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# Testicular Gene Expression in Cryptorchid Boys at Risk of Azoospermia

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**Key Words**
Ad spermatogonia · Affymetrix · Azoospermia · Cryptorchidism · Gene expression · Testis

**Abstract**
Despite timely and successful surgery, 32% of patients with bilateral and 10% with unilateral cryptorchidism will develop azoospermia. Cryptorchid boys at risk of azoospermia display a typical testicular histology of impaired mini-puberty at the time of the orchidopexy. During mini-puberty increased gonadotropin and testosterone secretion stimulate transformation of gonocytes into Ad spermatogonia. In the azoospermia risk group this transformation is to a great extent impaired. This study aimed to analyze data on whole genome expression signatures of undescended testes at risk of developing azoospermia. Twenty-three testicular biopsies from 22 boys were analyzed (19 testes from 18 boys with cryptorchidism and 4 contralateral descended testes from patients with testicular agenesis). Expression profiling identified 483 genes not or under-expressed in the azoospermia risk group compared with the control and low risk for azoospermia (LAZR) groups. Annotated loci were associated with spermatogenesis. Other significant genes were cellular defense response genes and hormone-controlled loci involved in spermatogenesis. Some genes transcribed in normal adult meiotic and post-meiotic germ cells are activated in healthy juvenile Ad spermatogonia. Thus, molecular events initiating the testicular expression program at the onset of puberty and maintaining it during adulthood occur very early in prepubertal testes. This molecular event is to a great extent impaired in the high risk for azoospermia (HAZR) group lacking Ad spermatogonia (stem cells for spermatozoa) indicating impaired mini-puberty.



Cryptorchidism is among the most common birth defects in humans and is one of the main etiologic causes of non-obstructive azoospermia in man. Twenty-seven (60%) of 45 men with otherwise unexplained non-obstructive azoospermia had a history of cryptorchidism [Fedder et al., 2004]. The main reasons for treating this pathology are to prevent development of infertility during adulthood and to decrease the risk of testicular cancer. However, despite successful orchidopexy, spermatogenesis ultimately may be dramatically impaired in a subgroup of patients showing disturbed testicular maturation during the period of mini-puberty, with as many as 20% developing azoospermia [Hadziselimovic and Herzog, 2001; Hadziselimovic et al., 2007]. A reliable indicator of this maturation process is the presence of Ad spermatogonia located in prepubertal testis [Seguchi and Hadziselimovic, 1974]. These cells display dark (electron-dense)

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cytoplasm and a bright spot within the nucleus that distinguishes them from fetal and A pale (Ap) spermatogonia [Seguchi and Hadziselimovic, 1974; Nistal and Paniagua, 1984; Paniagua and Nistal, 1984]. A surge in gonadotropin and testosterone levels during early postnatal development (mini-puberty) is required for the differentiation of gonocytes into Ad spermatogonia, a process that is proposed to be essential for normal adult fertility [Hadziselimovic et al., 1986, 2005; Hadziselimovic and Zivkovic, 2007].

Endocrine or primary end organ failure are 2 different etiological factors thought to be responsible for the increased incidence of azoospermia and subfertility in unilateral as well as bilateral cryptorchidism [Hadziselimovic et al., 1979, 2004, 2005; Job et al., 1987; Cortes et al., 1996; Gracia et al., 2000; Hamza et al., 2001; Toppari et al., 2007]. In this study, data from whole-genome expression signatures of undescended testes lacking Ad spermatogonia and at risk of developing azoospermia are provided and analyzed in the context of data obtained from descended and cryptorchid testes with Ad spermatogonia.

## Patients and Methods

*Testicular Biopsies and Pooling of Patients*

At our institution, it is routine practice to perform testicular biopsy during surgery for undescended testis. We find that testicular biopsy provides useful information about future semen quality and helps identify patients with atypical spermatogonia or carcinoma in situ.

Cryptorchid testis is defined as a testis localized outside the scrotum and incapable of being brought into a stable scrotal position. Twenty-three rice-grain-large testicular biopsies from 22 boys were analyzed (19 testes from 18 boys with cryptorchidism and 4 contralateral descended testes from patients with testicular agenesis). Not all contralateral testes in patients with testicular agenesis are expected to have normal histology and may require subsequent hormonal treatment [Huff et al., 1991]. Therefore, we routinely biopsy these testes during the period of testicular fixation performed to prevent testicular torsion. All contralateral testes were selected for normal histology according to age. The mean age of the patients at surgery was 3.4 (95% CI 0.6–6.1) years and 3.9 (95% CI 2.3–5.4) years for the controls. All patients had an extensive clinical examination. We could not find any clinical signs of developmental malformations or syndromes, and none had hypospadias. In particular no clinical sign for Kallmann syndrome was observed. Furthermore, all patients had normal thyroid screening and no features of hypopituitarism were discernible. Biopsies were fixed in 3% glutaraldehyde and embedded in Epon. Semi-thin 1-μm-thick sections were examined by Zeiss Axioscope phase-contrast and conventional light microscopy (Plan-Apochromat 63×/1.40 oil). An aliquot of each biopsy fraction, flash frozen into liquid nitrogen and stored at –80°C, was fixed in PBS containing 1% formaldehyde (Polio, France) and 1% fetal calf serum (Eurabbie, France) and analyzed by DNA flow cytometry to determine its relative DNA content. An aliquot was processed with Affymetrix target synthesis kits currently available for 3′ IVT arrays. Amplifications were based upon T7 RNA polymerase using oligo-dT priming and the same arrays were used to minimize variability. Cell pellets and tissues were sheared in RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol from which aqueous phases were taken out for RNA isolation. Total RNA isolation was performed using RNeasy Mini Kit according to standard protocols. RNA quality was monitored using RNA Nano 6000 Chips and the 2100 BioAnalyzer (Agilent). Targets were synthesized using the Affymetrix One-Cycle cDNA Synthesis Kit or Affymetrix 3′ IVT Express Kit. Following quality-check on BioAnalyzer, targets were hybridized onto GeneChip Human Genome U133 Plus 2.0 Arrays at 45°C for 17 h. Arrays were washed on a Fluidics Station 450 (Affymetrix) using the Hybridization Wash and Stain Kit (Affymetrix) and the Washing Procedure FS450_0007. Arrays were scanned on a Scanner 3000 7G (Affymetrix) and CEL files generated by AGCC software (Affymetrix). Raw data files are available at the EBI ArrayExpress repository [Parkinson et al., 2007] via the accession numbers E-TABM-130 and E-TABM-174 and GEO (GSE25518).

*Statistical Analysis and Interpretation of Microarray Data*

The data analysis and gene filtering was performed using R/Bioconductor [Gentleman et al., 2004]. Signal condensation was performed using only the RNA from the Bioconductor Affy package. Differentially expressed genes were identified using the Benjamin-Hochberg false discovery rate correction implemented in the LIMMA package and adjusted with the False Discovery Rate (FDR) method [Wettenhall and Smyth, 2004]. We selected these probe sets with a log2 average contrast signal of at least 5, an adjusted p value $<0.05$, and an absolute log2-fold change of $>0.585$ (1.5-fold in linear space). Hierarchical clustering and visualization was performed in R. The minimum number of genes in a cluster associated with a given annotation term was set at $\geq 3$. BioGRID, MINT, and IntAct (PSI MI 2.5 format) interactome data sets were uploaded and combined into AMEN. To improve gene annotation and interactome information, mouse, rat, and human protein entries were combined and converted into HomoloGene identifiers [Wheeler et al., 2007]. A data set containing gene identifiers and corresponding expression values was uploaded into the application. Gene lists from the resulting contrasts were further classified into regulatory networks and pathways with Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base.

These genes, called focus genes, were overlaid into a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Functional analyses associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered, and Fisher's exact test was used to calculate a p value, determining the probability that each biological function and/or disease assigned to that data set is due to chance and chance alone.

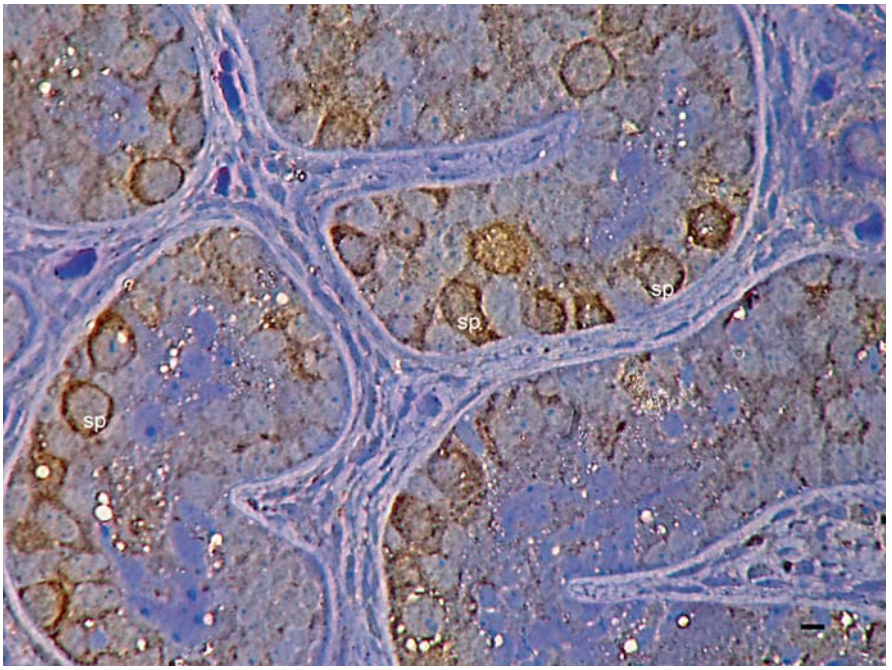


**Fig. 1.** Immunohistochemical staining for MAGEA4 in the LAZR group. In all cases antibody binding was visualized using peroxidase as a substrate to generate brown color. Sections were counterstained with toluidine blue. sp: Immunopositive spermatogonia. Horizontal bar = 0.1 mm.

*Quantitative Real-Time PCR*

Quantitative real-time PCR (qRT-PCR) was performed using a Corbett Research RG-6000 instrument. cDNAs were synthesized with the Reverse Transcriptase Core Kit (Eurogentec, cat# RT-RTCK-03) using random primers [Hadziselimovic et al., 2009]. Real-time PCR runs were performed using SYBR Fast Kit (Kapa Biosystems, cat# KK4602) with each gene-specific primer at 200 nM final concentration in a total volume of 17.5 μl. Wave lengths for source and detection were set at 470 and 510 nm, respectively. Gain was set at 8.33. The PCR program was set as follows: 95°C for 60 s, then 45 cycles at 95°C for 3 s, 60°C for 10 s, and 72°C for 4 s followed by a melting curve analysis (65°C to 95°C, rising by 0.65°C during each step) to attest to amplification specificity. Threshold cycles (crossing point) were determined using Rotor-Gene software version 6.1. Good PCR efficiency was checked by performing a dilution series of the cDNA. Minus RT controls were performed for each sample studied. Threshold cycles (crossing point) were determined using Rotor-Gene software version 6.1. Expression levels were normalized to *TFRC* and *GAPDH* using the geometric mean of their level of expression. These genes were selected because they showed the smallest variation between individual samples (both on microarrays and by qPCR). Fold differences were calculated using the ΔCt method (online supplement figure 1; for all online supplement material, see www.karger.com/doi/10.1159/000323955).

*Antibody Validation*

For immunohistochemical analysis, the Epon was removed from the tissue sections. The sections were treated with 2% bovine serum albumin to reduce non-specific binding and then incubated with the primary antibody overnight at 4°C. All samples were washed with PBS between incubations. We validated 8 antibodies: DDX25, EGR1, EGR4, CBL, ALDH1A2, KLF4, MAGEA4, and COL4A3 (fig. 1–3, online suppl. table 1). Secondary antibodies, labeled with horseradish peroxidase-polymer (HRP; goat polyclonal anti-rabbit IgG, mouse IgG and IgM, prediluted ab2891, Abcam, Cambridge, UK) were used to detect the binding of the primary antibody. The chromogenic reaction was developed by adding a freshly prepared solution of 3,3-diaminobenzidine solution (DAB + chromogen; DAKO). The DAB reaction was terminated by washing in Tris-buffered saline (0.05 M TBS and 0.85 M NaCl, pH 7.6). To visualize the histology of testicular cells, the samples were counterstained with toluidine blue. Antibody binding was indicated by a brown precipitate. Different cell types were identified based on their nuclear morphology and position within the developing gonad. Immunohistochemistry experiments were performed at least twice on at least 4 patients from each group, and only those with identical results between experiments for each sample were considered acceptable. Controls for non-specific binding of the secondary antibody were performed in all experiments by omitting the primary antibody; these consistently yielded no signal within the seminiferous epithelium or the interstitial space. The interstitial staining observed in the presence of the primary antibody was considered to be nonspecific because it was not associated with, or localized within, a particular cell type. However, staining of interstitial cells was recorded.

Experimental design, biomaterials and treatments, reporters, staining, imaging data, and image characterization were performed in compliance with the minimum information specification for immunohistochemistry experiments [Deutsch et al., 2008].

*Ethical Considerations and Approval*

In accordance with the Declaration of Helsinki, the Institutional Review Board and the Independent Ethics Committee of the Children's Clinic Liestal approved all aspects of this study. Approval was provided for research involving the use of material (data records or biopsy specimens) that had been collected for non-research purposes.

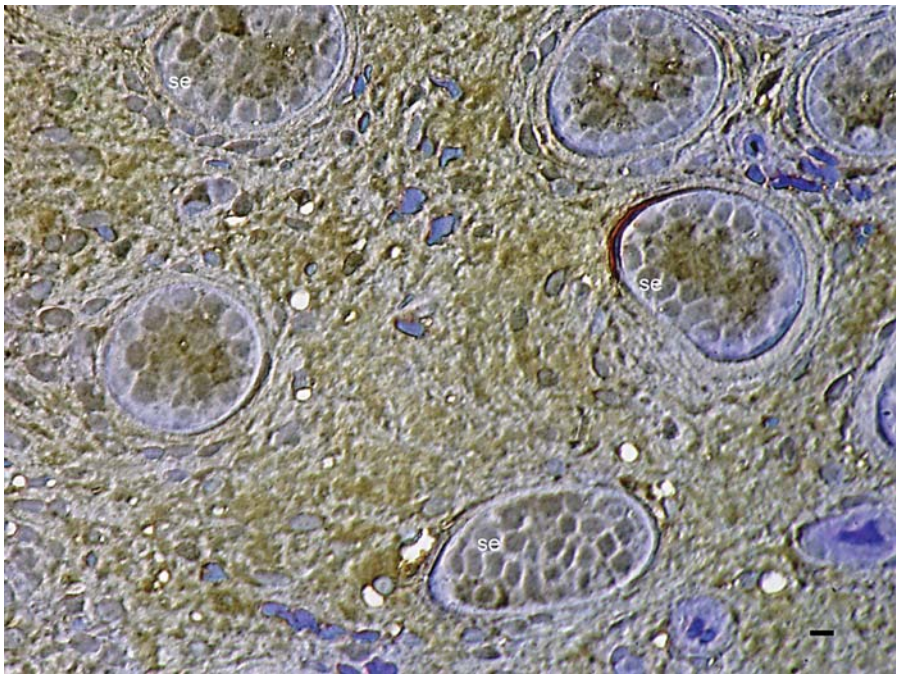


**Fig. 2.** Immunohistochemical COL4A3 staining in the HAZR group. Immunonegative tubules for COL4A3 contain Sertoli cells only (se). Background staining was performed with toluidine blue. Scale bar = 0.1 mm.

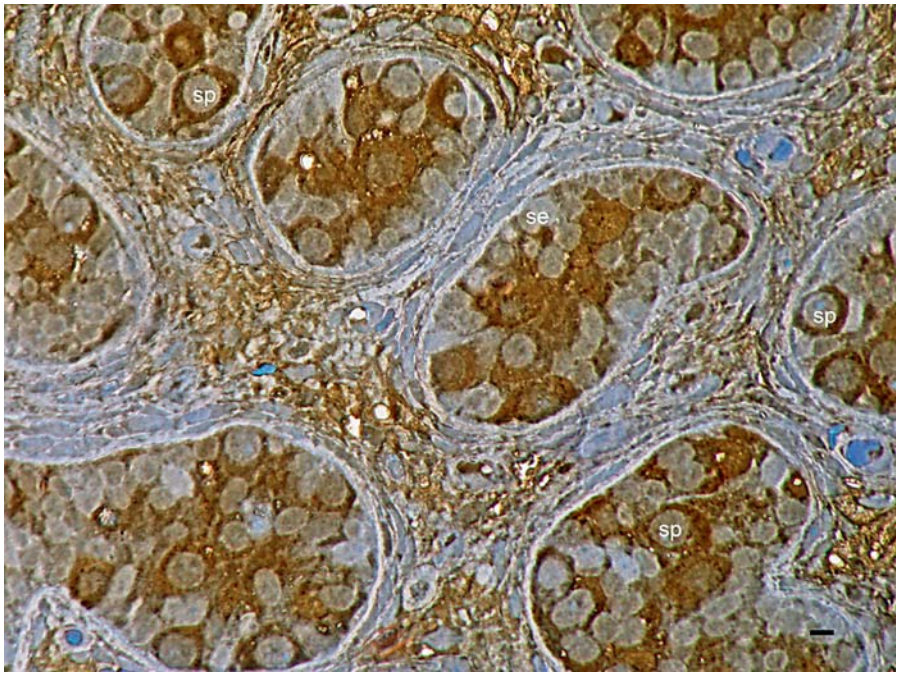


**Fig. 3.** Immunohistochemical COL4A3 staining in the control group. Germ cells localized at the basement membrane are stained brown (sp). Background staining was performed with toluidine blue. se: Sertoli cell. Scale bar = 0.1 mm.

## Results

*An Expression Signature Distinguishes High Azoospermia Risk from Control Patients*

Total RNA and cRNA samples were of high quality and the signal intensity distributions were similar and within the normal range. Our statistical filtration strategy identified 483 transcripts differentially expressed between the azoospermia risk group and control patients, and 370 transcripts differentially expressed between the high risk for azoospermia (HAZR) and low risk for azoospermia (LAZR) groups (fig. 4). Notably, 62 genes involved in germ cell development were equally expressed in the LAZR and control groups, whereas 46/62 genes

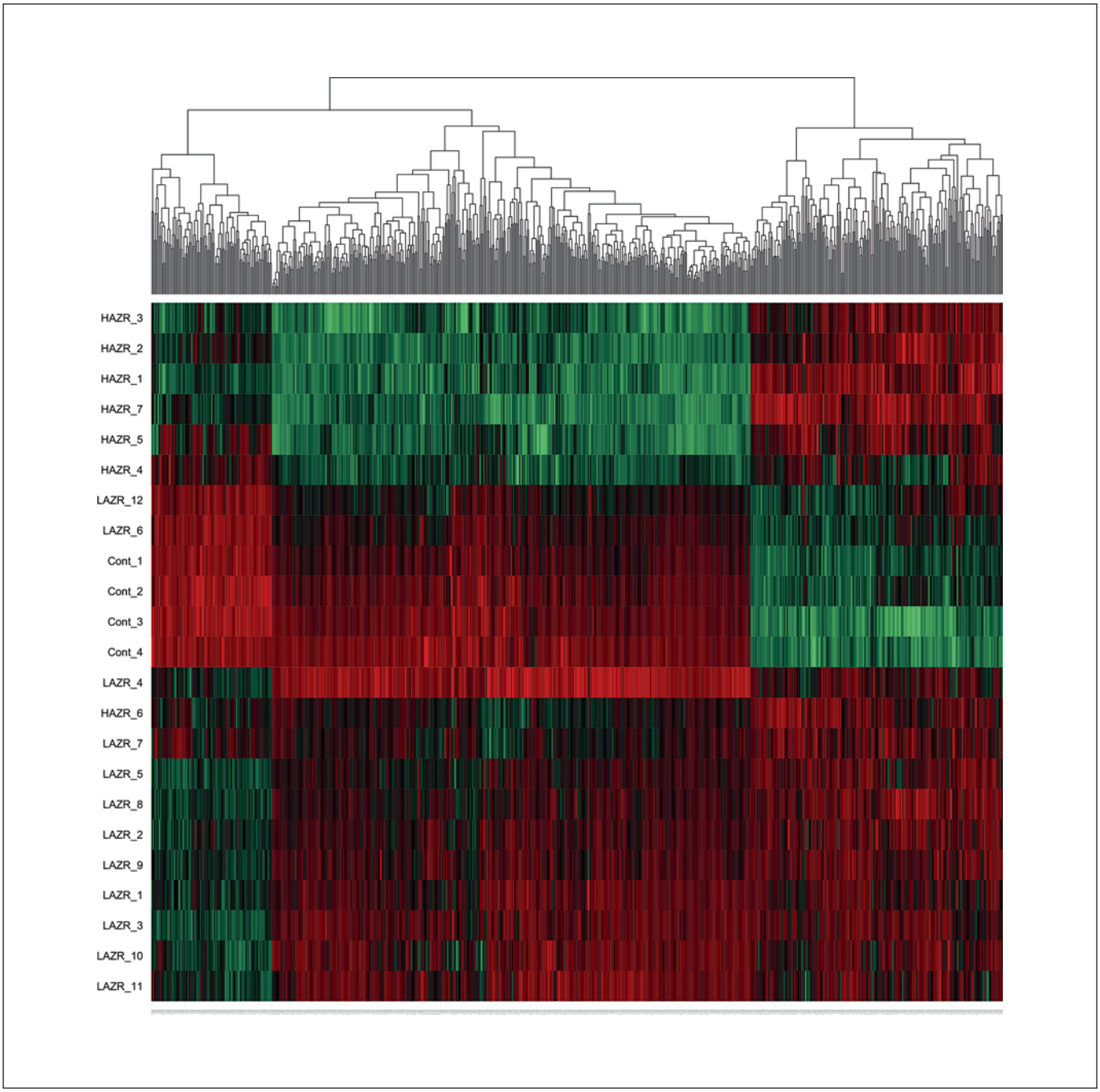


**Fig. 4.** Heat map (unsupervised) showing hierarchical clustering of gene expression profiles.

were not and 16/62 genes were expressed at lower levels in the HAZR group (table 1).

Furthermore, 18/27 genes proposed as being involved in A spermatogonia self-renewal [Wu et al., 2009] were not expressed in the HAZR group, but were expressed in the LAZR and control groups (online suppl. table 2). Neither *GDNF* (glial cell-derived neurotrophic factor) nor its regulatory genes, *BHLHB2* (basic helix-loop-helix domain containing, class B, 2), *LHX1* (LIM homeobox 1), *GFRA1* (GDNF family receptor alpha 1), and *TEC* (Tec protein tyrosine kinase), were found to be expressed in all groups studied. Compared with controls, we found that

**Table 1.** Transcript signature of differentially expressed genes involved in germ cell development in the high (HAZR) and the low azoospermia risk (LAZR) group as well as in the control group

| Gene | ID | HAZR | LAZR | Control | adjusted p | | Approved gene name |
|---|---|---|---|---|---|---|---|
| | | | | | HAZR/con. | HAZR/LAZR | |
| ***ALDH1A1*** | 207 016 s-at | 4.53 | 6.88 | 7.0 | 0.007 | 0.0006 | aldehyde dehydrogenase 1 family, member A1 |
| *AMPH* | 205 239 at | 3.42 | 4.67 | 5.33 | 0.01 | 0.02 | amphiphysin |
| *CACNA2D2* | 204 811 s-at | 5.63 | 6.95 | 6.85 | 0.01 | 0.0007 | calcium channel, voltage-dependent, alpha 2/delta subunit 2 |
| ***CBL*** | 225 234 at | 6.9 | 7.88 | 8.21 | 0.003 | 0.0004 | Casitas B-lineage lymphoma |
| *CDC20* | 202 870 s-at | 3.79 | 5.82 | 6.11 | 0.01 | 0.003 | cell division cycle 20 homolog *(S. cerevisiae)* |
| *CLGN* | 205 830 at | 4.51 | 5.54 | 5.83 | 0.01 | 0.01 | calmegin |
| *CSRP2* | 207 030 s-at | 5.83 | 7.54 | 7.38 | 0.02 | 0.002 | cysteine and glycine-rich protein 2 |
| *CXCL9* | 203 915 at | 4.49 | 6.07 | 6.03 | 0.009 | 0.0007 | chemokine (C-X-C motif) ligand 9 |
| ***DAZ1*** | 207 912s-at | 3.55 | 4.35 | 5.14 | 0.008 | n.s. | deleted in azoospermia 1 |
| ***DAZL*** | 206 588 at | 6.81 | 7.67 | 7.85 | 0.04 | 0.03 | deleted in azoospermia-like |
| ***DDX25*** | 219 945 at | 4.39 | 5.86 | 5.74 | 0.02 | 0.001 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 25 |
| ***DDX4*** | 221 630 s-at | 3.47 | 7.05 | 7.43 | 0.002 | 8.25E-05 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 |
| *DMRTB1* | 240313 at | 4.29 | 5.87 | 5.93 | 0.009 | 0.003 | DMRT-like family B with proline-rich C-terminal, 1 |
| *DPYSL4* | 205 493 s-at | 3.8 | 5.49 | 5.26 | 0.01 | 0.001 | dihydropyrimidinase-like 2 |
| *DTL* | 218 585 s-at | 5.04 | 6.5 | 6.5 | 0.008 | 0.0007 | denticleless homolog *(Drosophila)* |
| ***DUSP5*** | 209 457 at | 5.77 | 7.38 | 7.7 | 0.03 | 0.008 | dual specificity phosphatase 5 |
| ***EGR4*** | 207 768 at | 2.7 | 5.65 | 5.91 | 0.007 | 0.006 | early growth response 4 |
| *ESX1* | 155 2445 a-a | 3.46 | 6.48 | 6.75 | 0.01 | 8.63E-05 | ESX homeobox 1 |
| ***FGF9*** | 206 404 at | 4.64 | 5.97 | 5.75 | 0.01 | 0.0001 | fibroblast growth factor 9 (glia-activating factor) |
| ***FGFR3*** | 204 379 s-at | 4.52 | 8.6 | 8.65 | 0.003 | 0.0001 | fibroblast growth factor receptor 3 |
| *FOXG1B* | 206 018 at | 3.41 | 5.88 | 5.81 | 0.001 | 1.21E-05 | forkhead box G1b |
| ***FST*** | 204948 s-at | 5.53 | 6.42 | 6.76 | 0.003 | 0.001 | follistatin |
| *GTSF1* | 227711 at | 4.62 | 7.27 | 6.9 | 0.01 | 0.0001 | gametocyte-specific factor 1 |
| ***GAGE1*** | 207 086 x-at | 5.59 | 9.13 | 10.04 | 0.003 | 0.001 | G antigen 1 |
| ***ID4*** | 209 291 at | 5.19 | 7.6 | 8.37 | 0.001 | 0.0002 | inhibitor of DNA binding 4, dominant negative helix-loop-helix protein |
| ***ISL1*** | 206104 at | 3.53 | 5.68 | 6.22 | 0.003 | 0.0004 | ISL LIM homebox 1 |
| *KIF21B* | 204 411 at | 4.34 | 5.7 | 5.65 | 0.006 | 0.0001 | kinesin family member 21B |
| ***LIN28B*** | 229 349 at | 3.54 | 5.27 | 5.87 | 0.01 | 0.01 | lin-28 homolog B *(C. elegans)* |
| *LIN7B* | 246 290 x-at | 4.57 | 5.78 | 6.1 | 0.03 | 0.02 | lin-7 homolog B *(C. elegans)* |
| ***MAGEA4*** | 214 254 at | 3.78 | 5.95 | 6.53 | 0.006 | 0.009 | melanoma antigen family A, 4 |
| ***MAGEC1*** | 206 609 at | 3.8 | 6.77 | 6.92 | 0.001 | 8.24E-05 | melanoma antigen family C, 1 |
| ***MBD2*** | 214 397 at | 4.25 | 7.44 | 7.65 | 0.003 | 0.03 | methyl-CpG-binding domain protein 2 |
| *MND1* | 223 700 at | 2.89 | 5.29 | 5.23 | 0.03 | 0.004 | meiotic nuclear divisions 1 homolog *(S. cerevisiae)* |
| *MORC1* | 220 850 at | 3.49 | 5.26 | 5.55 | 0.01 | 0.0001 | MORC family CW-type zinc finger 1 |
| *NLRP2* | 221 690 s-at | 4.3 | 6.18 | 5.86 | 0.04 | 0.005 | NLR family, pyrin domain containing 2 |
| ***NMU*** | 206 023 at | 4.12 | 6.08 | 5.98 | 0.03 | 0.005 | neuromedin U |
| ***NRG1*** | 206 343 s-at | 3.55 | 5.13 | 5.28 | 0.03 | 0.004 | neuregulin 1 |
| ***PIWIL2*** | 231 371 at | 5.1 | 6.88 | 6.47 | 0.002 | 0.0001 | piwi-like 2 *(Drosophila)* |
| ***PIWIL4*** | 230 480 at | 4.7 | 7.15 | 7.57 | 0.001 | 0.0001 | piwi-like 4 *(Drosophila)* |
| *RBMY1A1* | 208 307 at | 4.39 | 6.45 | 6.37 | 0.008 | 0.0008 | RNA binding motif protein, Y-linked, family 1, member A1 |
| *RGS7* | 206 290 s-at | 2.76 | 5.27 | 5.39 | 0.01 | 0.001 | regulator of G-protein signaling 7 |
| ***RNF17*** | 220 270 at | 4.17 | 5.40 | 5.83 | 0.01 | 0.01 | ring finger protein 17 |
| *SH3GL2* | 205 751 at | 5.41 | 6.49 | 6.74 | 0.02 | 0.008 | SH3-domain GRB2-like 2 |
| *SIX1* | 228 347 at | 3.93 | 6.98 | 6.55 | 0.01 | 0.02 | SIX homeobox 1 |
| *SNAP91* | 204 953 at | 4.22 | 5.4 | 6.0 | 0.006 | 0.008 | synaptosomal-associated protein, 91 kDa homolog (mouse) |
| *SNRP* | 226587 at | 8.79 | 9.09 | 10.3 | 0.04 | n.s. | U1 snrp Snp1p. RRM domain containing protein |
| *SOX30* | 207 678 s-at | 3.13 | 5.20 | 6.02 | 0.002 | 0.002 | SRY (sex-determining region Y)-box 30 |
| ***SRY*** | 207678 s-at | 3.1 | 5.2 | 5.8 | 0.002 | 0.002 | sex-determining region Y |
| ***SPA17*** | 205 406 s-at | 4.3 | 6.02 | 6.18 | 0.02 | 0.01 | sperm autoantigenic protein 17 |
| ***SSX2*** | 207 493 x-at | 3.77 | 5.65 | 6.42 | 0.03 | 0.003 | synovial sarcoma, X breakpoint 2 |
| ***SYCP3*** | 155 3599a-a | 3.59 | 5.18 | 5.57 | 0.02 | 0.0002 | synaptonemal complex protein 3 |
| *TAF5* | 210 053 at | 6.92 | 7.91 | 8.08 | 0.02 | 0.005 | TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100 kDa |
| *TAF7L* | 220 325 at | 3.72 | 5.02 | 5.48 | 0.01 | 0.03 | TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 50 kDa |
| *TDRD10* | 231 371 at | 4.68 | 5.38 | 5.69 | 0.01 | n.s. | tudor domain containing 10 |
| *TDRD5* | 231 104 at | 3.78 | 4.92 | 5.36 | 0.01 | 0.01 | tudor domain containing 5 |
| *TDRD6* | 232 692 at | 5.1 | 6.79 | 7.26 | 0.001 | 0.0003 | tudor domain containing 6 |
| *TDRD9* | 118 285 at | 4.75 | 6.52 | 6.92 | 0.01 | 0.009 | tudor domain containing 9 |
| *TEX14* | 221035 s-at | 3.58 | 5.46 | 5.67 | 0.008 | 0.001 | testis expressed 14 |
| ***TLE1*** | 228 284 at | 5.48 | 7.24 | 7.47 | 0.01 | 0.0001 | transducin-like enhancer of split 1 (E(sp1) homolog, *Drosophila*) |
| ***TSPY1*** | 207 918 s-at | 5.03 | 9.98 | 10.28 | 0.001 | 8.24E-05 | testis-specific protein, Y-linked 1 |
| *UFT1* | 208 275 x-at | 3.61 | 5.59 | 5.71 | 0.003 | 0.0002 | undifferentiated embryonic cell transcription factor 1 |
| ***WNT3*** | 229 103 at | 2.98 | 5.16 | 4.65 | 0.03 | 0.0003 | wingless-related MMTV integration site 3 |

Genes interacting with hypothalamus-pituitary-testicular axis are indicated in bold. The limit of the essay detection is 5.

204 transcripts were more highly expressed in the HAZR group, whereas only 8 were more highly expressed in the LAZR group. Most of the over-expressed genes in the HAZR group were related to cell growth and proliferation as well as to apoptosis. In the azoospermia risk group, expression profiling identified genes not expressed or expressed at significantly lower levels at loci associated with spermatogenesis and testicular development, including *CBL, CDC20, DAZ1/2/3/4, DAZL, ESX1, LIN28B, MBD2, RBMY1A1, SRY, TAF5, TAF5L, TDRD5/6/9/10, TSPY1, UTF1* (table 1). Other significantly down-regulated genes included: cellular defense response genes *CXCL9*, G antigen family members *(GAGE)*, *NLRP2*, brain/pituitary development genes; *ISL1*, *NMU*, *NRG1*, *SIX1*, *TLE1* and hormone-controlled loci known to be involved in spermatogenesis; *DDX25, DDX4, DUSP5, EGR4, FGFR3, FGF9, ID4, NMU, NRG1, SYCP3*, *SPA17*, and *TLE7L*. We identified transcripts not expressed in the HAZR group that are specific for mitosis *(MORC1)* and meiosis *(MND1, GTSF1, PIWIL2/4, RNF17, SOX30*, *SYCP3* and *TAF5)* of germ cell lines (table 1). Recently, 6 genes were proposed to be specific biomarkers of adult human spermatogonia: fibroblast growth factor receptor 3 *(FGFR3)*, desmoglein 2 *(DSG2)*, E3 ubiquitin ligase c-CBL (casitas B-cell lymphoma), cancer/testis antigen NY-ESO-1 *(CTAG1A/B)*, undifferentiated embryonic cell transcription factor 1 *(UTF1)*, and synaptosomal-associated protein, 91-kDa homolog *(SNAP91)* [von Kopylow et al., 2010]. We found that all 6 genes were expressed in the prepubertal spermatogonia in the control and LAZR groups. In the HAZR group the transcription profile was altered; 2 genes, *DSG2* and *CTAG1A*, were equally expressed, whereas *CBL* and *SNAP91* were under-expressed and *FGFR3* and *UTF1* were not expressed (table 1).

*HIR-Signature Genes Encode Factors That Directly Interact with Each Other*

To further explore and extend the RNA profiling data, we next investigated if genes differentially expressed in the azoospermia risk group and in control patients encode proteins that are physically associated with other factors. Information on direct protein-protein interactions was retrieved from the BioGRID, MINT, and IntAct databases and processed using AMEN [Stark et al., 2006; Chatraryamontri et al., 2007; Kerrien et al., 2007]. We found 2 interaction networks comprising 9 genes *(AMPH, CACNA2D2, CSRP2, DPYSL4, KIF21B, LIN7B, RGS7, SH3GL2*, and *SNAP91)* and 4 genes *(TLE1, DTL, FOXG1B* and *SIX1)*, respectively. The proteins in this group were associated with diverse biological processes including microtubule-based movement and mitosis (KIF2C, KIF21B) and signal transduction (LIN7B, RGS7, SH3GL2). Proteins in the smaller complexes were annotated as DNA-dependent regulators of transcription (TLE1, SIX1, FOXG1B, SSX2) or proteins involved in DNA replication (DTL). We also identified a cancer/testis gene *(MAGEC1)* whose hallmark is to be actively transcribed in the germ line, repressed in normal somatic tissues, and de-repressed in various cancers [Simpson et al., 2005]. Finally, we found the *BRAF* gene which encodes a protein belonging to the raf/mil family of serine/threonine protein kinases to be under-expressed in the HAZR group. This protein plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation, and has a key role in transcriptional activation of GnRH-responsive genes. The third interaction network includes the *DAZ* (deleted in azoospermia) gene family (table 1). Proteins in this group are associated with spermatogenesis development and function. Furthermore, no testicular expression of *CDC20, ESX1, RBMY1A1* and *SYCP3* genes involved in azoospermia development were observed in the HAZR group (table 1).

*Immunohistochemical and qRT-PCR Validation*

We wanted to determine whether greater transcript expression corresponded with greater protein expression. Immunoperoxidase histochemistry, although not a quantitative method, is currently the only practical way to detect proteins in small fixed tissue samples. Identical cytoplasmic localization was found for all proteins analyzed (fig. 1–3). In addition to their expression in germ and Leydig cells, we found that the *CBL* gene was also expressed in Sertoli cells. Finally, we validated our microarray data by qRT-PCR for 4 genes that showed significant expression changes; all of these showed a high correlation with the obtained transcriptional profiles (online suppl. fig. 1).

## Discussion

The estimated frequency of azoospermia in the normal population is 0.4% (3/711) [Itoh et al., 2001]. Despite successful surgery, azoospermia was found 25 times more often in unilateral and 80 times more often in bilateral cryptorchidism (table 2). Thus cryptorchidism represents one of the most frequent causes of azoospermia in man. Furthermore, according to the testicular histology, 50% of cryptorchid patients in our cohort belonged to the high azoospermia risk group [Hadziselimovic et al., 2007]. Absence of Ad spermatogonia was a typical pathological

**Table 2.** Incidence of azoospermia in unilateral and bilateral cryptorchid males

| Reference | Unilateral cryptorchid | | | Bilateral cryptorchid | | |
|---|---|---|---|---|---|---|
| | Untreated | HCG | Surgery | Untreated | HCG | Surgery |
| Hansen, 1949 | 1/35 (3%) | | 3/37 (8%) | 9/9 (100%) | | 14/25 (56%) |
| Scott, 1961 | | | 5/38 (13%) | | | |
| Eisenhut and Hohenfellner, 1964 | | | 7/34 (20%) | | | |
| Canlorbe et al., 1966 | | 15/100 (15%) | 28/111 (25%) | | 22/59 (37%) | 38/87 (44%) |
| Madersbacher et al., 1972 | | | 5/36 (14%) | | | |
| Czaplicki et al., 1974 | | | | | 5/13 (38%) | 5/13 (38%) |
| Bramble et al., 1974 | | | | | | 5/21 (24%) |
| Richter et al., 1976 | | 4/43 (9%) | 1/28 (3%) | | 9/35 (26%) | 8/15 (53%) |
| Kleinteich et al., 1976 | | | 8/66 (12%) | | | 5/34 (15%) |
| Werder et al., 1976 | | | | | | 6/14 (43%) |
| Giarola and Agostini, 1979 | 15/99 (15%) | | 12/108 (11%) | 10/10 (100%) | | |
| Guillon, 1979 | | 6/55 (11%) | 15/105 (14%) | | 10/35 (29%) | 22/87 (25%) |
| Wojciechowski et al., 1979 | | | 10/106 (9%) | | | 16/24 (66%) |
| Duvie, 1984 | | | 1/10 (10%) | | | 22/48 (46%) |
| Ponchietti and Grechi, 1986 | | | 8/104 (8%) | | | |
| Puri and O'Donnell, 1988 | | | 4/119 (3%) | | | |
| Okuyama et al., 1989 | | | 10/37 (27%) | | | |
| Mandat et al., 1994 | | | 12/112 (11%) | | | 2/23 (9%) |
| Taskinen et al., 1996 | | | 0/39 (0%) | | | 3/12 (25%) |
| Cortes et al., 1996 | | | 2/56 (4%) | | | |
| Gracia et al., 2000 | | | 8/196 (4%) | | | 5/55 (9%) |
| Paasch et al., 2004 | | | 17/130 (13%) | | | 11/37 (30%) |
| Hadžiselimović et al., 2004 | | | 16/182 (9%) | | | |
| Moretti et al., 2007 | | | 4/30 (13%) | | | 3/12 (25%) |
| Trsinar and Muravec, 2009 | | | 2/49 (4%) | | | 2/19 (10%) |
| | 16/134 (12%) | 25/198 (12.6%) | 178/1733 (10.3%) | 19/19 (100%) | 46/142 (32%) | 167/526 (32%) |

finding for this group of patients. During testicular maturation, gonocytes from cryptorchid boys undergoing abnormal mini-puberty fail to differentiate into Ad spermatogonia [Hadziselimovic and Herzog, 2001; Hadziselimovic et al., 2007; Hadziselimovic and Hoecht, 2008]. This developmental process requires increased gonadotropin and testosterone secretion [Hadziselimovic et al., 1986, 2005]. Several different physiological functions have been attributed to mini-puberty [Hutanhemi, 2003]. Our findings, however, suggest that the male fertility potential is established during this period [Hadziselimovic et al., 2005]. Furthermore, analyzing the whole genome expression profiling of testicular tissue, we found that cryptorchid boys lacking Ad spermatogonia had decreased expression of most of the genes essential for hypothalamo-pituitary-testicular axis function (table 1). In particular, *EGR4*, which is involved in regulating the secretion of luteinizing hormone, was virtually silent in the HAZR group [Hadziselimovic et al., 2009].

In the present study, the whole genome expression profiling analysis of prepubertal Ad-positive spermatogonia (Ad+) and Ad-negative spermatogonia (Ad–) testes yielded the unexpected result that several genes important for the meiotic and post-meiotic stages of spermatogenesis can be detected in Ad+ prepubertal testes but not in Ad– samples. A critical question arises as to which cell types express the mRNAs identified as being present in Ad+ but not in Ad– testes.

One would expect transcripts that are missing in samples from the azoospermia risk group patients to be predominantly expressed in Ad spermatogonia because these cells are absent in gonads with this pathology. Indeed, we found that a large group of genes for which no mRNA is detected in Ad– samples show peak expression in the LAZR as well as in the control testis group (table 1). Thus, it is conceivable that some of the molecular events initiating the testicular expression program at the onset of puberty and maintaining it during adulthood actually take place very early in prepubertal testes.

Moreover, 6 genes proposed to be specific biomarkers of adult human spermatogonia [von Kopylow et al., 2010] were expressed in the prepubertal Ad+ testes, while 4 genes were lacking or had lower expression in Ad– testes. This is further evidence that molecular events initiating the testicular expression program at the onset of puberty take place very early in infant testes, and that this program is impaired in the HAZR group.

We observed that many of the genes for which transcriptional activity was detected in Ad+ but not in Ad– testes are implicated in spermatogenesis and fertility. This group includes *MORC1*, encoding an ATPase-like protein required for the transition through meiosis [Watson et al., 1998; Inoue et al., 1999], *CLGN*, a factor required for sperm function [Ikawa et al., 2001], and the transcriptional inhibitor *ID4*, which was shown to be FSH- or cAMP-inducible in Sertoli cells [Norton et al., 1998; Chaudhary et al., 2001] as well as the tudor *(TDRD)* genes, which are also necessary for gametogenesis and rule the PIWI-interacting RNA pathway, helping to maintain the integrity of the genome and the development of the germ cells [Siomi et al., 2010] (table 1). Noticeably, expression of *Tdrd5* was absent in testes isolated from We/We mouse embryos, which completely lack germ cells [Smith et al., 2004]. Furthermore, a group of genes was identified that appear not to be expressed in juvenile Ad– gonads, although they display peak transcription in adult Sertoli cells. Since both Ad+ and Ad– gonads contain juvenile Sertoli cells, the differences might be caused by an absence of spermatogonia in SCO-type gonads or, more likely, impaired maturation of Sa-type Sertoli cells. It is noteworthy that this group includes *DUSP5*, a member of the dual-specificity protein phosphatase subfamily involved in MAP kinase and IL-2 signaling, the cell cycle and cell adhesion as well as influencing GnRH effect [Kovanen et al., 2003; Jeong et al., 2007; Armstrong et al., 2009], and *DMRTB1* from the DMRT family (doublesex and mab-3 related transcription factor) involved in the regulation of postnatal testis differentiation [Raymond et al., 2000]. The important testis developmental gene *SRY* (sex-determining region of Y chromosome), implicated in sexual development of the reproductive system and specification of Sertoli and Leydig cell lineages [Barsoum and Yao, 2006], was not expressed in the HAZR group (table 1). Furthermore, genes known to be essential for mouse stem cell self-renewal such as *Ret, Gfra1* and *Bcl6b* were more highly expressed in human prepubertal spermatogonia than in somatic cells [Wu et al., 2009]. A hypothesis was proposed that glial cell line-derived neurotrophic factor (GDNF) is essential for human stem cell (A spermatogonia) renewal [Wu et al., 2009]. Neither *GDNF* nor its regulatory genes *BHLHB2, LHX1, GFRA1*, and *TEC* were expressed in all groups studied. Additionally, 2 regulatory genes, *BCL6B* and *RET*, were equally expressed in all 3 groups. This contradictory observation may result from the different methods utilized: analysis of isolated germ cells versus in situ analysis. The strength of the current study is that spermatogonia were analyzed directly in their cognate microenvironment. Thus, our result seriously questions the hypothesis of GDNF-driven regulation of human Ad spermatogonia self-renewal.

Microdeletions of the long arm of the Y chromosome (Yq) involving 3 azoospermia factor (AZF) loci, AZFa, AZFb, and AZFc, represent one of the main molecular determinants of male infertility, and account for about 10% of cases of non-obstructive azoospermia or severe oligozoospermia [Stuppia et al., 1998]. The most frequent deletion of the Y chromosome is AZFc, b2/b4 [Kuroda-Kawaguchi et al., 2001]. However, among the 32 men with a history of cryptorchidism, only 1 had Y microdeletions [Fedder et al., 2004]. This result is consistent with additional studies in which it was not possible to detect Y microdeletions in any of 66 men treated for cryptorchidism in childhood [Fagerli et al., 1999; Vutyavanich et al., 2007]. Therefore, Y microdeletions as a cause of azoospermia in cryptorchidism are rather unlikely.

Among the deleted genes involved in azoospermia development, the main candidates for spermatogenesis failure are the *DAZ* gene, a testis-specific gene present in 4 copies within AZFc, encoding an RNA-binding protein, and the *RBMY* gene, which is declared to be the major AZFb gene [Yen, 2004; Vogt et al., 2005]. Furthermore, the cell division cycle molecule *CDC20* was found to be down-regulated in azoospermic testes [Yang et al., 2009]. Azoospermia associated with a decrease in *DAZ* gene function in humans may, in part, be a consequence of failure at synapses caused by reduced levels of SYCP3 protein [Reynolds et al., 2007]. We found that specimens from the azoospermia risk group did not exhibit *CDC20, DAZ1,2,3,4/DAZL*, *RBMY1A1*, and *SYCP3* gene expression (table 1). Recently, up-regulated expression of the *EGR4* gene was described in azoospermic men with AZFc deletion and lack of *DAZ* gene expression [Gatta et al., 2010]. The observed lack of *DAZ1* and *EGR4* gene expression in the HAZR group implies that the *EGR4* gene functions as an upstream regulator of the *DAZ/DAZL* gene pathway. It should be emphasized that prepubertal treatment of boys at risk for azoospermia and infertility (aged 6–36 months) with buserelin (luteinizing hormone releasing hormone analogue) restored full fertility in 86% of the cases (none had azoospermia), whereas all boys re-

ceiving surgery who were not treated became oligospermic, and 20% developed azoospermia [Hadziselimovic, 2006]. These data are in keeping with hypogonadotropic hypogonadism being critically involved in cryptorchidism [Hadziselimovic et al., 1986, 2004, 2009; Job et al., 1987; Hamza et al., 2001] and argue against primary testicular failure (testicular dysgenesis) [Cortes et al., 1996; Gracia et al., 2000; Toppari et al., 2007; Virtanen et al., 2007] as the major cause of azoospermia and infertility.

Taken together, our results support the notion that azoospermia and infertility induced by cryptorchidism are predominantly an endocrine disease of impaired mini-puberty rather than the mutation of the *CDC20, DAZ/DAZL/SYCP3* or *RBMY/RBMY1A1* genes. Finally, we present additional evidence that molecular events initiating the adult testicular expression program, occurring very early in prepubertal testes, were abnormal in cryptorchid boys at risk for azoospermia development. Thus, we found a transcript expression signature that distinguishes the high azoospermia and the low azoospermia risk groups.

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