Piwi-Pathway Alteration Induces LINE-1 Transposon Derepression and Infertility Development in Cryptorchidism

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Abstract

Spermatogonia contain processing bodies that harbor P-element-induced wimpy testis (Piwi) proteins. Piwi proteins are associated specifically with Piwi-interacting RNAs to silence transposable DNA elements. Loss-of-function mutations in the Piwi pathway lead to derepression of transposable elements, resulting in defective spermatogenesis. Furthermore, deletion of gametocyte-specific factor 1 *(GTSF1)*, a factor involved in Piwi-mediated transcriptional repression, causes male-specific sterility and derepression of LINE-1 (L1) retrotransposons. No previous studies have examined *GTSF1*, L1 and *PIWIL4* expression in cryptorchidism. We examined transposon-silencing genes and L1 transposon expression in testicular biopsies with Affymetrix microarrays and immunohistology. Seven members of the Tudor gene family, 3 members of the DEAD-box RNA helicase family, and the *GTSF1* gene were found to show significantly lower RNA signals in the high-infertility-risk group. In the immunohistochemical analysis, patients from the low-infertility-risk group showed coherently stronger staining for GTSF1 and PIWIL4 proteins and weaker staining for L1 transposon when compared to the high-infertility-risk samples. These new findings provide first evidence consistent with the idea that infertility in cryptorchidism is a consequence of alterations in the Piwi pathway and transposon derepression induced by the impaired function of mini-puberty.

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Forty years ago, the ultrastructure of processing bodies (P-bodies), previously known as intermitochondrial cement, in spermatogonia of normal and cryptorchid boys was described [Seguchi and Hadziselimovic, 1974; Hadziselimovic, 1977]. At that time, no adequate interpretation was given regarding the observed P-body malformation in cryptorchid spermatogonia [Hadziselimovic, 1977].

Today we know that P-bodies are cytoplasmic organelles containing dynamic aggregates of specific mRNAs and proteins [Castañeda et al., 2011]. P-bodies exhibit a complex mechanism regulating the organization and function of P-element-induced wimpy testis (Piwi) proteins and small Piwi-interacting RNAs in transposon-silencing pathways. This P-body mechanism includes a methylation-directed protein-protein interaction, mediated by germline Tudor domain proteins and methylated Piwi family proteins [Chen et al., 2009].

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Also, a recent report has shown that the gametocyte-specific factor 1 *(GTSF1)* is an essential factor in Piwi-mediated transcriptional repression [Dönertas et al., 2013]. In addition, the androgen-dependent RNA helicase, DEAD (Asp-Glu-Ala-Asp)-box polypeptide 4 *(DDX4)* [Stanton et al., 2012], was found to be essential for the organization and presumably for the function of P-bodies [Kuramochi-Miyagava et al., 2010]. Thus, silencing transposable DNA elements requires intact P-bodies that harbor Piwi proteins specifically associated with Tudor family genes *(TDRD)*, DEAD-box RNA helicase *(DDX)*, and *GTSF1*. This transposon-silencing mechanism maintains the integrity of the genome and promotes the development of gametes. Malfunction of the Piwi-interacting RNA pathway leads to derepression of multiple transposon families in mouse germ cells and is associated with sterility [Aravin et al., 2007]. It is postulated that sterility is most likely caused by the occurrence of double-strand DNA breaks generated by transposition events [Klattenhoff et al., 2007]. In a previous study, we found impaired expression of *DDX4*, *MAEL*, *MOV10L1*, *PIWIL2*, *PIWIL4*, and *TDRD9* genes in boys with cryptorchidism, who exhibited a high infertility risk (HIR) due to altered mini-puberty function [Hadziselimovic et al., 2011a]. That finding indicated that gene instability is induced by impairment in the endogenous system that defends against transposons; we hypothesized that this instability may contribute to the development of azoospermia.

The objective of the present study was to determine whether alterations in transposon-silencing gene expression and LINE 1 (L1) transposon derepression were correlated to infertility in cryptorchidism. Therefore, we analyzed gene expression and protein levels in testicular biopsies from boys with cryptorchidism and compared the expression patterns between HIR and LIR groups.

Patients and Methods

Testicular Biopsies and Patient Groups

All patients had an extensive clinical examination, and we could not find any clinical signs of developmental malformations or syndromes. Furthermore, all patients had normal thyroid screening, and no features of hypopituitarism were discernible. At surgery, the testicular position in HIR patients was 1 abdominal and 6 tubercle, and in low-infertility-risk (LIR) patients, 1 inguinal and 11 tubercle. One patient from the HIR group and one from the LIR group had hormone treatments with LH-RH 5 months prior to surgery. At our institution, it is routine practice to perform a testicular biopsy during surgery for undescended testes. We have found that testicular biopsies provide useful information about the future quality of semen, and this information may be used to identify patients with atypical spermatogonia or carcinoma in situ. A cryptorchid testis is defined as a testis localized outside the scrotum, which is not amenable to being moved into a stable scrotal position. In the present study, for microarray analysis, we included 19 rice-grain-sized testicular biopsies. The HIR group (n = 7) is characterized by a lack of Ad spermatogonia and a total germ cell count <0.2 spermatogonia/tubule. The LIR group (n = 12) is characterized by ≥0.1 Ad spermatogonia per tubular cross section and a total germ cell count ≥0.6 spermatogonia/tubule. The 2 groups were comparable in age, with mean ages of 2.9 years (95% CI, 0.5–5.84) for the HIR group and 3.6 years (95% CI, 2.78–4.41) for the LIR group. The biopsy specimens were immediately fixed in 3% glutaraldehyde containing PBS (pH 7.6). Post-fixation was performed in 1% OsO_4. Biopsies were embedded in Epon 812. Semithin sections were stained with toluidine blue and analyzed with a Zeiss-Axio-Scope light microscope. Six-hundred-ångström-thick silver sections were cut with a Reichert ultramicrotome, and stained with uranyl acetate and lead citrate. Ultrathin sections were analyzed with a Zeiss EM 9A electron microscope. RNA isolation, microarray analysis and quantitative real-time PCR examination were previously published [Hadziselimovic et al., 2009, 2011b].

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 log_2 average contrast signal of at least 4.1, an adjusted p value <0.05, and an absolute log_2-fold change >0.585 (1.5-fold in linear space). Complete statistical analysis details were previously published [Hadziselimovic et al., 2011b].

Antibody Analysis

For immunohistochemical analysis, Epon was removed from the tissue sections. The sections were treated with 2% bovine serum albumin to reduce nonspecific binding and then incubated with primary antibody overnight at 4°C. All samples were washed with PBS. We analyzed L1 (sc-67197), GTSF1 (sc-240545), PIWIL4 (sc-67593) primary antibody (Santa Cruz Biotechnology Inc.), and INSL3 (ab65981) (Abcam). Secondary antibodies, labeled with polymer-horseradish peroxidase (goat polyclonal, anti-rabbit IgG, mouse IgG and IgM) (Abcam) were used to detect 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 (0.005 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. 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. The interstitial and intratubular 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.

Table 1. Transcript signature of differentially expressed genes involved in the Piwi pathway and germ cell development in HIR and LIR groups

Gene[a]	Affymetrix No.	HIR	LIR	p value[b]
TDRD1	221018 s-at	3.08	4.1	0.0003
TDRD2 (TDRKH)	223530-at	4.85	6.14	0.01
TDRD3	214028-at	6.6	7.05	n.s.
TDRD4 (RNF17)	220270-at	4.17	5.40	0.015
TDRD5	231104-at	3.78	4.92	0.015
TDRD6	232692-at	5.1	6.79	0.0003
TDRD7	213361-at	6.78	7.66	n.s.
TDRD8 (STK31)	223883 s-at	4.4	8.25	$8.24\,E^{-0.5}$
TDRD9	228285-at	4.7	6.52	0.009
TDRD10	231371-at	5.25	6.17	n.s.
TDRD11	2022-at16	8.51	8.6	n.s.
TDRD12	215356-at	4.69	4.54	n.s.
DDX4	221630-at	3.47	7.05	$8.24\,E^{-2}$
DDX25	219945-at	4.39	5.86	0.001
DDX43	220004-at	4.76	7.24	0.0002
GTSF1	227711-at	4.6	7.27	0.0001

[a] log$_2$ signal intensity; average contrast signal median values. [b] Adjusted for all values. Significance was accepted at $p < 0.05$. n.s. = Not significant.

Fig. 1. PIWIL4. **A** Weak PIWIL4 protein staining localized in the germ cells of HIR testis (arrow). In all cases, antibody binding was visualized using peroxidase as a substrate to generate brown color. Sections were counterstained with toluidine blue. **B** LIR testis. Intensive PIWIL4 staining is observed in the cytoplasm of spermatogonia (arrows). Scale bars = 10 μm.

For immunohistochemical analysis, 12 biopsies from the LIR group and 7 from the HIR group were analyzed. In addition, we analyzed 6 biopsies from contralateral descended testis, 3 from each group. Two examiners (F.H. and G.K.) independently analyzed the stain intensity in blind fashion. The incidence of weak (+ or ++) and strong (+++ or ++++) stain intensity was recorded and compared. 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].

Ethics

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

Results

RNA Profiling

GTSF1 gene expression was at the limit of detection in HIR samples, but was strongly expressed in LIR samples. Twelve *TDRD* transcripts (table 1) are expressed in prepubertal spermatogonia. We found only weak *TDRD1* and *TDRD5* expression in the LIR group and no *TDRD1* or *TDRD5* expression in the HIR group. *TDRD1* is known
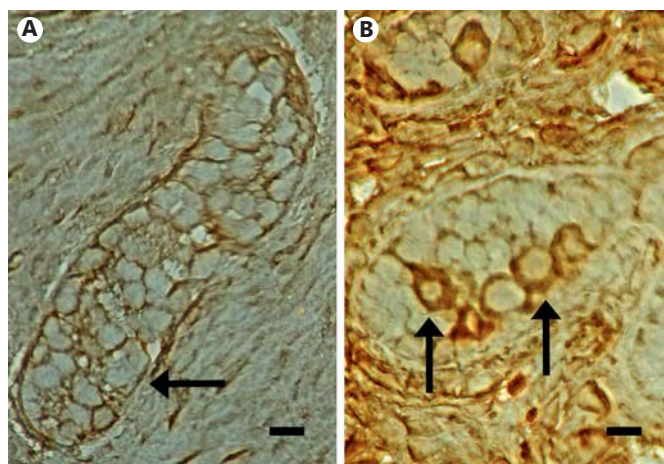
to be required for spermatogenesis, and it is strongly expressed in the testis after puberty [Lardenois et al., 2010]. The *TDRD2* (or *TDRKH*) gene was expressed in the LIR group but showed only weak expression in the HIR group. *TDRD3*, the ubiquitously expressed, transcriptional coactivator that binds methylarginine to histone tails, showed strong expression in both HIR and LIR groups. *TDRD4* (or *RNF17*) was expressed at the limit of assay detection in the HIR group, but they were reasonably expressed in the LIR group. The largest difference between the groups was in the expression of *TDRD8* (or *STK31*). Clear differences between the groups were also observed in the expression levels of *TDRD6* and *TDRD9*. Notably, the 2 groups showed equivalent expression levels for *TDRD10*, *TDRD11* (or *SND1*) and *TDRD12*. The *TDRD7* gene is essential for early spermatid differentiation, and it is a master regulator in the biogenesis of chromatoid bodies [Tanaka et al., 2011]. *TDRD7* showed strong expression in all testes studied.

The testosterone-dependent RNA helicase, *DDX4*, which is essential for the organization of intermitochondrial cement, was not expressed in the HIR group, but was strongly expressed in the LIR group. Furthermore, gonadotropin-dependent *DDX25* helicase [Tsai-Morris et al., 2004] as well as *DDX43* RNA helicases were differentially expressed in the HIR and LIR groups (table 1).
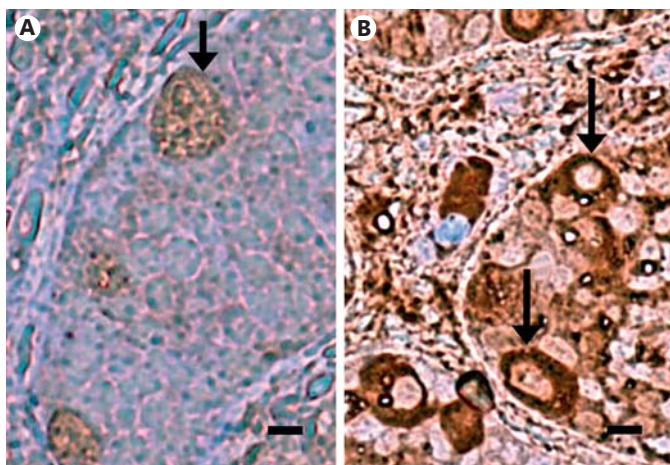
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Fig. 2. GTSF1. **A** In contrast to strong accumulation of the GTSF1 protein in LIR germ cells, a weak brown staining of the GTSF1 protein is found in the germ cells of HIR testis (arrow). **B** GTSF1 is strongly stained in the germ cells of LIR testis (arrows). For staining, see figure 1. Scale bars = 10 μm.
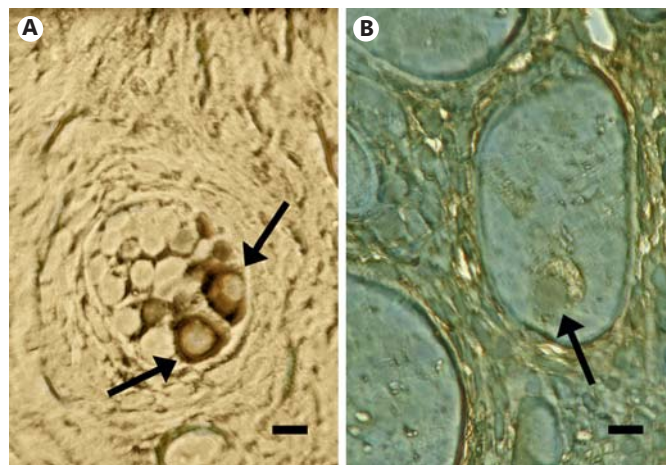

Fig. 3. LINE-1. Cryptorchid testis. **A** Intra-abdominal (HIR) testis with small atrophied tubule and extensive interstitial fibrosis. Spermatogonia show a strong immunohistochemical staining for LINE-1 retrotransposons (arrows). **B** In contrast, spermatogonia from LIR testes show a weak immunohistochemical staining (arrow). For staining, see figure 1. Scale bars = 10 μm.
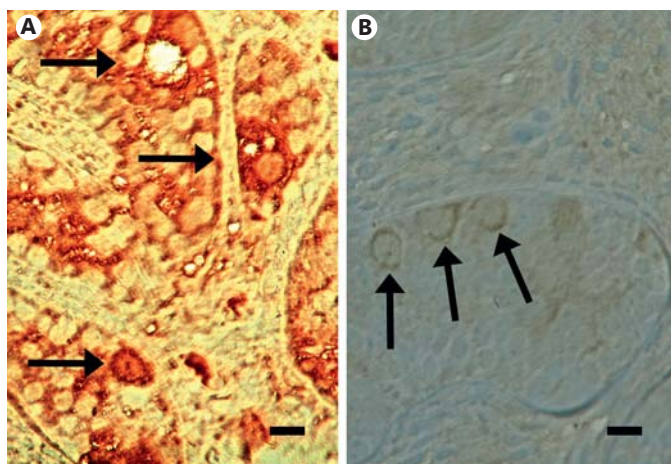

Fig. 4. LINE-1. Contralateral descended testis in unilateral cryptorchid boy. **A** Strong LINE-1 peroxidase staining (arrows) in spermatogonia from contralateral descended testis (HIR group). **B** Weak immunohistochemical staining for LINE-1 proteins in the germ cells of contralateral descended LIR testis (arrows). For staining, see figure 1. Scale bars = 10 μm.

Immunohistochemical Validation

PIWIL4 protein was strongly expressed in the LIR group and weakly stained in the HIR group (fig. 1). The GTSF1 protein was found in both groups of cryptorchid testes, but the staining was stronger in the LIR group than in the HIR group (fig. 2). The L1 protein was detected in the germ cell cytoplasm in all testes analyzed. However, more intense staining (+++ and ++++) was observed in HIR than in LIR spermatogonia (+ or ++) (fig. 3, 4). Immunohistochemical data are credible because the control gene *INSL3*, which shows no differential mRNA expression, remains also constant at protein level (fig. 5). Moreover, altered P-body morphology was found in most spermatogonia in the HIR group (fig. 6).

Discussion

This study shows that males with cryptorchidism and impaired mini-puberty have alterations in Piwi pathway gene expression, which are different in HIR and LIR individuals; thus, these alterations are implicated in the development of infertility. In turn, altered function of malformed P-bodies failed to repress transposon activity.

It is widely accepted that exposure of the cryptorchid testis to abnormally high temperatures induces germ cell loss and infertility [Setchell, 1998]. However, the temperature effect cannot explain several findings. For example, both HIR and LIR males with unilateral cryptorchidism have one descended testis that escapes the temperature influence; however, in identically aged groups, surgery produced completely different fertility outcomes in HIR and LIR individuals [Hadziselimovic and Hoecht, 2008]. On the other hand, early orchidopexy, performed before
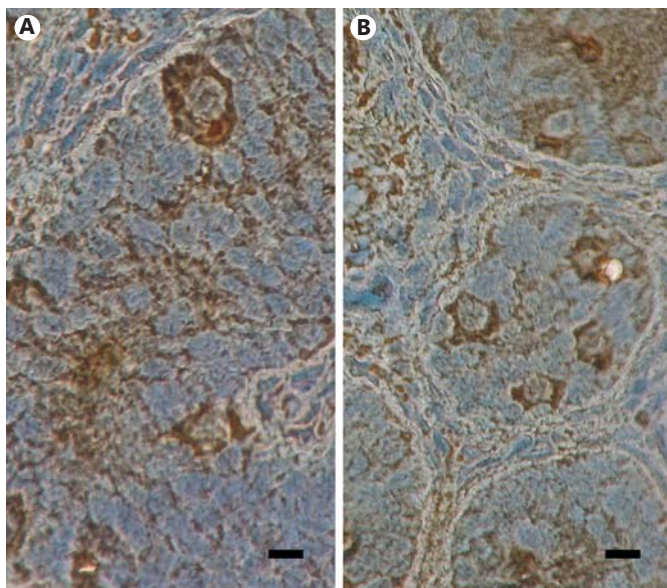
Fig. 5. INSL3. Identical INSL3 immunohistochemical protein staining in HIR (**A**) and LIR (**B**) spermatogonia (brown) validates identical INSL3 transcripts levels observed in both groups. The $\log_2$ intensity of *INSL3* gene expression was 8.96 in HIR and 8.59 in LIR testes. Scale bars = 10 μm.

Fig. 6. P-body ultrastructure. **A** Ap spermatogonium from testis of an HIR boy. P-bodies are missing (arrow). Scale bar = 1 μm. **B** Normal ultrastructure of P-body of a 5-year-old boy showing intermitochondrial cement regularly distributed between mitochondria of a crysta type (arrow). Scale bar = 0.1 μm.

assumed temperature-induced testicular damage, did not improve fertility in one third of cryptorchid males [Hadziselimovic and Herzog, 2001]. These failures were explained by impaired transformation of gonocytes into Ad spermatogonia, which resulted from alterations in *EGR4* gene expression and gonadotropin as well as testosterone secretion during mini-puberty [Hadziselimovic et al., 2005, 2009]. Another example of infertility that could not be explained by temperature exposure was evidenced by the development of boys with deficiencies in the steroid 5-alpha reductase gene, *SRD5A2*, which leads to bilateral cryptorchidism. These boys exhibited normal spermatogonia numbers and normal differentiation during the entire prepubertal period. Those findings were in sharp contrast to observations in boys with normal *SRD5A2* levels that developed isolated bilateral cryptorchidism and showed identical testicular malpositioning [Hadziselimovic and Dessouky, 2008]. Because the testicular positions in these 2 groups of boys are identical, their testes would be expected to have experienced the same temperature exposure. Therefore, if temperature were the only explanation for a massive germ cell loss in bilateral cryptorchidism, the 2 groups should have exhibited identical germ cell loss. However, this was not the case [Hadziselimovic and Dessouky, 2008]; thus, at the
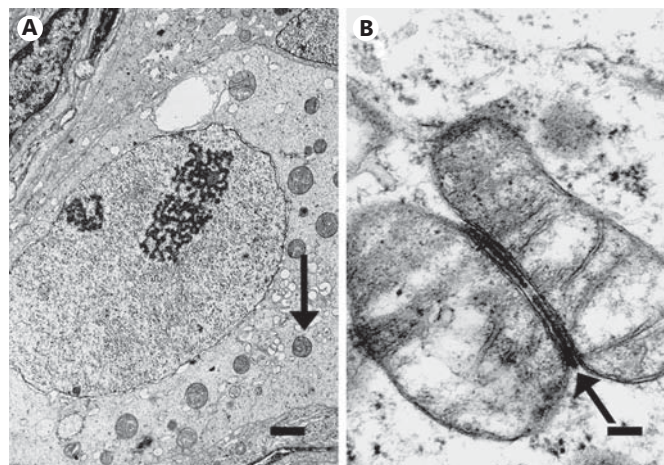
level of spermatogonia, temperature appears to have only a negligible effect.

Based on our results, we propose that an insufficient endogenous defense system against transposons may be responsible for massive germ cell death and infertility.

Selfish mobile elements, or transposons, subject the genome to severe biological threat by moving to positions that alter important genome information and organization [Kazazian, 2004]. Epigenetic reprogramming, including the erasure and resetting of DNA methylation, takes place in primordial germ cells. This reprogramming is likely to be important in preventing the passage of DNA methylation defects from one generation to the next [Castañeda et al., 2011; Bortvin, 2013]. However, the most important role for epigenetic reprogramming is to restore the developmental potency of the germline and to prepare the genome for the establishment of male-specific DNA methylation patterns in imprinted genes [Bortvin, 2013].

PIWI, *DDX* and *TUDOR* family genes are implicated in germline development because they participate in controlling transposons through the Piwi-small RNA silencing pathway. This pathway for transposon control is intimately integrated into germ cell development [Tanaka et al., 2011; Chuma and Nakano, 2012]. Piwi-associated

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small RNAs can act independently or they can cooperate with chromatin modifications to achieve long-lasting effects necessary for cellular memory and transgenerational inheritance [Stuwe et al., 2014]. PIWIL4 is predominantly expressed in the mouse spermatogonia during the period of de novo DNA methylation [Bortvin, 2013]. Earlier studies have revealed that the loss of Piwi-like genes, *MILI/PIWIL2* and *MIWI2/PIWIL4*, or the loss of *TUDOR* genes causes the overexpression of L1 transposons to levels much higher than the levels detected in wild-type testes [Aravin et al., 2007; Kuramochi-Miyagawa et al., 2008; Shoji et al., 2009]. Testosterone plays a key role in the regulation of spermatogenesis [Walker, 2010]. Recently it has been shown that testosterone alters testicular function through regulation of the Piwi-interacting RNA expression [Kang et al., 2014]. Mutant mice with insufficient testosterone secretion expressed less *Miwi/Piwil1*, a transposon-silencing gene [Gill-Sharma et al., 2012]. Moreover, mice deficient in each of the genes essential for silencing the L1 retrotransposons were sterile [Chuma and Nakano, 2012; Bortvin, 2013].

We found that HIR individuals with altered hypothalamic-pituitary-gonadal axis function show little or no expression of the PIWIL4 protein [Hadziselimovic et al., 2011a] and 7 of the 12 *TDRD* family members that are important for spermatogenesis (table 1). This deficiency is accompanied by underexpression of *DDX4* and *DDX25* genes, which are dependent on gonadotropin and testosterone stimulation. Interestingly, sterile mice with *Vasa (Ddx4)* deficiencies had malformed P-bodies with an ultrastructural appearance identical to that observed in our previous study [Hadziselimovic, 1977; Kuramochi-Miyagawa et al., 2010]. Accordingly, we confirm that impaired expression of key genes in the Piwi pathway and derepression of L1 retrotransposons accompanied by decreased PIWIL4 protein synthesis in the HIR group is involved in the development of infertility in human cryptorchidism.

GTSF1 is a member of the evolutionarily conserved *UPF0224* gene family, which is involved in retrotransposon suppression and has been found to be expressed predominantly in germ cells. It has also been demonstrated that *GTSF1*-null mice exhibited male-specific sterility. Cells lacking *GTSF1* contained nuclear Piwi proteins that were loaded with small RNAs, but the transposon-silencing capacity was ablated [Dönertas et al., 2013]. Our observation that HIR testes show insufficient *GTSF1* expression suggests that infertility may be additionally associated with a lack of *GTSF1* expression contributing to the development of L1 overexpression.

In conclusion, our results suggest that germ cell maldevelopment in cryptorchidism is accompanied by a hormonal imbalance and a perturbation in germ cell-specific gene expression during mini-puberty. An HIR group of cryptorchid boys exhibited altered Piwi-pathway gene expression, which permits overexpression of transposons and culminates in the development of infertility or sterility. This novel observation implies that the growth and formation of P-bodies during mini-puberty are regulated by hormones, and that P-bodies contribute to the establishment of germ cell memory and male-specific DNA methylation pathways.

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