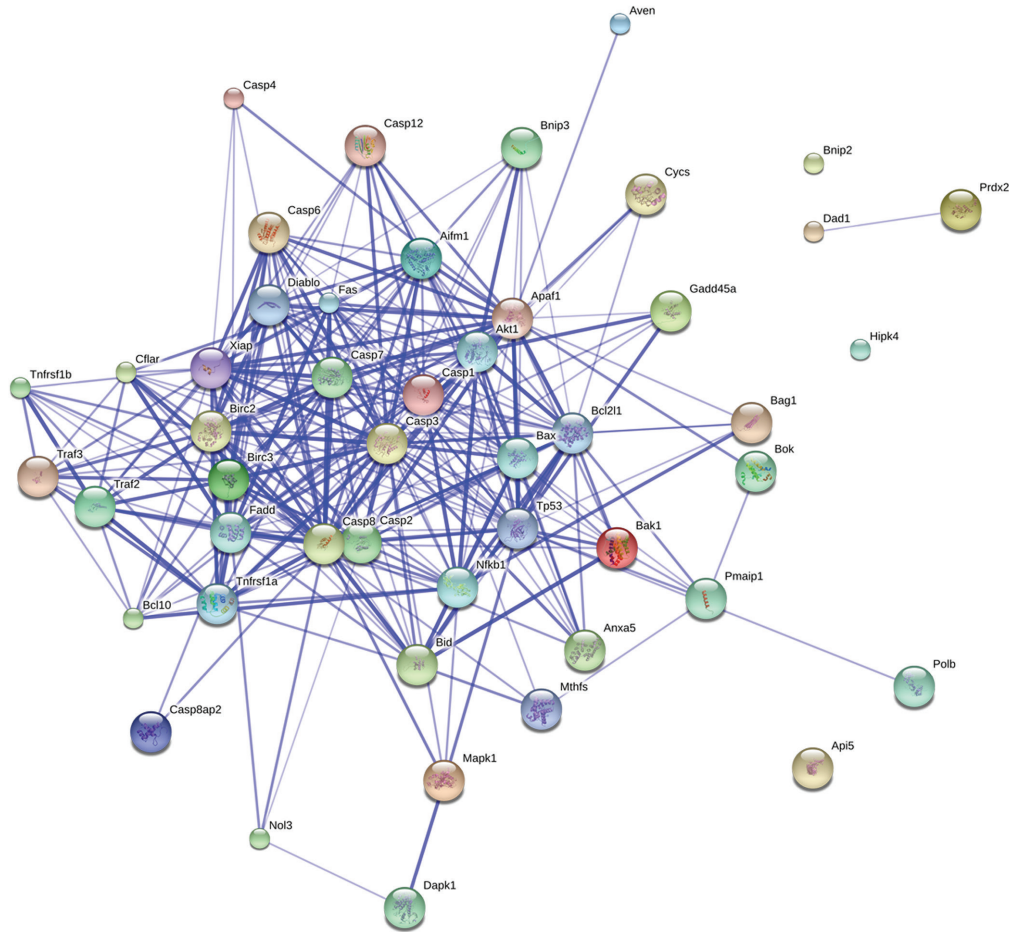
**Figure 4:** STRING analysis of predicted functional partners for genes upregulated in retinoic acid (RA)-treated gonocytes. STRING analysis of the 48 genes upregulated in RA-treated gonocytes (compared to untreated cells). Functional partner predictions were based on available experimental data, databases, text-mining, and homology. The thickness of the blue lines connecting various genes represents the level of confidence with which functional partners can be predicted.

in rat, while *Aven*, defender against cell death 1 (*Dad1*), *Prdx 2*, and *Polb* had minimal interacting partners, indicating a lack of knowledge on the function of these genes in rat. The increased expression of both pro- and anti- apoptotic genes in RA-treated gonocytes suggested that the balance was maintained, a likely requirement to prevent gonocyte apoptosis at this stage of development.

### Changes in the mRNA expression of apoptosis-related genes between postnatal day 3 gonocytes and postnatal day 8 spermatogonia

In order to further select genes related to the transition from gonocyte to spermatogonia among the 48 genes identified in the Profiler arrays, we examined the differential expression of apoptosis-related genes between PND3 gonocytes and PND8 spermatogonia using Illumina gene array analysis. Of the 48 genes found upregulated in RA-treated gonocytes, 43 were also present in the Illumina array at some level. Of those, 20 genes were highly expressed (relative abundance of at least 100) in either germ cell type, and 11 of these genes had slightly higher expression in PND8 spermatogonia than in gonocytes (**Table 2**). These genes were *Gadd45a*, *Dad1*, caspase 8 associated protein 2 (*Casp8ap2*), *Cycs*, *Birc2*, *Casp2*, *Mapk1*, *Prdx 2*, *Mcl1*, *Api5*, and *Anxa5*. Changes in these genes were further validated by qPCR analysis of PND3 gonocytes and PND8 spermatogonia. Although not significant probably due to spermatogonial heterogeneity, the spermatogonial marker *Stra8* showed a trend for higher expression in spermatogonia compared to gonocytes. Only few of the

genes showed significant changes between gonocytes and spermatogonia, including the anti-apoptotic gene *Birc2* and pro-apoptotic *Anxa5*, while others showed increasing or decreasing trends (**Figure 5**). The variability observed for some of the genes could be due to the fact that the cells are not developmentally synchronized, PND3 gonocytes representing a mixture of proliferative and quiescent neonatal germ cells, while PND8 germ cells correspond to a mixture of undifferentiated type A spermatogonia, including a subset of stem cells, committed progenitors (paired, aligned) and types A1 to A4 spermatogonia. Thus, changes occurring in only one specific type of germ cell could potentially be masked by the absence of such changes in other subsets of germ cells. Moreover, as shown in **Figure 1b**, PND8 germ cells also include gonocytes that failed to differentiate and are targeted for apoptosis.

We then examined by qPCR analysis whether these genes were altered during RA-induced gonocytes differentiation (**Figure 6**). Of these 11 genes, 3 were significantly upregulated in gonocytes treated with RA. This included the pro-apoptotic genes *Gadd45a and Cycs*, which were also increased in PND8 spermatogonia in comparison to gonocytes, suggesting that the induction of these genes might be regulated by RA. RA also increased the expression of the anti-apoptotic gene *Birc2* in differentiating gonocytes, suggesting again a balance between anti- and pro-apoptotic genes in differentiating gonocytes. *Stra8* mRNA levels were also increased by RA, confirming the induction of gonocyte differentiation (**Figure 6**).
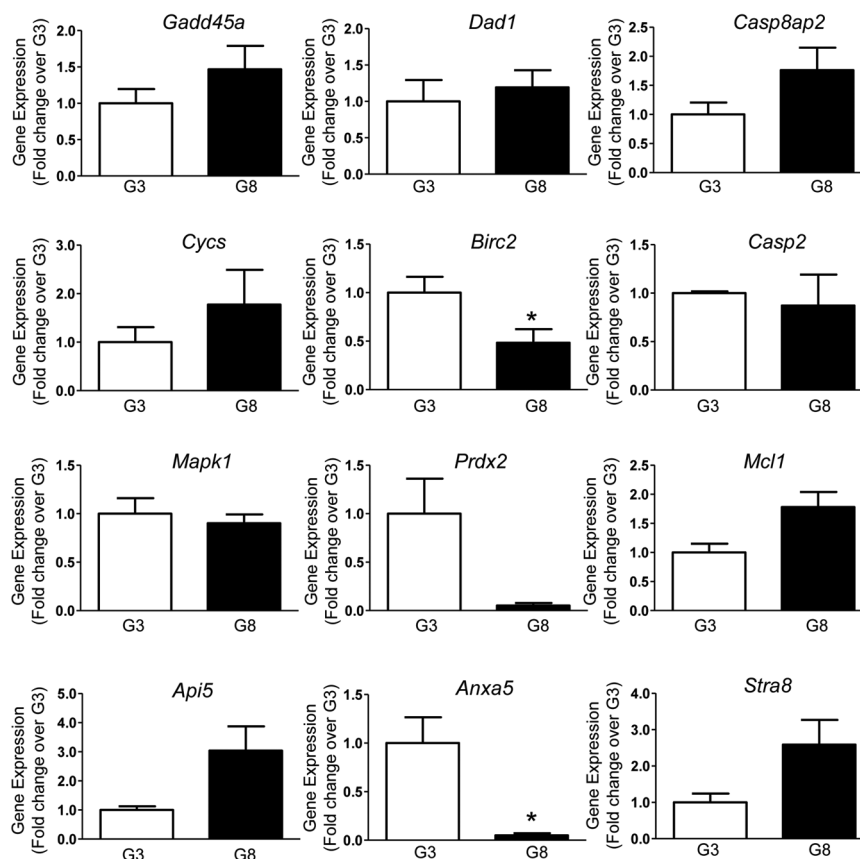
**Figure 5:** mRNA expression of apoptosis-related genes in postnatal day 3 (PND3) gonocytes and PND8 spermatogonia. mRNA levels of apoptosis-related genes were measured by quantitative polymerase chain reaction analysis in isolated PND 3 gonocytes (G3) and PND8 spermatogonia (G8). Results are fold change values over the expression levels in PND3 gonocytes and are expressed as means ± SEM of data obtained from 3 to 5 independent germ cell preparations (each containing 30–40 pups [PND3] and 10 pups [PND8]), with each sample performed in triplicate (*$P < 0.05$).

### Changes in the mRNA expression of apoptosis-related genes in seminomas compared to normal human testis specimen and rat germ cells

As previously mentioned, studies have suggested that an improper gonocyte development is at the origin of Carcinoma in Situ (CIS), the precursor stage of TGCTs.[13] Moreover, one of the required steps in tumor formation is the ability of transformed cells to evade apoptosis. Thus, having identified pro- and anti-apoptotic genes altered during the progression from gonocytes to spermatogonia, we examined if any of these genes were dysregulated in seminomas (the most common type of TGCTs) compared to normal testicular tissues. We found that five of the genes were significantly downregulated in seminomas, including *Gadd45a*, *Casp8ap2*, *Cycs*, *Birc2,* and *Dad1* (**Figure 7**). Potential interactions between the genes in human and rat were further examined by STRING analysis (**Figure 8**). In both species, *Cycs* and *Birc2* clustered together, with other apoptosis-related genes such as *DIABLO, Casp3, Casp7, Casp9, and XIAP* (*Birc4*). Interestingly, *Gadd45a* located in a separate gene cluster containing genes involved in DNA repair and replication, including *PCNA* and *FEN1* for both species, *Tp53* in rat and several replication factors such as *RFC1* in human. In addition, *Gadd45a* was shown to interact with several cell cycle proteins and kinases associated with mitosis and/or meiosis (e.g., Cdc2, Cdk1, Ccnb1) in rat (**Figures 4** and **8**).

Gene array analysis in PND3 and 8 rat testes showed that *Gadd45a* transcript was 3.3- and 3.9-fold more abundant in germ cells than in the corresponding Sertoli/myoid cell extracts, respectively; while the relative expression of the other genes, including *Cycs* and *Birc2* in germ cells was closer to their levels in Sertoli/myoid cells (**Table 2**). Gene array analysis showed that *Casp3* and *9* were not altered in gonocytes and spermatogonia, but that *Casp3* was significantly increased and *Casp9* significantly decreased in seminoma in comparison to normal human testicular tissues (**Supplemental Table 2**). The mRNA expression of *DIABLO* was not altered in rat germ cells or human samples while *XIAP* showed a small but not significant trend toward increase between normal testis and seminomas (**Supplemental Table 2**). In adult humans, available data from the gene annotation portal BioGPS (biogps.org) comparing the relative expression levels of these genes in various adult human tissues as well as within the testis, indicated a preferential expression of *Cycs* and *Birc2,* but not *Gadd45a,* in germ cells in comparison to the interstitium and Leydig cells (data not shown). Taken together with our results, these data suggest that these genes are differentially expressed in early postnatal and adult germ cells, with *Gadd45a* preferentially expressed in early postnatal germ cell development, while *Cycs* and *Birc2* appear to be more specific for adult germ cells.

### DISCUSSION

The present study analyzed the expression profiles of apoptosis-related genes between neonatal rat testicular gonocytes and type A spermatogonia, in order to identify genes that would be dynamically regulated during the transition from gonocytes to spermatogonia.
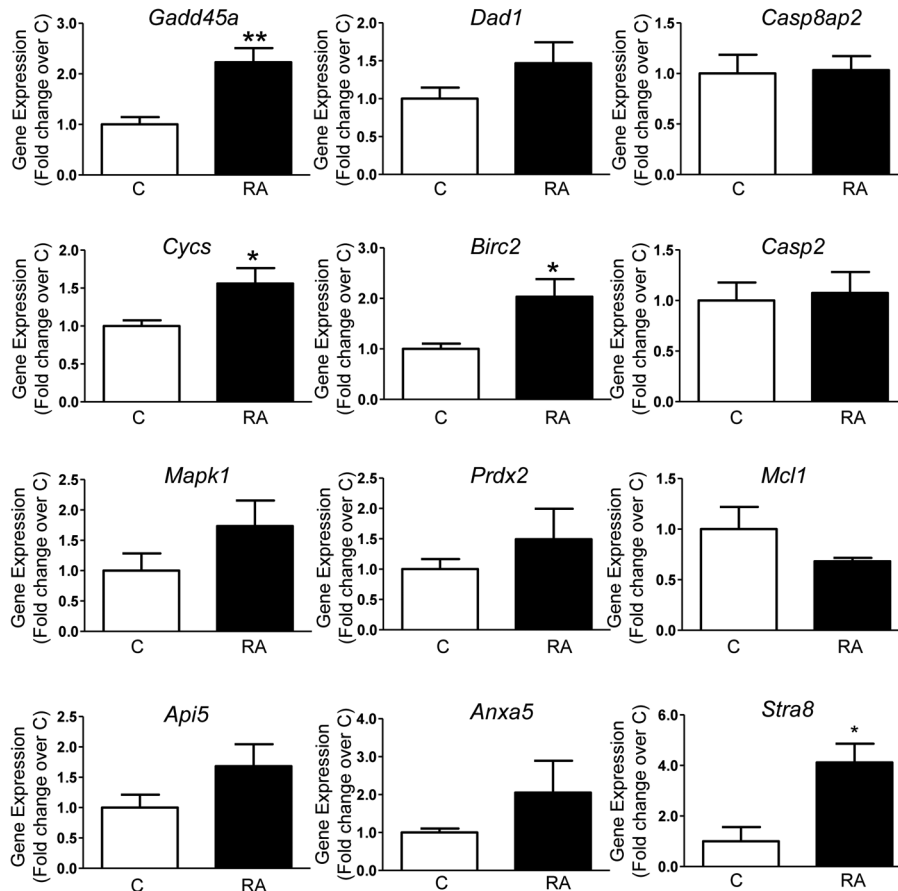
**Figure 6:** mRNA expression of apoptosis-related genes in gonocytes treated with or without retinoic acid (RA). mRNA expression of apoptosis-related genes was measured by quantitative polymerase chain reaction analysis of gonocytes treated with or without RA ($10^{-6}$ M). *Stra8* mRNA levels were also measured as an internal control for differentiation. Results are fold change values overexpression levels in untreated gonocytes and are expressed as means ± SEM of data obtained from 3 to 5 independent cell preparations, each sample performed in triplicate (*$P < 0.05$, **$P < 0.001$).

Our finding that *Casp3* and *9* were not active in PND3 gonocytes but were strongly activated in misplaced germ cells in PND8 testes is in agreement with published studies reporting that minimal apoptosis takes place before the end of the first postnatal week in rodents.[11,21] The absence of apoptosis in neonatal gonocytes at the periods during which they proliferate and initiate differentiation[1–6] is in sharp contrast with the active phases of apoptosis observed in fetal gonocytes (between gestation days 13 and 18 in rat), and during the 2nd week after birth.[1,31] It has been proposed that germ cells undergo apoptosis in order to maintain a ratio of Sertoli cells to germ cells ensuring an optimal microenvironment and nourishment from Sertoli cells to the germ cells.[32] Germ cell apoptosis is also believed to represent a "self-preservation" mechanism, by which improperly developed, chromosomally abnormal germ cells are eliminated, including defective fetal gonocytes and postnatal gonocytes that failed to become spermatogonia.[33,34]

In the present study, we used a Qiagen RT² Profiler array to screen among the 84 most common apoptosis-related genes (as determined by a Qiagen literature search), for genes that would present changes in expression levels during RA-induced gonocytes differentiation. As previously described by us and others, RA treatment recapitulates some aspects of gonocyte differentiation *in vitro*, such as to induce increased expression of *Stra8*.[5,6,35] Our results showed that RA treatment also up-regulated several known pro-apoptotic gene transcripts,

but that it did not trigger *Casp3* and *9* activation, suggesting the absence of apoptosis in gonocytes. Indeed, the simultaneous increase in anti-apoptotic gene transcripts in RA-treated cells suggests that gonocyte survival is insured by a balance between pro- and anti-apoptotic signals during gonocytes differentiation. In this context, an additional signal might be required to tilt the response toward survival or apoptosis, depending on the intra-tubule location and health of the cell at the end of the first postnatal week. The highly phagocytic Sertoli cells have been shown to play a role in germ cell apoptosis by secreting anti- and pro-apoptotic factors, and thus, they could be at the origin of such signal.[36]

Among the genes increased in RA-treated gonocytes, the pro-apoptotic gene *Gadd45a* (growth arrest and DNA-damage-inducible, alpha) is particularly interesting because it was preferentially expressed in germ cells and upregulated between the gonocyte and spermatogonia phases, suggesting that it might be a key regulator of apoptosis in juvenile spermatogonia. Moreover, the finding that *Gadd45a* was significantly decreased in seminomas suggests that its down-regulation might be one of the processes involved in the prevention of apoptosis in tumor cells. *Gadd45a* belongs to a family of small signaling proteins that can be regulated at both the transcriptional and posttranscriptional levels.[37,38] Originally cloned as one of many UV-induced genes in Chinese hamster ovary cells, *Gadd45a* is more commonly known as a stress sensor, positioned downstream of both p53 and *BRCA*, and leading to growth
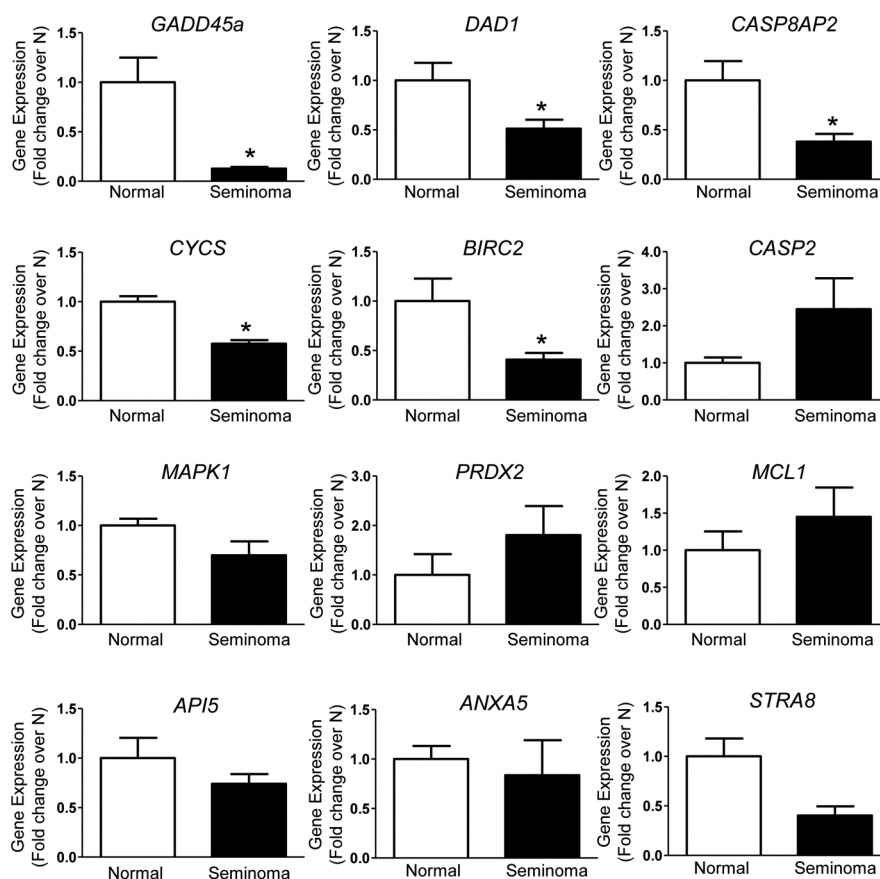
**Figure 7:** mRNA expression of apoptosis-related genes in normal testicular tissues and seminomas. The mRNA expression of apoptosis-related genes was measured by quantitative polymerase chain reaction analysis of normal human testicular tissues and seminomas. Results are fold change values overexpression levels in normal testicular tissue and are expressed as means ± SEM of data obtained from 3 to 4 independent human samples, each sample performed in triplicate (*$P < 0.05$).

arrest and/or apoptosis.[37–40] Similar to its common interacting partner p53, $Gadd45a^{-/-}$ mice have shown genomic instability, including aneuploidy and gene amplification, associated with the tendency to develop tumors following genotoxic stress.[37,41] Studies have also shown that $Gadd45a$ promotes DNA demethylation, whereas others have shown that this is not the case.[42,43] With regards to testicular development, although $Gadd45g^{-/-}$ mice have problems with sexual development, including male infertility and an intersex phenotype, $Gadd45a$ and $b^{-/-}$ mice did not have problems with testis development or sex determination.[44] While $Gadd45a$ has not been studied in testicular cancers, it is known to be involved in a variety of tumors. In breast cancer, $Gadd45a$ expression was found to be hormone receptor-dependent, being highly expressed in cells expressing ER and progesterone receptors (PR), but at low levels in triple-negative tumors (ER−, PR−, and Her2/Neu−).[45] This is interesting, considering that testis development and function, including spermatogenesis, are hormone-dependent processes. Our finding that $Gadd45a$ expression is greatly decreased in seminomas in comparison to normal testis is consistent with its role in other cancer types, where $Gadd45a$ repression was associated with uncontrolled cell proliferation, increased survival due to the ability of cancer cells to evade apoptosis, and increased tumorigenicity.[38,40]

Another apoptosis-related gene that emerged from our studies is $Cycs$ (somatic cytochrome C), a pro-apoptotic factor commonly involved in the respiratory chain and electron transfer.[46] Once released into the cytosol, $Cycs$ becomes a vital part of the apoptosis pathway by activating the aspartate-specific cysteine proteases $Casp9$ and $3$.[46,47]

Studies have shown that inactivation of $Casp9$ is embryonic lethal, whereas $Casp3$ knockout mice died in the weeks following birth, preventing the study of their potential role in spermatogenesis.[48] Since $Cycs$ and activated $Casp3$ were found in spermatogenic cell, they could play a role in apoptosis.[49,50] Bcl-2 is known to inhibit $Cycs$ translocation, blocking caspase activation and apoptosis.[51] Studies have shown that Bcl-2 inactivation does not have a drastic effect on spermatogenesis, indicating either that Bcl-2 is not involved in germ cell apoptosis or that the system is redundant.[31,52] However, Bcl-2 and Bcl-XL overexpression both result in abnormal spermatogenesis, similarly to the phenotype of BAX knockout models, suggesting a role for these genes in the regulation of spermatogenic cell apoptosis.[1,21] Furthermore, studies in leukemogenesis have shown that transcription factor $Sall4$ binds the promoters of several apoptosis-inducing genes, including $Cycs$.[53] Interestingly, $Sall4$ is a marker for undifferentiated spermatogonia, while $Sall4a$ is upregulated at PND7,[54] a period at which germ cells should have relocated to the basement membrane and when postnatal germ cell apoptosis rises.[1] $Sall4b$ was also positively expressed in PND0 quiescent gonocytes.[54] Finally, although $Cycs$ has not yet been described in TGCTs, it is expressed in other types of tumors and is used as a biomarker for prostate cancer.[55] Our present findings that similarly to $Gadd45a$, $Cycs$ expression was increased in differentiating gonocytes but significantly downregulated in seminomas compared to normal testicular tissues, suggest that $Cycs$ may play a role in early germ cell apoptosis, and that it could be one of the genes suppressed during germ cell cancer formation.
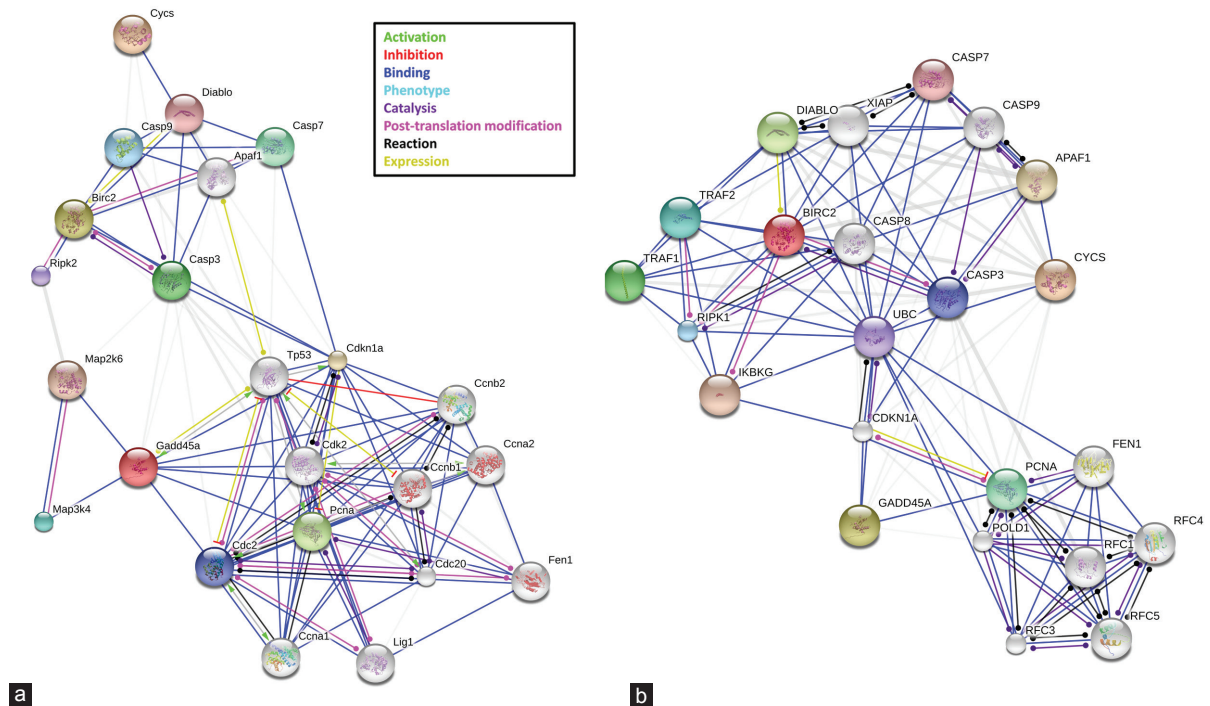
**Figure 8:** Search tool for the retrieval of interacting genes/proteins analysis indicating the predicted functional partners for *Gadd45a*, *Cycs*, and *Birc2* in rat (**a**) and human (**b**). Functional partner predictions were based on available experimental data, databases, text-mining, and homology. The colors of the lines connecting various genes represent different types of interactions between genes.

*Birc2,* also known as cellular inhibitor of apoptosis 1 (*cIAP1*), was one of the genes significantly altered during rat gonocyte differentiation and in human seminomas. *Birc2* has been found in various types of adult rat germ cells, including type B spermatogonia and primary spermatocytes, where it is believed to play a role in the prevention of apoptosis.[56] However, a study in rats where testicular germ cell apoptosis was induced by chronic crude garlic feeding reported that *Birc2* increased expression was not sufficient to prevent germ cell apoptosis, implying that several anti-apoptotic genes might be required for the prevention of apoptosis.[57] In the present study, the fact that *Birc2* expression increased in RA-treated gonocytes may correspond to a survival mechanism during differentiation. Moreover, the lower levels in PND8 spermatogonia in comparison to unstimulated gonocytes suggest that *Birc2* might be downregulated once the window of gonocyte differentiation has closed, in order to allow for the apoptosis of germ cells that failed differentiation. Surprisingly, analysis of *Birc2* expression in seminomas revealed that it was decreased in these tumors, rather than increased as would be expected of anti-apoptotic genes in cancer cells.[55] Although IAPs have been proposed as potential drug targets for cancer treatment due to their inhibitory effects on caspases,[58,59] recent preclinical studies have revealed increased risk of metastasis in patients treated with IAP antagonists for the treatment of bone metastasis.[60] Other studies have reported examples of tumors in which cIAPs acted as tumor-suppressing genes, their inhibition aggravating rather than improving disease outcome.[61] These unexpected results were attributed to the existence in metastatic cells of an IAP-induced alternate nuclear factor-κB (NF-κB) signaling pathway, different from the canonical pro-survival pathway. In this model, the presence of IAP antagonist would promote IAP proteasomal degradation, leading to an aberrant NF-κB activation and increased tumor cell survival and proliferation.[60,61] These findings further stress the complexity of IAPs role and the importance of the tumor/cell type context. Thus, it will be interesting to determine whether seminomas express both canonical and non-canonical NF-κB pathways and whether *Birc2* acts as an anti-apoptotic or tumor suppressor gene in this type of cancer.

Although not significantly altered during gonocyte differentiation, the mRNA expression of the anti-apoptotic gene *Dad1* was significantly decreased in seminomas. Mating studies to establish a rat knockout model of *Dad1* have shown that *Dad1*^{−/−} embryos were not detected past E3.5, suggesting that this gene is needed for developing past the late blastocyst stage during embryonic development.[62] In addition, although *Dad1* expression has not been analyzed in seminomas before the current study, *Dad1* has been shown to be upregulated in cisplatin-treated ovarian cancer cells, positioning it as a potential therapeutic target in cisplatin-resistant tumors.[63] Testicular tumors are also treated with cisplatin, combined in a standard cocktail with bleomycin and etoposide.[64,65] Why this anti-apoptosis gene is downregulated in seminomas remains to be determined. While it is possible that a fine tuning between both pro- and anti-apoptotic genes is necessary during tumor formation and/or growth, it is also possible that *Dad1* might have pro-apoptotic properties in specific tumor types or tissue context, by analogy to IAPs.

Another gene that was not altered during gonocyte differentiation but significantly downregulated in seminomas is *Casp8ap2*, a pro-apoptotic gene commonly activated in the programmed cell death pathway.[66] To our knowledge, *Casp8ap2* has not been previously described in seminomas, but studies have shown that measuring *Casp8ap2* expression levels in leukemia patients can help diagnose and characterize patients more highly susceptible to chemotherapy.[66] The present finding that *Casp8ap2* expression is repressed in seminomas is in agreement with the common observation of downregulated pro-apoptotic genes in cancer.

STRING analysis indicated that *Casp3* was at the center of a network comprising *Cycs*, *Birc2*, *Casp9*, *7*, *DIABLO* and *XIAP* both in rat and human, with *Cycs* positioned upstream of *Casp3* and *Birc2* being

negatively regulated by *Casp3*. In early postnatal rat germ cells, *Casp2* and *7* showed preferential expression in gonocytes and spermatogonia, being 10- to 20-fold higher than *Casp3* and *9*, while in normal human testis and seminoma, *Casp9* and *2* were the most abundant caspases. The high levels of *Casp2* observed in early postnatal germ cells are consistent with a study where *Casp2* was reported to participate to the postnatal surge of germ cell apoptosis taking place during the first spermatogenic wave.[67] Moreover, *Casp2* was shown to play a role in cell cycle arrest at the G2/M DNA-damage checkpoint.[68] Here, we found that *Casp3* and *9* are strongly activated at PND8 in germ cells that failed migration to the basement membrane. This implies that the 3 caspases are activated during the same time-frame of germ cell development. Thus, it will be interesting to determine their respective roles in this process. With regards to testicular cancer, our findings that *Casp3* was increased simultaneously to the decreased expression of *Casp9* in seminoma are interesting, in view of a study that identified a failure of *Casp9* activation as possibly implicated in the cisplatin resistance of a human testicular germ cell line.[69] However, the higher cisplatin-sensitivity threshold could be overcome by the activation of a *Casp9* independent pathway, further highlighting the multiplicity and complexity of apoptotic mechanisms in tumor cells. Further studies will be needed to determine the regulation of these caspases at the posttranslational level in germ cell tumors.

Two other genes found to increase by gene profiling arrays in differentiating gonocytes were the pro-apoptotic gene *DIABLO* and the anti-apoptotic gene *XIAP*. Gene array analysis showed that *DIABLO* and *XIAP* expressions were constant between gonocytes and spermatogonia. In human biopsies, *XIAP* expression showed a trend towards increase in seminomas, whereas *DIABLO* levels remained unchanged. This difference resulted in a small increase in the *XIAP/DIABLO* ratio, which is reminiscent of a study reporting an increase in the *XIAP/DIABLO* ratio between normal and CIS as well as seminomas.[70] However, in that study, most of the changes were attributed to decreases in *DIABLO* levels, whereas in the present study, *XIAP* was responsible of the changes in ratio. In the published study as well as in the present work, there were large variations within some of the gene expression values. This might be due to the fact that most TGCTs are heterogeneous, corresponding to a mixture of phenotypes rather than to a pure tumor type. Therefore, different percentages of seminoma cells within tumors could result in seminomas samples with variable proportions of apoptosis-related genes.

## CONCLUSION
Our study characterized apoptosis-related genes that are significantly altered during the transition from neonatal gonocyte to spermatogonia, as well as in seminomas compared to normal human testis biopsies. Two of these genes, *Gadd45a and Cycs*, are likely to play a role in the wave of germ cell apoptosis that occurs during the 2nd week after birth in rodent testes. Moreover, the reduction of these genes in seminomas, as well as that of *Casp9*, fit with the frequent observation of repressed pro-apoptotic genes in tumors. The results obtained for *Birc2* suggest that this gene might behave as a tumor promoter in seminomas rather than an anti-apoptotic gene as previously found in other types of tumors. Further studies will be needed to clarify this fact and to determine the exact relationship between the genes identified in developing germ cells and in seminomas.

## AUTHOR CONTRIBUTIONS
G.M. was involved in study design, execution, data collection, analysis, and wrote the manuscript. M.C played a critical role in the study design, data analysis, and manuscript preparation.

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**Supplement Table 1: List of all genes analyzed in qiagen RT2 profiler PCR array**

| Well | Refseq | Symbol | Description |
|---|---|---|---|
| A01 | NM_001100850 | Abl1 | C-abl oncogene 1, receptor tyrosine kinase |
| A02 | NM_031356 | Aifm1 | Apoptosis-inducing factor, mitochondrion-associated 1 |
| A03 | NM_033230 | Akt1 | V-akt murine thymoma viral oncogene homolog 1 |
| A04 | NM_013132 | Anxa5 | Annexin A5 |
| A05 | NM_023979 | Apaf1 | Apoptotic peptidase activating factor 1 |
| A06 | NM_001127379 | Api5 | Apoptosis inhibitor 5 |
| A07 | NM_001107757 | Aven | Apoptosis, caspase activation inhibitor |
| A08 | NM_022698 | Bad | BCL2-associated agonist of cell death |
| A09 | NM_001106647 | Bag1 | BCL2-associated athanogene |
| A10 | NM_053812 | Bak1 | BCL2-antagonist/killer 1 |
| A11 | NM_017059 | Bax | Bcl2-associated X protein |
| A12 | NM_031328 | Bcl10 | B-cell CLL/lymphoma 10 |
| B01 | NM_016993 | Bcl2 | B-cell CLL/lymphoma 2 |
| B02 | NM_133416 | Bcl2a1d | B-cell leukemia/lymphoma 2 related protein A1d |
| B03 | NM_031535 | Bcl2l1 | Bcl2-like 1 |
| B04 | NM_022612 | Bcl2l11 | BCL2-like 11 (apoptosis facilitator) |
| B05 | NM_021850 | Bcl2l2 | Bcl2-like 2 |
| B06 | NM_022684 | Bid | BH3 interacting domain death agonist |
| B07 | NM_053704 | Bik | BCL2-interacting killer (apoptosis-inducing) |
| B08 | NM_021752 | Birc2 | Baculoviral IAP repeat-containing 2 |
| B09 | NM_023987 | Birc3 | Baculoviral IAP repeat-containing 3 |
| B10 | NM_022274 | Birc5 | Baculoviral IAP repeat-containing 5 |
| B11 | NM_001106835 | Bnip2 | BCL2/adenovirus E1B interacting protein 2 |
| B12 | NM_053420 | Bnip3 | BCL2/adenovirus E1B interacting protein 3 |
| C01 | NM_017312 | Bok | BCL2-related ovarian killer |
| C02 | NM_001130554 | Card10 | Caspase recruitment domain family, member 10 |
| C03 | NM_012762 | Casp1 | Caspase 1 |
| C04 | NM_130422 | Casp12 | Caspase 12 |
| C05 | XM_234878 | Casp14 | Caspase 14 |
| C06 | NM_022522 | Casp2 | Caspase 2 |
| C07 | NM_012922 | Casp3 | Caspase 3 |
| C08 | NM_053736 | Casp4 | Caspase 4, apoptosis-related cysteine peptidase |
| C09 | NM_031775 | Casp6 | Caspase 6 |
| C10 | NM_022260 | Casp7 | Caspase 7 |
| C11 | NM_022277 | Casp8 | Caspase 8 |
| C12 | NM_001107921 | Casp8ap2 | Caspase 8 associated protein 2 |
| D01 | NM_031632 | Casp9 | Caspase 9, apoptosis-related cysteine peptidase |
| D02 | NM_134360 | Cd40 | CD40 molecule, TNF receptor superfamily member 5 |
| D03 | NM_053353 | Cd40lg | CD40 ligand |
| D04 | NM_057138 | Cflar | CASP8 and FADD-like apoptosis regulator |
| D05 | NM_001170467 | Cidea | Cell death-inducing DFFA-like effector a |
| D06 | NM_001108869 | Cideb | Cell death-inducing DFFA-like effector b |
| D07 | NM_012839 | Cycs | Cytochrome c, somatic |
| D08 | NM_138910 | Dad1 | Defender against cell death 1 |
| D09 | NM_001107335 | Dapk1 | Death associated protein kinase 1 |
| D10 | NM_053679 | Dffa | DNA fragmentation factor, alpha subunit |
| D11 | NM_053362 | Dffb | DNA fragmentation factor, beta polypeptide |
| D12 | NM_001008292 | Diablo | Diablo homolog (Drosophila) |
| E01 | NM_152937 | Fadd | Fas (TNFRSF6)-associated via death domain |
| E02 | NM_080895 | Faim | Fas apoptotic inhibitory molecule |
| E03 | NM_139194 | Fas | Fas (TNF receptor superfamily, member 6) |

*Contd...*

**Supplement Table 1: Contd...**

| Well | Refseq | Symbol | Description |
|---|---|---|---|
| E04 | NM_012908 | Faslg | Fas ligand (TNF superfamily, member 6) |
| E05 | NM_024127 | Gadd45a | Growth arrest and DNA-damage-inducible, alpha |
| E06 | NM_057130 | Hrk | Harakiri, BCL2 interacting protein (contains only BH3 domain) |
| E07 | NM_012854 | Il10 | Interleukin 10 |
| E08 | NM_080769 | Lta | Lymphotoxin alpha (TNF superfamily, member 1) |
| E09 | NM_001008315 | Ltbr | Lymphotoxin beta receptor (TNFR superfamily, member 3) |
| E10 | NM_053842 | Mapk1 | Mitogen activated protein kinase 1 |
| E11 | NM_053777 | Mapk8ip1 | Mitogen-activated protein kinase 8 interacting protein 1 |
| E12 | NM_021846 | Mcl1 | Myeloid cell leukemia sequence 1 |
| F01 | XM_226742 | Naip2 | NLR family, apoptosis inhibitory protein 2 |
| F02 | XM_342346 | Nfkb1 | Nuclear factor of kappa light polypeptide gene enhancer |
| F03 | NM_053516 | Nol3 | Nucleolar protein 3 (apoptosis repressor with CARD domain) |
| F04 | NM_017141 | Polb | Polymerase (DNA directed), beta |
| F05 | NM_017169 | Prd×2 | Peroxiredoxin 2 |
| F06 | NM_012630 | Prlr | Prolactin receptor |
| F07 | NM_172322 | Pycard | PYD and CARD domain containing |
| F08 | XM_342810 | Ripk2 | Receptor-interacting serine-threonine kinase 2 |
| F09 | NM_001012066 | Sphk2 | Sphingosine kinase 2 |
| F10 | NM_012675 | Tnf | Tumor necrosis factor (TNF superfamily, member 2) |
| F11 | NM_001108873 | Tnfrsf10b | Tumor necrosis factor receptor superfamily, member 10b |
| F12 | NM_012870 | Tnfrsf11b | Tumor necrosis factor receptor superfamily, member 11b |
| G01 | NM_013091 | Tnfrsf1a | Tumor necrosis factor receptor superfamily, member 1a |
| G02 | NM_130426 | Tnfrsf1b | Tumor necrosis factor receptor superfamily, member 1b |
| G03 | NM_145681 | Tnfsf10 | Tumor necrosis factor (ligand) superfamily, member 10 |
| G04 | NM_001001513 | Tnfsf12 | Tumor necrosis factor ligand superfamily member 12 |
| G05 | NM_030989 | Tp53 | Tumor protein p53 |
| G06 | XM_223012 | Tp53bp2 | Tumor protein p53 binding protein, 2 |
| G07 | NM_019221 | Tp63 | Tumor protein p63 |
| G08 | NM_001108696 | Tp73 | Tumor protein p73 |
| G09 | NM_001100480 | Tradd | TNFRSF1A-associated via death domain |
| G10 | NM_001107815 | Traf2 | Tnf receptor-associated factor 2 |
| G11 | NM_001108724 | Traf3 | Tnf receptor-associated factor 3 |
| G12 | NM_022231 | Xiap | X-linked inhibitor of apoptosis |
| H01 | NM_031144 | Actb | Actin, beta |
| H02 | NM_012512 | B2m | Beta-2 microglobulin |
| H03 | NM_012583 | Hprt1 | Hypoxanthine phosphoribosyltransferase 1 |
| H04 | NM_017025 | Ldha | Lactate dehydrogenase A |
| H05 | NM_001007604 | Rplp1 | Ribosomal protein, large, P1 |
| H06 | U26919 | RGDC | Rat genomic DNA contamination |
| H07 | SA_00104 | RTC | Reverse transcription control |
| H08 | SA_00104 | RTC | Reverse transcription control |
| H09 | SA_00104 | RTC | Reverse transcription control |
| H10 | SA_00103 | PPC | Positive PCR control |
| H11 | SA_00103 | PPC | Positive PCR control |
| H12 | SA_00103 | PPC | Positive PCR control |

Listed 8 are all the 84 apoptosis related genes, the housekeeping genes, and the internal controls 9 contained in the Qiagen array.

**Supplement Table 2: Gene expression array analyses of 4 genes altered in PND3 gene 12 profiling**

| | Gene expression (mean±sem) | | | |
|---|---|---|---|---|
| Gene | Gonocyte | Spermatogonia | Normal | Seminoma |
| Casp3 | 61±12 | 55±11 | 56±9 | 96±7* |
| Casp9 | 27±5 | 31±0 | 157±32 | 62±13* |
| Diablo | 81±3 | 88±1 | 110±8 | 119±34 |
| XIAP | 8±1 | 8±1 | 59±13 | 81±6 |

These genes were not selected for qPCR validation because their expression levels in 13 gene arrays was below an intensity value of 100. However, 3 of the genes were expressed at relatively high levels (>35 in at least one type of sample). Gene arrays data were obtained using 3 independent samples for each type of rat cell/human tissue. Means±SEM are show. $P<0.05$