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Molecular Pathology of Cryptorchidism-Induced Infertility

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Key Words
Cryptorchidism · *EGR4* · Infertility · Molecular pathophysiology · Piwi pathway · Transposon

Abstract
Cryptorchidism is the most common cause of non-obstructive azoospermia in man. In contrast to the general belief that temperature-dependent effects on the undescended gonad damage cryptorchid testes before sexual maturation is complete, molecular pathology strongly supports the theory that impaired mini-puberty is responsible for azoospermia and infertility in cryptorchidism. Molecular biological observations favor LH deficiency, with *EGR4* as a master regulatory gene in Leydig cell dysgenesis, as the reason for impaired mini-puberty, and recent evidence supports the idea that infertility in cryptorchidism is a consequence of alterations in the Piwi pathway.

Cryptorchidism is considered as a disease with complex etiology, in which hormonal, genetic, anatomical, and environmental factors are involved. Some evidence is consistent with a genetic component playing a role in the etiology of cryptorchidism. Familial cases have been described, and a family history of undescended testes represents a risk factor [Foresta et al., 2008]. Syndromic cryptorchidism, usually accompanied by other genital and/or extragenital features, often results from single gene abnormalities. In the case of isolated cryptorchidism, however, the evidence is weaker due to the multifactorial nature of the trait, its heterogeneous manifestation and the existence of potential gene-environment interactions. In experimental animal models, deletion of genes involved in testicular descent, such as *Insl3* and its receptor *Rxfp2*, *Hoxa10*, *Hoxa11*, or *Ar* causes cryptorchidism. However, mutations in their human orthologs cannot explain cryptorchidism since they are infrequent and also present in the normal population [Foresta et al., 2008]. Moreover, isolated cryptorchidism was found not to be commonly associated with defects in genes involved in hypogonadotropic hypogonadism, such as *FGRF1*, *GNRHR*, *PROK2*, *TAC3*, or *TACR3* [Laitinen et al., 2011]. A summary of gene mutations associated with isolated congenital cryptorchidism is shown in table 1 [Tannour-Louet et al., 2010].

The incidence of azoospermia in unilateral cryptorchidism is as high as 13%, regardless of treatment, and increases to 89% in untreated bilateral cryptorchidism

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Table 1. Single gene mutations associated with cryptorchidism

Gene	Name
SOS1	son of sevenless homolog 1 *(Drosophila)*
HOXD13	homeobox D13
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
SPATA12	spermatogenesis associated 12
SOX2	SRY (sex-determining region Y)-box 2
ESR1	estrogen receptor 1
HOXA10	homeobox A10
FGFR1	fibroblast growth factor receptor 1
NR5A1	nuclear receptor subfamily 5, group A, member 1
ZNF215	zinc finger protein 215
ZNF214	zinc finger protein 214
KRAS	Kirsten rat sarcoma viral oncogene homolog
PTPN11	protein tyrosine phosphatase, non-receptor type 11
RXFP2	relaxin/insulin-like family peptide receptor 2
PWCR	small nuclear ribonucleoprotein polypeptide N
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
INSL3	insulin-like 3
PROKR2	prokineticin receptor 2
KAL1	anosmin 1
ARX	aristaless-related homeobox
AR	androgen receptor

Adapted from Tannour-Louet et al. [2010].

[Hadziselimovic and Herzog, 2001]. Thus, cryptorchidism is the most common cause of non-obstructive azoospermia in man [Fedder et al., 2004]. In 2005, impaired mini-puberty was proposed to be responsible for azoospermia and adult infertility in cases of cryptorchidism [Hadziselimovic et al., 2004, 2005].

The Physiological Meaning of Mini-Puberty

Eleven years ago, we found that the potential for male fertility is established in infancy during a period of 30 to 90 postnatal days, a period we designated as mini-puberty [Hadziselimovic et al., 2004, 2005]. Due to a transient increase in gonadotropins and testosterone during mini-puberty, gonocytes differentiate into Ad spermatogonia, which establish male germ cell memory and male-specific DNA methylation pathways [Hadziselimovic et al., 2015].

The question as to whether impaired testosterone secretion is a result of defective mini-puberty is controversial. Two studies indicated that cryptorchid boys may have a mild primary testicular dysfunction. Pierik et al. [2009] found testosterone and free-androgen deficiency in cryptorchid infants, indicating disturbed testicular function evident early after birth. Scandinavian results support the hypothesis that cryptorchidism is associated with a primary testicular disorder, which could be a cause or consequence of cryptorchidism. Hormonal malfunction in 3-month-old boys is reflected in low inhibin B production in a Finnish cohort and a high gonadotropin level in Finnish and Danish cohorts [Suomi et al., 2006].

However, cryptorchid boys have low or even undetectable levels of luteinizing hormone (LH) and testosterone surges [Gendrel et al., 1977], atrophic Leydig cells, and abrogated differentiation of gonocytes into Ad spermatogonia [Hadziselimovic et al., 1986] (fig. 1). The vast majority of data available support the conclusion that in many children with undescended testes the response of Leydig cells to human chorionic gonadotropin (hCG) is diminished as compared to normal boys [for review, see Jockenhovel and Swerdloff, 1989]. Nevertheless, some cryptorchid boys show a normal response, with a higher incidence among unilateral cryptorchidism. Except for a blunted testosterone response to hCG, there is no evidence of altered steroidogenesis in cryptorchid testes prior to puberty [Jockenhovel and Swerdloff, 1989]. Pretreatment of cryptorchid boys with hCG cancelled out the differences in their response to a stimulation test as compared to a control population [Gendrel et al., 1977]. Thus, the cause of the lower testosterone response seems to be at the pituitary or hypothalamic level, and may be a result of insufficient Leydig cell stimulation. Numerous LH-RH tests have demonstrated a lower LH response by gonadotropin-releasing hormone [Job et al., 1974; Gendrel et al., 1977; Jacobelli et al., 1979; Mazzi et al., 1979; Bollerslev et al., 1986; Hamza et al., 2001].

During the last 40 years, histology has highly contributed to better understand the etiology of cryptorchidism. In 1975, we proposed pronounced Leydig cell atrophy starting in early infancy as evidence to support endocrinopathy as an etiological factor in cryptorchidism [Hadziselimovic et al., 1975]. Development of Ad spermatogonia from gonocytes, which occurs during the first months of life, has been shown to be testosterone-dependent and is disturbed in cryptorchid boys [Hadziselimovic et al., 2005; Zivkovic et al., 2007]. Semi-thin section analysis of the contralateral descended testis in unilateral cryptorchidism confirmed several studies from the late 1960s that suggested cryptorchidism is a bilateral disease [Salle et al., 1968; Hedinger, 1971; Huff et al., 2001]. Cryptorchid boys with severely impaired mini-puberty and typical testicular histology will develop azoospermia or infertility irrespective of early and successful surgery [Hadziselimovic and Herzog, 2001; Hadziselimovic and Hoecht, 2008]. The high azoospermia risk (HAZR) group

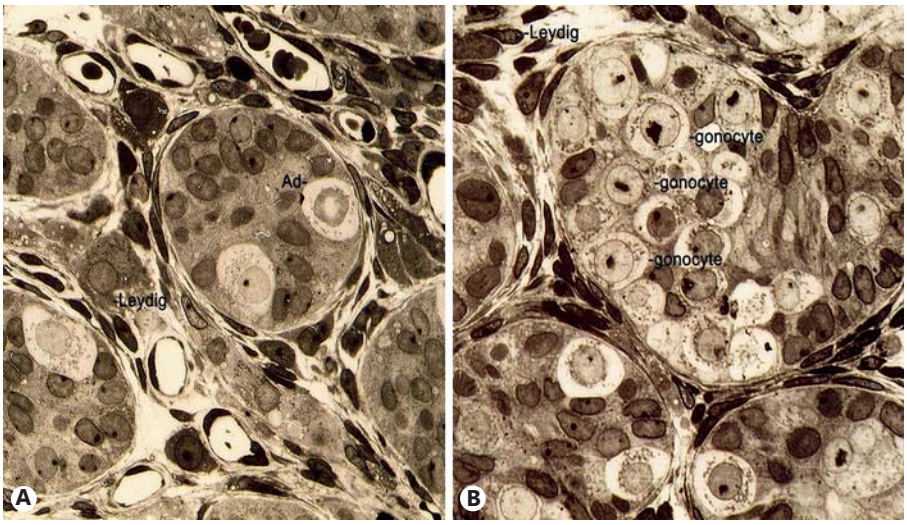

Fig. 1. A Normally differentiated germ cells in infants with intact mini-puberty. One Ad and 2 Ap spermatogonia are present. The fetal stem cell (gonocyte) pool has disappeared. **B** Impaired mini-puberty results in a defective transformation of gonocytes into Ad spermatogonia. Between tubules, atrophic Leydig cells are present, which is a sign of impaired gonadotropin stimulation.

has significantly lower basal plasma LH levels compared to the low azoospermia risk (LAZR) group, indicating hypogonadotropic hypogonadism [Hadziselimovic, 1982]. Furthermore, a prospective study 20 years post-surgery found that sperm concentrations correlate with the number of Ad spermatogonia found at the time of orchidopexy: 80% of males in the HAZR group were oligospermic and 20% were azoospermic (fig. 2). Since these patients had 25-times fewer sperm than the group with Ad spermatogonia in both testes, the presence of Ad spermatogonia in juvenile testes is positively correlated with adult fertility [Hadziselimovic and Hoecht, 2008] (fig. 3). Our observation explains the findings of Gilhooly et al. [1984] of 2 different subsets of unilaterally cryptorchid boys: one with normal spermatogenic potential and the other exhibiting germinal deficiency unresponsive to surgical treatment in both testes. Correlations between testicular histology and postpubertal hormone levels confirmed a relative gonadotropin deficiency in the majority of adult cryptorchid men [Hadziselimovic and Hoecht, 2008]. A recent study estimated that the incidence of defective mini-puberty in cryptorchid boys is as high as 50% [Bilius et al., 2015]. More than one-third of males in the HAZR group will develop azoospermia; Nistal et al. [2007] reported 38% and Hadziselimovic [2007] 33%. However, none of the males with identical testicular pathology at surgery who received treatment with Buserelin (LH-RHa) following orchidopexy developed azoospermia. Thus, Buserelin treatment completely rescued the development of cryptorchidism-induced azoospermia, validating the theory that impaired mini-puberty results in adult infertility [Hadziselimovic, 2007].

Molecular Insight into Cryptorchidism-Induced Infertility

Germline genome integrity is essential for ensuring successful gametogenesis and reproduction, and the maintenance of genomic integrity is largely epigenetically regulated. During their development, gametes undergo an epigenetic reprogramming that, although necessary, represents a permissive period for the propagation of deleterious transposable elements. Defensive transposon silencing mechanisms include methylation by DNA methyltransferases, the piRNA pathway, as well as histone modifications [Castañeda et al., 2011]. Thus, epigenetic defects have arisen as a potential cause of male infertility, in addition to mutations in specific genes and chromosomal abnormalities.

Regarding the infertility induced by cryptorchidism, recent microarray data have shed some light on the molecular basis of its pathogenesis.

EGR4, Master Regulatory Gene for Fertility Development

Analysis of gene expression patterns has revealed absent or low expression of genes involved in the control of hypothalamus-pituitary-gonadal axis function in the HAZR group of cryptorchid testes (table 2). These patients had virtually no early growth response 4 *(EGR4)* expression [Hadziselimovic et al., 2009] (fig. 4). EGR4 belongs to the EGR family of zinc-finger transcription factors (EGR1–4), which control cell growth and differentiation. EGR4 is

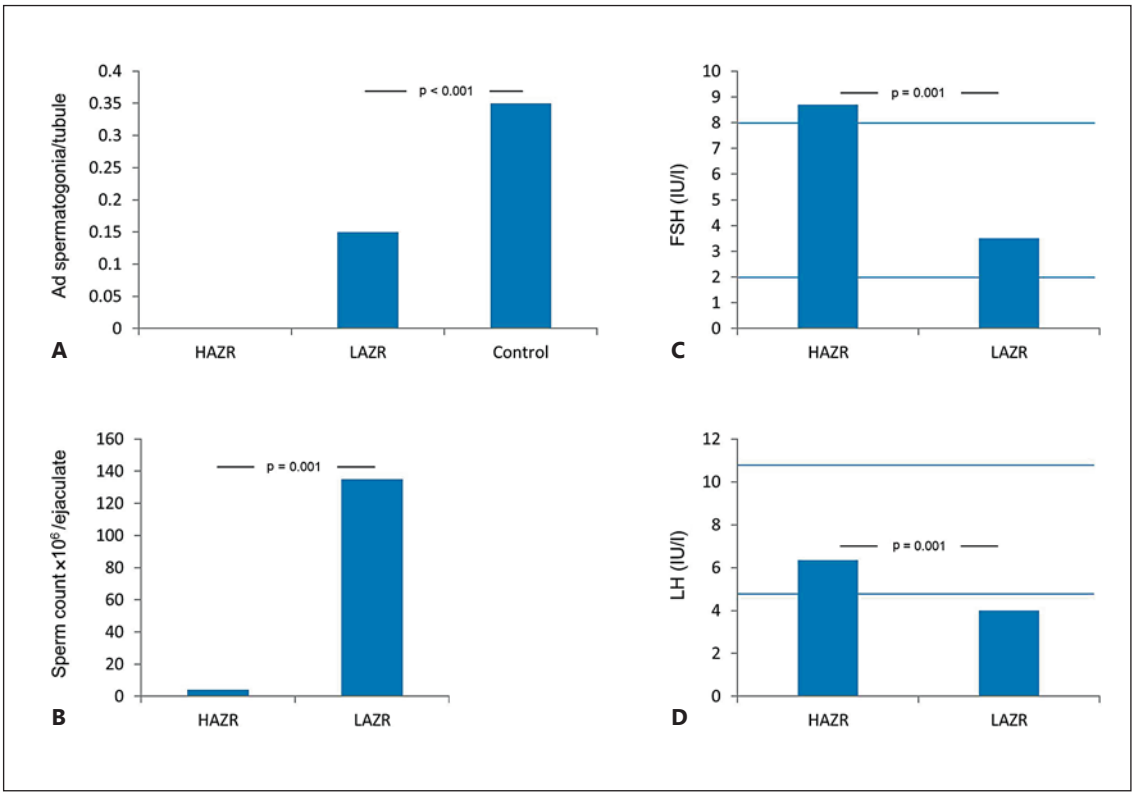

Fig. 2. Summarized results from Hadziselimovic and Hoecht [2008], prospective study. **A** Numbers of Ad spermatogonia vary among the HAZR (Ad-negative/both testes) and LAZR (Ad-positive/both testes) groups. The lowest germ cell count was observed in the HAZR group and the highest was observed in the LAZR and control group. **B** Sperm count, after 20 years of the follow-up study. In the HAZR group the mean sperm count was 25 times less than that in the LAZR group. **C** FSH levels were minimally increased in the HAZR group (normal range in adult males: 2–8 IU/l). **D** The LAZR patients with the healthiest histology have LH levels in the hypogonadotropic range, while the HAZR group has normal LH values despite more severe testicular pathology, indicating LH deficiency (normal range in adult males: 4.8–10.8 IU/l).

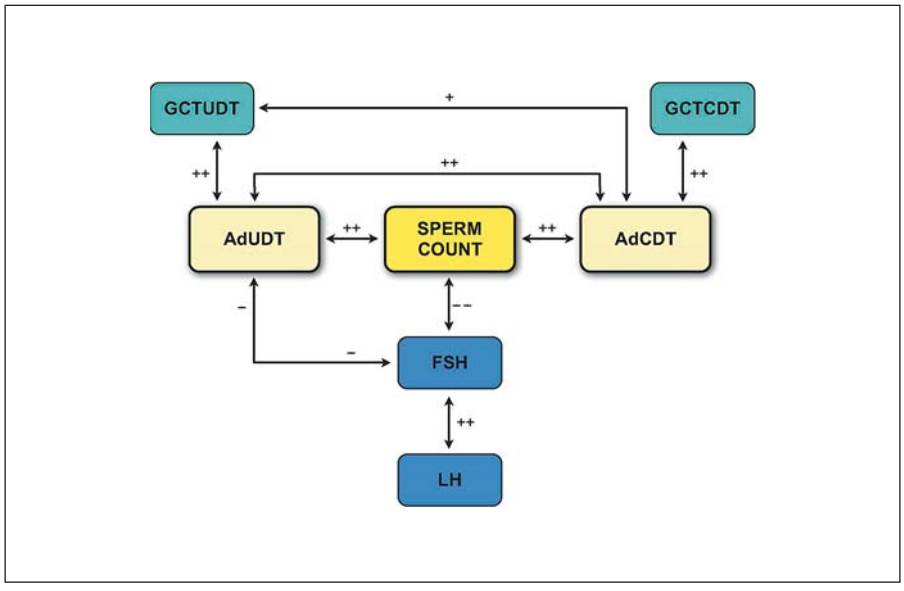

Fig. 3. Schematic model showing a dominant role of Ad spermatogonia in predicting fertility outcome and the importance of plasma FSH levels (++/−− strong correlation; +/− significant correlation). AdCDT (scrotal testis) is the best predictor of future fertility. AdUDT (undescended testis) is a decisive factor for supporting an FSH negative feedback mechanism. GCTUDT (total germ cell count in undescended testis) and GCTCDT (total germ cell count in scrotal testis) have no direct influence either on the sperm count or on the plasma FSH level.

Table 2. Genes involved in the hypothalamus-pituitary-testicular axis downregulated in HAZR

Gene	Name	p HAZR/control	p HAZR/LAZR
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	0.007	0.0006
AMPH	amphiphysin	0.01	0.02
CACNA2D2	calcium channel, voltage-dependent, alpha 2/delta subunit 2	0.01	0.0007
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase	0.003	0.0004
CDC20	cell division cycle 20	0.01	0.003
CLGN	calmegin	0.01	0.01
CSRP2	cysteine and glycine-rich protein 2	0.02	0.002
CXCL9	chemokine (C-X-C motif) ligand 9	0.009	0.0007
DAZ1	deleted in azoospermia 1	0.008	n.s.
DAZL	deleted in azoospermia-like	0.04	0.03
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	0.002	8.25E-05
DDX25	DEAD (Asp-Glu-Ala-Asp) box polypeptide 25	0.02	0.001
DMRTB1	DMRT-like family B with proline-rich C-terminal, 1	0.009	0.003
DPYSL4	dihydropyrimidinase-like 4	0.01	0.001
DTL	denticleless E3 ubiquitin protein ligase homolog *(Drosophila)*	0.008	0.0007
DUSP5	dual specificity phosphatase 5	0.03	0.008
EGR4	early growth response 4	0.007	0.006
ESX1	ESX homeobox 1	0.01	8.63E-05
FGF9	fibroblast growth factor 9	0.01	0.0001
FGFR3	fibroblast growth factor receptor 3	0.003	0.0001
FOXG1B	forkhead box G1	0.001	1.21E-05
FST	follistatin	0.003	0.001
GTSF1	gametocyte-specific factor 1	0.01	0.0001
GAGE1	G antigen 1	0.003	0.001
ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	0.001	0.0002
ISL1	ISL LIM homeobox 1	0.003	0.0004
KIF21B	kinesin family member 21B	0.006	0.0001
LIN28B	lin-28 homolog B *(C. elegans)*	0.01	0.01
LIN7B	lin-7 homolog B *(C. elegans)*	0.03	0.02
MAGEA4	melanoma antigen family A4	0.006	0.009
MAGEC1	melanoma antigen family C1	0.001	8.24E-05
MBD2	methyl-CpG binding domain protein 2	0.003	0.03
MND1	meiotic nuclear divisions 1 homolog *(S. cerevisiae)*	0.03	0.004
MORC1	MORC family CW-type zinc finger 1	0.01	0.0001
NLRP2	NLR family, pyrin domain containing 2	0.04	0.005
NMU	neuromedin U	0.03	0.005
NRG1	neuregulin 1	0.03	0.004
PIWIL2	piwi-like RNA-mediated gene silencing 2	0.002	0.0001
PIWIL4	piwi-like RNA-mediated gene silencing 4	0.001	0.0001
RBMY1A1	RNA binding motif protein, Y-linked, family 1, member A1	0.008	0.0008
RGS7	regulator of G-protein signaling 7	0.01	0.001
RNF17	ring finger protein 17	0.01	0.01
SH3GL2	SH3-domain GRB2-like 2	0.02	0.008
SIX1	SIX homeobox 1	0.01	0.02
SNAP91	synaptosomal-associated protein, 91 kDa	0.006	0.008
SNRP	U1 snrp Snp 1p. RRM domain-containing protein	0.04	n.s.
SOX30	SRY (sex-determining region Y)-box 30	0.002	0.002
SRY	sex-determining region Y	0.002	0.002
SPA17	sperm autoantigenic protein 17	0.02	0.01
SSX2	synovial sarcoma, X breakpoint 2	0.03	0.003
SYCP3	synaptonemal complex protein 3	0.02	0.0002
TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	0.02	0.005
TAF7L	TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.01	0.03
TDRD5	tudor domain containing 5	0.01	0.01
TDRD6	tudor domain containing 6	0.001	0.0003
TDRD9	tudor domain containing 9	0.01	0.009

Table 2 (continued)

Gene	Name	p HAZR/control	p HAZR/LAZR
TDRD10	tudor domain containing 10	0.01	n.s.
TEX14	testis expressed 14	0.008	0.001
TLE1	transducin-like enhancer of split 1 (E(sp1) homolog, *Drosophila*)	0.01	0.0001
TSPY1	testis-specific protein, Y-linked 1	0.001	8.24E-05
UTF1	undifferentiated embryonic cell transcription factor 1	0.003	0.0002
WNT3	wingless-type MMTV integration site family, member 3	0.03	0.0003

Genes interacting with hypothalamus-pituitary-testicular axis are indicated in bold.

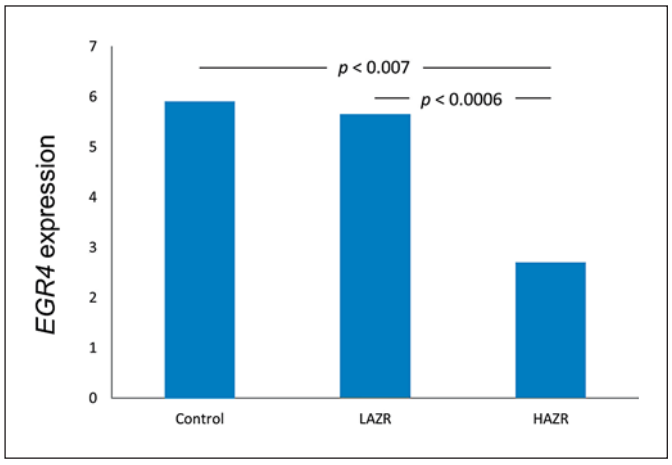

Fig. 4. mRNA levels of *EGR4* analysed by microarrays in HAZR and LAZR cryptorchid testes and control testes. Median values and average absolute deviation from median are presented (log2).

important for the secretion of LH and plays a crucial role in male fertility by regulating germ cell maturation. *Egr4* mutant male mice develop severe oligozoospermia due to incomplete germ cell maturation arrest and apoptosis during the early-mid pachytene stage of meiosis [Tourtellotte et al., 1999]. Both EGR1 and EGR4 regulate the secretion of LHb in the pituitary gland, exhibiting functional redundancy. Double *Egr4*/*Egr1* mutant mice are infertile due to defective LH regulation and display severe Leydig cell atrophy and androgen insufficiency. Moreover, administration of hCG or testosterone restores androgen-dependent organs [Tourtellotte et al., 2000]. In *Egr4* mutant mice, *Egr1* expression is upregulated in Leydig cells, presumably via a compensatory mechanism [Tourtellotte et al., 1999]. In contrast to expected increases in *EGR1* expression to compensate for *EGR4* deficiency, relative *EGR1* insufficiency was found in the HAZR group [Hadziselimovic et al., 2009]. *EGR* genes control LH secretion and steroidogenesis, and *EGR4* is thought to be a master regulatory gene for fertility development. This observation underscores the finding that boys with cryptorchidism who lack Ad spermatogonia have low basal and stimulated plasma gonadotropin values, compatible with hypogonadotropic hypogonadism [Hadziselimovic, 1982].

DDX4 and *DDX25*

Testosterone produced by Leydig cells is essential for the onset and maintenance of spermatogenesis. The androgen-responsive gene, gonadotropin-regulated testicular RNA helicase *(GRTH)/DDX25*, was also expressed at lower levels in the HAZR group. DDX25 is a testis-specific member of the DEAD-box family of RNA helicases expressed in germ cells and Leydig cells [Dufau and Tsai-Morris, 2007] and an essential post-transcriptional regulator of spermatogenesis [Sheng et al., 2006; Tsai-Morris et al., 2010]. In Leydig cells, *DDX25* is directly regulated by androgens, whereby testosterone enhances its transcription in an autocrine manner [Villar et al., 2012]. In contrast, androgen regulation of *GRTH/DDX25* expression in germ cells likely occurs via a paracrine mechanism involving testosterone/AR action in Sertoli cells [Mendelson, 2013]. In germ cells, GRTH/DDX25 is associated with ribonuclear protein particles and involved in the transport of transcripts from the nucleus to cytoplasmic chromatoid bodies [Sheng et al., 2006]. DDX25 is thought to promote the survival of meiotic germ cells by regulating the temporal translation of transcripts critical for sperm maturation, such as anti-apoptotic proteins [Tsai-Morris et al., 2004; Gutti et al., 2008]. Targeted disruption of *Ddx25* in mice causes infertility due to azoospermia, with increased apoptosis of spermatocytes and arrested spermatogenesis, and these mice have significantly smaller chromatoid bodies in spermatids [Tsai-Morris et al., 2004].

DDX4 (VASA) is a gonadotropin-dependent gene underexpressed in the HAZR as compared to the LAZR group and control testes. Interestingly, sterile mice with *Vasa (Ddx4)* deficiency have malformed processing bodies (P-bodies) with an ultrastructural appearance identical to that observed in our previous study [Hadziselimovic, 1977; Kuramochi-Miyagawa et al., 2010].

Germ Cell Development Genes

In addition to alterations in the hypothalamus-pituitary-testicular axis, RNA profiling analysis revealed a gene expression signature that distinguishes HAZR patients from control and LAZR patients [Hadziselimovic et al., 2011]. Concomitant with the reduction in germ cells, genes involved in germ cell development and fertility and spermatogonial stem cell self-renewal show lower or even undetectable expression in HAZR testes. Some of the genes for which low levels of mRNA are found in testes lacking Ad spermatogonia are involved in meiotic and post-meiotic stages of adult spermatogenesis. If juvenile Ad spermatogonia express these genes in normal prepubertal testes, some of the molecular events that initiate the testicular expression program at the onset of puberty and maintain it during adulthood occur very early in prepubertal testes and are likely altered in the HAZR group of cryptorchid patients. This idea is further corroborated by the fact that genes proposed as biomarkers of adult human spermatogonia *(FGFR3*, *UTF1*, *CBL*, and *SNAP91)* [Von Kopylow et al., 2010] are present in LAZR, but missing in HAZR testes [Hadziselimovic et al., 2011]. Several genes transcribed mainly in Sertoli cells, such as *DUSP5* and *DMRTB1*, appeared to be expressed at lower levels in testes lacking Ad spermatogonia. This finding supports the idea that the proliferation of Sertoli cells is also impaired after defective mini-puberty, and that fertility potential is established during this period through both the maturation of Ad spermatogonia and proliferation of Sertoli cells [Zivkovic and Hadziselimovic, 2009], underscoring the need for a normal testicular environment in spermatogenesis.

Other genes for which no mRNA is detected in the HAZR group included genes whose deletion is classically involved in azoospermia: *DAZ1*, *DAZL*, *RBMY1A1*, *SYCP3*, and *CDC20* [Hadziselimovic et al., 2011]. Deleted in azoospermia (DAZ) family proteins and mammalian RNA-binding motif protein Y chromosome (RBMY) are encoded by the male-specific region of the Y chromosome. DAZ and DAZ-like protein (DAZL) are expressed in germ cells and are essential for normal spermatogenesis [Yen, 2004]. DAZ members are necessary for late-stage meiosis and the development of haploid spermatids, whereas DAZL functions primarily in primordial germ cell formation [Kee et al., 2009]. Azoospermia associated with decreased DAZ may go along with reduced levels of SYCP3 [Reynolds et al., 2007]. Although they cause azoospermia, microdeletions of the long arm of the Y chromosome do not seem to be associated with cryptorchidism [Fedder et al., 2004; Vutyavanich et al., 2007]. Furthermore, in azoospermic men with AZFc deletion and no DAZ gene expression, *EGR4* is upregulated [Gatta et al., 2010]. Thus, a lack of both *EGR4* and *DAZ1* mRNAs in the HAZR subset of cryptorchid patients suggests that DAZ/DAZL acts downstream of EGR4 function, reinforcing the notion that cryptorchidism-induced azoospermia is predominantly the result of an endocrine condition rather than being caused directly by mutations in these genes.

Role of Transposon-Silencing Genes and P-Bodies

Epigenetic reprogramming, which includes the erasure and resetting of DNA methylation, occurs in primordial germ cells and is important for preventing the passage of DNA methylation defects from one generation to the next, restoring the developmental potency of the germline, and preparing the genome for the establishment of male-specific DNA methylation patterns in imprinted genes [Castañeda et al., 2011; Bortvin, 2013]. De novo methylation of germ cells begins in prenatal life and is completed by the onset of meiosis [Oakes et al., 2007]. Nearly 40% of the mammalian genome are constituted by endogenous transposable elements (transposons), which are deleterious and cause genomic instability and cell death. In order to prevent their activity, the promoters of the genes are constitutively repressed by hypermethylation [Smith and Meissner, 2013]. However, this repression is lost in male germ cells during the developmental window of epigenetic reprogramming, requiring a robust defense system comprised of the P-element-induced wimpy testis (Piwi)-interacting RNA (piRNA) pathway and de novo DNA methylation machinery [Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008]. piRNAs constitute a unique mode of epigenetic regulation, as they are a type of small non-coding RNA prominently expressed in the germline that constitute an innate defense system against the activity of genetic mobile elements. piRNAs are bound by Piwi-like proteins (Piwil1, Piwil2 and Pi-

wil4), which belong to the Argonaute family of proteins [Siomi et al., 2010]. piRNA-Piwi protein complexes allow selective silencing of transposons by both post-transcriptional and transcriptional mechanisms, such as the degradation of transposon RNA or DNA methylation of the transposon (i.e. epigenetic silencing). Since they maintain genomic stability in the germline, piRNAs are indispensable for fertility [Aravin et al., 2007]. The 3 members of the Piwi protein family in the mouse are required for spermatogenesis [Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007]. Inactivation of Miwi2 (Piwil4) or Mili (Piwil2) leads to loss of DNA methylation, derepression of transposons, defects in spermatogenesis, and sterility [Aravin and Hannon, 2008].

Members of the piRNA pathway need other proteins to function, including Maelstrom (MAEL), DEAD box polypeptide 4 (DDX4; mammalian VASA), Moloney leukemia virus 10-like 1 (MOV10L1), GASZ, and members of the Tudor family of proteins [Aravin et al., 2009]. Tudor proteins regulate the biological functions of Piwi proteins by acting as a scaffold to organize the components of the piRNA pathway [Siomi et al., 2010]. Mice deficient in each of the genes essential for transposon silencing are sterile [Soper et al., 2008; Shoji et al., 2009; Frost et al., 2010; Kuramochi-Miyagawa et al., 2010]. This intricate genome defense machinery is localized in specialized cytoplasmic granules or nuage, which are considered to be germline analogs of somatic P-bodies [Aravin et al., 2009]. P-bodies contain aggregates of specific mRNAs and proteins and are sites of RNA degradation and silencing [Eulalio et al., 2007; Castañeda et al., 2011]. P-bodies exhibit a complex mechanism for regulating the organization and function of Piwi proteins and piRNAs in transposon-silencing pathways. This P-body mechanism includes a methylation-directed protein-protein interaction mediated by germline Tudor domain proteins and Piwi proteins [Chen et al., 2009].

RNA profiling data are consistent with the notion that cryptorchidism-induced azoospermia is due to germ cell death stemming from defects in the epigenetic regulatory pathways essential for genome stability and spermatogenesis. Results have shown alterations in mRNA levels for genes involved in the piRNA-Piwi pathway in the HAZR group: RNA levels for 2 members of the Piwi protein family, as well as other related genes important for transposon silencing, are decreased as compared to LAZR patients *(PIWIL2*, *PIWIL4*, *DDX4*, *MAEL*, *MOV10L1*, 7 members of the Tudor family of proteins, and *GTSF1)* [Hadziselimovic et al., 2012, 2015]. Thus, males with cryptorchidism and impaired mini-puberty show alterations in Piwi-pathway gene expression that appear to result in the deregulation of LINE1 transposons [Hadziselimovic et al., 2015], which ultimately causes apoptosis of germ cells. Notably, morphological changes consistent with altered function of P-bodies in the HAZR group were found at the ultrastructural level [Hadziselimovic et al., 2015]. Recently, testosterone was shown to influence testicular functions by regulating the piRNA-pathway [Kang et al., 2014]. This finding is consistent with the observation that gonadotropin and testosterone insufficiency during mini-puberty induces alterations in the Piwi-piRNA pathway.

Microrchidia-1 *(MORC1)* is another gene involved in male fertility. *Morc1*-deficient male mice are sterile and develop micro-testes due to germ cell apoptosis prior to meiosis [Watson et al., 1998]. MORC1 was recently shown to silence transposable elements, including LINEs, in the mouse male germline by facilitating DNA methylation in a piRNA-independent manner [Pastor et al., 2014]. Interestingly, *MORC1* signals are also decreased in HAZR cryptorchid testes [Hadziselimovic et al., 2011]. Thus, the failure of different transposon repression mechanisms and subsequent genomic instability are likely involved in the development of azoospermia in cryptorchid patients. Multiple mechanisms would not be surprising, as L1 has been shown to be silenced in at least 3 different ways, including piRNA-mediated and piRNA-independent methylation [Di Giacomo et al., 2013].

Novel Implications for Treatment

Successful scrotal relocation of the testis reduces, but does not prevent, infertility in certain individuals [Kolon et al., 2014]. Since abnormal mini-puberty is responsible for adult-onset infertility in cryptorchidism, post-surgical hormonal treatment is highly recommended in HAZR cryptorchid boys who underwent successful early orchidopexy but remain at risk to become infertile as adults.

Concluding Remarks

Whole-genome expression analysis strongly supports the theory that impaired mini-puberty is responsible for azoospermia and adult infertility in cryptorchidism. Multiple differences in gene expression between HAZR and LAZR groups underscore the importance of an intact hypothalamus-pituitary-testicular axis in fertility develop-

ment. Molecular biological observations support LH deficiency, with *EGR4* as a master gene in Leydig cell dysgenesis, as the reason for impaired mini-puberty.

In contrast to the general belief that temperature-dependent effects on cryptorchid gonads damage undescended testes before sexual maturation is complete, recent evidence is consistent with the idea that infertility in cryptorchidism is a consequence of alterations in the Piwi pathway and transposon derepression. Thus, abnormal germ cell development in cryptorchidism is preceded by a hormone imbalance and perturbation in germ cell-specific gene expression during mini-puberty. In addition, intact function of P-bodies during mini-puberty contributes to the establishment of germ cell memory and male-specific DNA methylation pathways.

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