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Deficient Expression of Genes Involved in the Endogenous Defense System against Transposons in Cryptorchid Boys with Impaired Mini-Puberty

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Adult/dark spermatogonia · Azoospermia · Cryptorchidism · Germ cell · Transposon

Abstract
Mini-puberty is the period between 30 and 80 days after birth when testosterone and gonadotropin surges occur in male infants to induce the transformation of gonocytes into adult/dark spermatogonia. Cryptorchid boys with impaired mini-puberty develop infertility despite timely and successful surgical treatment. The decreased germ cell count found in this group of boys could be the result of uncontrolled transposon activity inducing genomic instability and germ cell death. A genome-wide analysis of 18 cryptorchid and 4 control testes was performed with Affymetrix chips. We found that 5 of 8 genes that are important for transposon silencing were not expressed in the high azoospermia risk group of cryptorchid boys but were expressed in the low azoospermia risk and control groups. Two genes, *CBX3* and *DNMT1*, were equally expressed in all 3 groups. Impaired expression of the *DDX4, MAEL, MOV10L1, PIWIL2, PIWIL4,* and *TDRD9* genes in the group of cryptorchid boys at high risk of infertility indicates that gene instability induced by impaired expression of transposon silencing genes contribute to the development of azoospermia. Intact mini-puberty appears to be essential for the development of the endogenous defense system mediated by transposon silencing.

Cryptorchidism represents one of the most frequent causes of azoospermia in men [Fedder et al., 2004]. Typical pathological findings in testicular biopsies obtained during surgery from boys at risk of developing azoospermia is a complete lack of type Ad (adult/dark) spermatogonia [Hadziselimovic et al., 2011]. During testicular maturation, gonocytes from cryptorchid infants undergoing abnormal mini-puberty fail to differentiate into Ad spermatogonia due to insufficient gonadotropin and testosterone stimulation [Hadziselimovic et al., 1986, 2005]. Whole-genome expression profiling has shown that boys lacking Ad spermatogonia (i.e. stem cells for spermatozoa) show a lack or decrease in expression of most of the genes essential for hypothalamo-pituitary-testicular axis function [Hadziselimovic et al., 2009, 2011]. In particular, *EGR4*, which is involved in regulating the secretion of luteinizing hormone, was not expressed [Hadziselimovic et al., 2009]. In addition, our recent results support the notion that azoospermia and

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infertility induced by cryptorchidism are predominantly an endocrine disease of impaired mini-puberty rather than being due to a mutation of *CDC20, DAZ* and *DAZL, SCP3,* or *RBMY* genes [Hadziselimovic et al., 2011]. Vast territories of animal genomes are populated by numerous types of transposons (mobile genetic elements) that act predominantly as parasites, without being influenced by the impact on the host [Aravin and Hannon, 2008; Cordaux and Batzer, 2009]. Uncontrolled retrotransposon activity results in genomic instability and germ cell death [Frost et al., 2010]. In response to the danger posed by transposons, organisms have evolved an endogenous defense system that employs a particular class of small RNAs known as piwi-interacting RNAs (piRNAs) to identify and selectively silence transposons [Frost et al., 2010]. From the perspective of germ cells, transposon derepression is an extremely dangerous and highly undesirable event [Van der Heijden and Bortvin, 2009]. Transient relaxation of transposon silencing intrinsic to meiosis is viewed as either a prerequisite, or an unavoidable consequence of the reconfiguration of the chromatin structure of the meiotic chromosome [Van der Heijden and Bortvin, 2009]. In mutant animals, defective expression of one or more of the genes involved in transposon silencing, e.g. DEAD box polypeptide 4 *(DDX4)* [Kuramochi-Miyagawa et al., 2010], maelstrom *(MAEL)* [Soper et al., 2008], Moloney leukemic virus 10-like 1 *(MOV10L1)* [Frost et al., 2010], piwi-like 2 *(PIWIL2)*, *PIWIL4 (Drosophila)*, tudor domain containing 9 *(TDRD9)* [Shoji et al., 2009], chromobox homolog 3 *(CBX3)* [Brown et al., 2010], and DNA (cytosine-5)-methyltransferase 1 *(DNMT1)* [Bourc'his et al., 2001] resulted in the development of infertility. Whole genome expression analysis confirmed that the genes were expressed in normal prepubertal testes. Here, we describe deficient expression of 75% of genes involved in transposon silencing in cryptorchid boys at high risk of developing azoospermia (HAZR).

Patients and Methods

Testicular Biopsies

At our institution, it is routine practice to perform testicular biopsy during surgery for undescended testis. We think that testicular biopsy provides useful information about future semen quality and helps identify patients with atypical spermatogonia or carcinoma in situ. A 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 sized testicular biopsies from 22 boys were analyzed (19 testes from 18 boys with cryptorchidism and 4 contralateral descended testes from patients with one-sided testicular agenesis). However, not all contralateral testes in patients with one-sided 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 testicular fixation performed to prevent testicular torsion. Contralateral testes were considered to be normal if they exhibited accepted normal histology.

The mean age of the patients at surgery was 3.4 (95% CI 0.6–6.1) years, and for the controls, 3.9 (95% CI 2.3–5.4) years. All patients had an extensive clinical examination. We could not identify any clinical signs of developmental malformation or syndromes, and none of the patients had micropenis or hypospadias. We did not perform mutational analysis of main genes involved in the pathogenesis of Kallmann syndrome, however, none of the following genes, *INSL3, RXFP2/LGR8/GREAT, NR4A1, NR5A1, CYP19A1* (previously *CYP19*), *NR0B1/DAX1/AHC, KAL1, GNRHR, PROK2, LHB, ESR1, SOX2, KISS1R,* and *HOXA10* were found to be pathologically expressed in boys with isolated cryptorchidism [Hadziselimovic et al., 2010]. According to the histological analysis, biopsies were categorized into 3 groups: HAZR (n = 5), characterized by no Ad spermatogonia and total germ cell count per tubule <0.2; low azoospermia risk group (LAZR, n = 14), characterized by being Ad spermatogonia positive and germ cell count >0.2; and controls (n = 4). 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).

RNA Isolation and Microarray Analysis

An aliquot of each biopsy fraction flash frozen in liquid nitrogen and stored at –80°C was fixed in PBS containing 1% formaldehyde (Polio, France) and 1% fetal calf serum (Eurabbie, France). The sample was analyzed by DNA flow cytometry to determine its relative DNA content. An aliquot was processed using the Affymetrix target synthesis kits currently available for 3′ IVT arrays. Amplification was based on T7 RNA polymerase using oligo-dT primers, and the same arrays were used to minimize variability. Cell pellets and tissues were sheared in RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol. The aqueous phases were removed for RNA isolation.

Total RNA isolation was performed using the 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 a quality check on the 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 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 accession numbers E-TABM-130, E-TABM-174, and GEO (GSE25518).

Statistical Analysis and Interpretation of Microarray Data

Data analysis and gene filtering were 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 Benjamini-Hochberg false discovery rate correction implemented in the LIMMA package and adjusted with the false discovery rate method [Wettenhall and Smyth, 2004]. We selected probe sets with a log2 average contrast signal of at least 4.8, 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 >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 data 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.

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) using random primers. Real-time PCR runs were performed using the SYBR Fast Kit (Kapa Biosystems) with each gene-specific primer at 200 nM final concentration in a total volume of 17.5 µl. Wavelengths for source and detection were set at 470 and 510 nm, respectively. Gain was set at 8.33. The PCR program was 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 (65–95°C, rising by 0.65°C during each step) to confirm 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 expression level. These genes were selected because they showed the smallest variation between individual samples (both on microarrays and qPCR). Fold differences were calculated using the ΔCt method.

Correlation of microarray data with data obtained by qRT-PCR on 4 genes that displayed significant expression changes, showing a high correlation in the obtained transcriptional profiles. Spearman's correlation coefficient was used for data comparison; *DLX2*: rs = 0.918, p = 0.00043; *DDX25*: rs = 0.945, p = 0.00014; *MAGEA4*: rs = 0.880, p = 0.000026; *EGR4*: rs = 0.838, p = 0.0038.

Antibody Validation

For immunohistochemical analysis, the Epon was removed from the tissue sections. The sections were treated with 2% bovine serum albumin to reduce nonspecific binding and then incubated with the primary antibody overnight at 4°C. All samples were washed with PBS between incubations. We validated 10 antibodies: DDX25, EGR1, EGR4, CBL, ALDH1A2, KLF4, MAGEA4, MOV10L1, COL4A3, and TDRD9 (online suppl. table 1, www.karger.com/doi/10.1159/000335188). 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 primary antibody binding. The chromogenic reaction was developed by adding a freshly prepared 3,3-diaminobenzidine solution (DAB + Chromogen; DAKO). The DAB reaction was terminated by washing the sections in Tris-Buffered Saline (0.05 M TBS and 0.85 M NaCl, pH 7.6).

To visualize the histology of the 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 nonspecific 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. Interstitial staining observed in the presence of the primary antibody was considered 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 specified for immunohistochemistry experiments [Deutsch et al., 2008].

Ethical Considerations and Approval

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

Results

Whole genome expression analysis indicated differential expression of genes responsible for control of the endogenous defense system among the 3 groups analyzed (table 1).

Among 8 genes known to participate in transposon silencing and fertility in male animals, 5 *(DDX4, MAEL, MOV10L1, PIWIL4,* and *TDRD9)* were not expressed in the HAZR group of cryptorchid boys (table 1). Furthermore, *PIWIL2* was weakly expressed in the HAZR group, but it was significantly less expressed than in the LAZR and control groups (table 1). Two genes known to silence transposons, *CBX3* and *DNMT1*, were strongly expressed in all 3 groups (table 1). MOV10L1 and TDRD9 proteins were localized in the cytoplasm of the germ and Leydig cells, whereas Sertoli cells showed no histochemical reaction to MOV10L1 and TDRD9 antibodies and did not

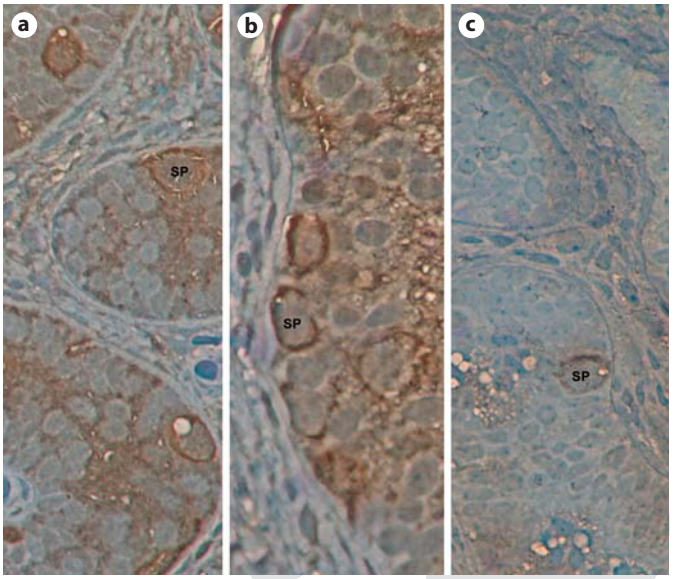

Fig. 1. Immunohistochemical staining for *MOV10L1* in the LAZR (**a**), control (**b**) and HAZR (**c**) group. In all cases antibody binding was visualized using peroxidase as a substrate to generate brown color. Sections were counterstained with toluidine blue. SP = Immunopositive spermatogonia. Magnification 63×.

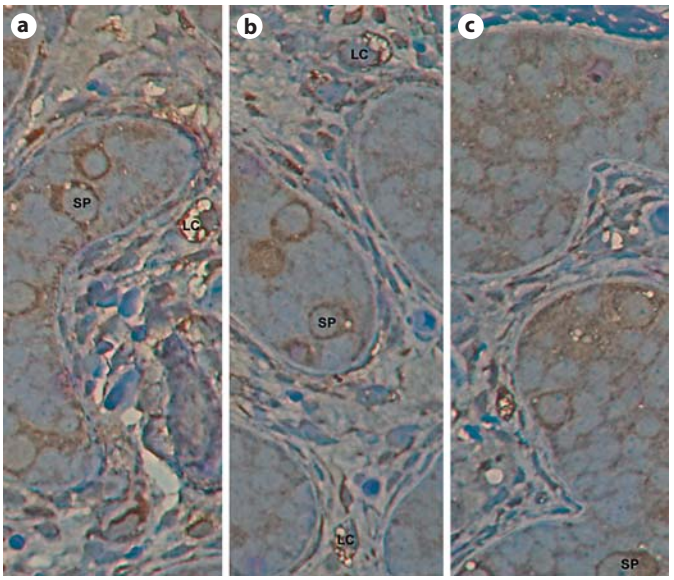

Fig. 2. Immunohistochemical *TDRD9* staining in LAZR (**a**), control (**b**) and HAZR (**c**) group. Cytoplasm of both spermatogonia (SP) and Leydig cells (LC) was immunopositive. Background staining was performed with toluidine blue. Magnification 63×.

Table 1. Differentially expressed genes (log2 average contrast signal-median values) involved in transposon silencing in HAZR (high azoospermia risk), LAZR (low azoospermia risk) and control group (p = adjusted p)

	DDX4	*MAEL*	*MOV10L1*	*PIWIL2*	*PIWIL4*	*TDRD9*	*CBX3*	*DNMT1*
HAZR	3.47	4.6	3.8	5.1	4.7	4.75	10.18	7.23
Control	7.43	7.6	4.9	6.47	7.57	6.92	10.45	7.48
	p < 0.001	p < 0.004	p < 0.003	p < 0.002	p < 0.001	p < 0.01	n.s.	n.s.
LAZR	7.05	7.5	4.1	6.88	7.15	6.52	10.4	7.46
HAZR	3.47	4.6	3.8	5.1	4.7	4.75	10.18	7.23
	p = 8.25E-05	p < 0.0001	n.s.	p < 0.0001	p < 0.0001	p = 0.009	n.s.	n.s.
Control	7.43	7.6	4.9	6.47	7.57	6.92	10.45	7.48
LAZR	7.05	7.5	4.1	6.88	7.15	6.52	10.4	7.46
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

stain with peroxidase (fig. 1, 2). In the HAZR group, only weak staining for TDRD9 and MOV10L1 was observed in the cytoplasm of the germ cells and Leydig cells (fig. 1, 2). *TP63* (tumor protein 63) gene, which is responsible for germline stability, was not expressed in the HAZR group while strong expression was found in the control group ($p < 0.007$).

Discussion

During the last 40 years histology has contributed significantly to our understanding of the etiology of cryptorchidism. In 1975 we described pronounced Leydig cell atrophy starting in early infancy as the evidence to support endocrinopathy as an etiologic factor of cryptorchi-

dism [Hadziselimovic et al., 1975]. Development of Ad spermatogonia from gonocytes, which takes place during the first months of life, has been shown to be testosterone dependent and is impaired in the majority of cryptorchid boys [Hadziselimovic et al., 2005; Zivkovic et al., 2007; Hadziselimovic and Hoecht, 2008]. A testosterone and free-androgen deficiency was observed in cryptorchid infants, indicating a disturbance of testicular function that was evident early after birth [Pierik et al., 2009]. However, the analysis of peripheral blood leukocytes showed no evidence for involvement of genes underlying idiopathic hypogonadotropic hypogonadism in isolated cryptorchidism [Laitinen et al., 2011]. As the authors used DNA obtained from peripheral blood leukocytes, they could not rule out, however, the possibility of tissue mosaics of *FGFR1* mutations, previously described in a family with idiopathic hypogonadotropic hypogonadism [Sato et al., 2006]. This is defined by absent or incomplete puberty and characterized biochemically by low levels of sex steroids, with low or inappropriately normal gonadotropin hormones [Mitchell et al., 2011]. Boys at high risk of infertility according to testicular histology had decreased basal and stimulated plasma levels of luteinizing and follicle stimulating hormone [Hadziselimovic et al., 1975]. Notably, in testicular tissue from boys with isolated cryptorchidism, we found decreased expression of *FGFR1, SOS1* and *RAF1* genes [Hadziselimovic et al., 2010]; all boys in the high infertility group have had a normal pubertal development, indicating a major gene defect was not present [Hadziselimovic and Hoecht, 2008]. Nevertheless, cryptorchidism represents a symptom of different clinical disorders involving classical syndromes, ascended secondary cryptorchidism, and congenital isolated cryptorchidism.

The hallmark of successful treatment of cryptorchid boys is fertility. Ad spermatogonia, the accepted stem cells for mature spermatogenesis, have been identified as a key marker of potential fertility; 94% of men who have Ad spermatogonia in biopsies taken at the time of orchidopexy have normal sperm counts, whereas 92% of men without Ad spermatogonia at the time of orchidopexy have abnormal spermiograms [Hadziselimovic and Herzog, 2001; Hadziselimovic et al., 2007]. Thus, differences in sperm production can be predicted by the presence or absence of Ad spermatogonia. A typical histological finding in the HAZR group is a severe reduction of the total number of germ cells including Ad spermatogonia as early as the second year of life. Undescended testicular position is unlikely to be a reason for massive germ cell loss since *SRD5A2* deficient boys with bilateral cryptorchid testicular position have normal numbers and differentiation of spermatogonia during the whole prepubertal period [Hadziselimovic and Dessouky, 2008]. An alternate explanation for massive germ cell death in the HAZR group could be an insufficient endogenous defense system against transposons. Approximately 45% of the human genome is derived from transposable elements, the majority of which originate from retrotransposons [Cordaux and Batzer, 2009]. In somatic cells, retrotransposons are suppressed by DNA methylation, but they are not suppressed in male germ cells when, during a short period of intensive epigenetic reprogramming, DNA methylation is temporarily lost [Aravin and Bourc'his, 2008]. An endogenous piRNA-based defense system that silences retrotransposon activity in germ cells through both transcriptional and posttranscriptional mechanisms was discovered [Aravin and Hannon, 2008]. Recent studies revealed a crucial role of *MOV10L1* in male fertility and piRNA-directed retrotransposon silencing in male germ cells, suggesting that *MOV10L1* functions as a key component of a safeguard mechanism for the genetic information in the male germ cells of mammals [Frost et al., 2010]. In addition, TDRD9 has been shown to be essential for silencing the Line-1 retrotransposon in the mouse male germline. *Tdrd9* encodes an ATPase/DExH-type helicase, and its mutation causes male sterility with meiotic failure [Shoji et al., 2009]. In fetal testes, *Tdrd9* mutation causes Line-1 desilencing and an aberrant piRNA profile in prospermatogonia, followed by cognate DNA demethylation [Shoji et al., 2009]. Both *Piwil4/Miwi2* and *Dnmt3l* are involved in the DNA methylation of interspersed repeats during spermatogenesis, and mutation of either *Miwi2* or *Dnmt3l* results in a Sertoli cell-only phenotype and the loss of DNA methylation of transposons, resulting in their ectopic expression [Bourc'his et al., 2001; Carmell et al., 2007]. *VASA/DDX4* is an evolutionarily conserved RNA helicase essential for germ cell development [Kuramochi-Miyagawa et al., 2010]. Retrotransposon expression is elevated in *Mvh* (mouse *VASA* homolog)-deficient mice [Kuramochi-Miyagawa et al., 2010]. Studies of *MAEL* mutant testes suggest a close functional relationship of MAEL with MIWI2 and MILI/PIWIL2, two of the 3 murine piwi-like proteins [Kuramochi-Miyagawa et al., 2004; Cordaux and Batzer et al., 2009]. Disruption of *MAEL* results in a profound defect in the synapses of homologous chromosomes in male meiosis, indicating that MAEL, a component of the germ-cell-specific organelle nuage, is indispensable for transposon silencing and identifies the initiation of meiosis as an important

step in transposon control in the male germline [Soper et al., 2008]. We found that *MOV10L1, TDRD9*, *PIWIL2*, *PIWIL4, DDX4* and *MAEL* are activated in prepuberty before the onset of meiosis in control and cryptorchid testes from the LAZR group. In the HAZR group, weak expression of *PIWIL2* and the lack of expression of *PIWIL4, MOV10L1, TDRD9, DDX4* and *MAEL* indicate defective transposon silencing activity for these genes. Loss of expression and weak cytoplasmic staining in cryptorchid testes indicate that, in humans, *MOV10L1* is important for both stem cell maintenance and renewal. Integration of an endogenous retrovirus upstream of the *TP63* locus increased germline stability [Beyer et al., 2011]. In contrast to controls, HAZR patients lacked expression of the *TP63* gene.

Noticeably, the *DNMT1* and *CBX3* genes known to participate in transposon silencing, were equally expressed in all groups. The *Cbx3hypo/hypo* spermatogenesis defect is comparable to that found in *Miwi2* and *Dnmt3l* mutants [Brown et al., 2010]. Furthermore, identical expression of the *CBX3* and *DNMT1* genes in all groups studied indicates gonadotropin and testosterone epigenetic independence in contrast to 6 other genes that are seemingly dependent on intact mini-pubertal gonadotropin and testosterone increases.

In conclusion, we observed that the majority of genes responsible for transposon silencing were not expressed in the HAZR group of cryptorchid boys, indicating that this altered expression may be responsible for the massive germ cell loss in these patients.

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