matogonia or Sertoli cells from immature to adult testes, suggesting that they have a role in germ cell differentiation and in the maintenance of stem cell potential. The present findings might enable novel evaluation of spermatogenic failure caused by cryptorchidism.

APPENDIX

Identification of Differentially Expressed Transcripts in the Testes of Patients With Cryptorchidism

(1) 18 higher expressed genes

Gene symbol	Gene name	Function	Accession No.
TPT1	Tumor protein, translationally controlled 1	Protein binding	NM_003295
FTH1	Ferritin, heavy polypeptide 1	Cell proliferation	NM_002032
LOC441581	FSHD region gene 2 protein	Unknown	NM_001080998
ZSCAN2	Zinc finger and SCAN domain containing 2, transcript variant	Cell differentiation	NM_181877
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	Translational elongation	NM_001402
NUMA1	Nuclear mitotic apparatus protein 1	Cell cycle	NM_006185.2
NBPF1	Neuroblastoma breakpoint family, member 1	Unknown	NM_017940
UBC	Ubiquitin C	Protein modification	NM_021009
NBPF14	Neuroblastoma breakpoint family, member 14	Unknown	NM_015383
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	Unknown	NR_002819
TTTY6	Testis-specific transcript, Y-linked 6	Unknown	AF332237
RPL3	Ribosomal protein L3, transcript variant 1	Translation	BC_107711
RPL7A	Ribosomal protein L7a	Translation	NM_000972
RPL32	Ribosomal protein L32	Translation	NM_000994
RPS9	Ribosomal protein S9	Translation	NM_001013
RPS14	Ribosomal protein S14	Translation	NM_001025071
RPS20	Ribosomal protein S20	Translation	NM_001023
RPS25	Ribosomal protein S25	Translation	NM_001028

(2) 16 lower expressed genes

Gene symbol	Gene name	Function	Accession No.
HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA splicing	NM_002137.2
MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle	Muscle development	NM_021019.3
IFITM3	Interferon-induced transmembrane protein 3	Immune response	NM_021034.1
IFITM2	Interferon-induced transmembrane protein 2	Immune response	NM_006435.1
ZC3H13	Zinc finger CCCH-type containing 13	Nucleic acid binding	NM_015070.2
C3orf28	Chromosome 3 open reading frame 28	Unknown	NM_014367.3
SOD1	Superoxide dismutase 1	Antioxidant activity	NM_000454.4
PTGDS	Prostaglandin D2 synthase 21 kDa	PGD2 synthesis	NM_000954.5
CLU	Clusterin, transcript variant 2	Protein binding	NM_203339.1
RPL7A	Ribosomal protein L7a	Translation	NM_000972.2
RPL17	Ribosomal protein L17	Translation	NM_001035006.1
RPL24	Ribosomal protein L24	Translation	NM_000986.3
RPL28	Ribosomal protein L28	Translation	NM_000991.3
RPS2	Ribosomal protein S2	Translation	NM_002952.3
RPS18	Ribosomal protein S18	Translation	NM_022551.2
RPS25	Ribosomal protein S25	Translation	NM_001028.2

REFERENCES

1. Lee PA, Coughlin MT and Bellinger MF: Inhibin B: comparison with indexes of fertility among formerly cryptorchid and control men. J Clin Endocrinol Metab 2001; **86:** 2576.

2. Ritzen EM, Bergh A, Bjerknes R, Christiansen P, Cortes D, Haugen SE et al: Nordic consensus on treatment of undescended testes. Acta Paediatr 2007; **96:** 638.

3. Hadziselimovic F and Herzog B: The importance of both an early orchiopexy and germ cell maturation for fertility. Lancet 2001; **358:** 1156.

4. Lue YH, Hikim AP, Swerdloff RS, Im P, Taing KS, Bui T et al: Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. Endocrinology 1999; **140:** 1709.

5. Li YC, Hu XQ, Xiao LJ, Hu ZY, Guo J, Zhang KY et al: An oligonucleotide microarray study on gene expression profile in mouse testis of experimental cryptorchidism. Front Biosci 2006; **11:** 2465.

6. Politoff L, Hadziselimovic F, Herzog B and Jenni P: Does hydrocele affect later fertility? Fertil Steril 1990; **53:** 700.

7. Mizuno K, Tokumasu A, Nakamura A, Hayashi Y, Kojima Y, Kohri K et al: Genes associated with the formation of germ cells from embryonic stem cells in cultures containing different glucose concentrations. Mol Reprod Dev 2006; **73:** 437.

8. Yenofsky R, Cereghini S, Krowczynska A and Brawerman G: Regulation of mRNA utilization in mouse erythroleukemia cells induced to differentiate by exposure to dimethyl sulfoxide. Mol Cell Biol 1983; **3:** 1197.

9. Guillaume E, Pineau C, Evrard B, Dupaix A, Moertz E, Sanchez JC et al: Cellular distribution

of translationally controlled tumor protein in rat and human testes. Proteomics 2001; **1:** 880.

10. Lamberti A, Caraglia M, Longo O, Marra M, Abbruzzese A and Arcari P: The translation elongation factor 1A in tumorigenesis, signal transduction and apoptosis: review article. Amino Acids 2004; **26:** 443.

11. Taimen P, Parvinen M, Osborn M and Kallajoki M: NuMA in rat testis—evidence for roles in proliferative activity and meiotic cell division. Exp Cell Res 2004; **298:** 512.

12. Chen SH, Wu PS, Chou CH, Yan YT, Liu H, Weng SY et al: A knockout mouse approach reveals that TCTP functions as an essential factor for cell proliferation and survival in a tissue- or cell type-specific manner. Mol Biol Cell 2007; **18:** 2525.

13. Koziol MJ, Garrett N and Gurdon JB: Tpt1 activates transcription of oct4 and nanog in transplanted somatic nuclei. Curr Biol 2007; **17:** 801.

14. Pesce M, Wang X, Wolgemuth DJ and Scholer H: Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech Dev 1998; **71:** 89.

15. Fessenden JM and Moldave K: Studies on aminoacyl transfer from soluble ribonucleic acid to ribosomes. Resolution of two soluble transferring activities. J Biol Chem 1963; **238:** 1479.

16. Rho SB, Park YG, Park K, Lee SH and Lee JH: A novel cervical cancer suppressor 3 (CCS-3) interacts with the BTB domain of PLZF and inhibits the cell growth by inducing apoptosis. FEBS Lett 2006; **580:** 4073.

17. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M et al: Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 2004; **36:** 653.

18. Plotton I, Sanchez P, Durand P and Lejeune H: Decrease of both stem cell factor and clusterin mRNA levels in testicular biopsies of azoospermic patients with constitutive or idiopathic but not acquired spermatogenic failure. Hum Reprod 2006; **21:** 2340.

19. Hadziselimovic F, Hocht B, Herzog B and Buser MW: Infertility in cryptorchidism is linked to the stage of germ cell development at orchidopexy. Horm Res 2007; **68:** 46.

20. Schwentner C, Oswald J, Kreczy A, Lunacek A, Bartsch G, Deibl M et al: Neoadjuvant gonadotropin-releasing hormone therapy before surgery may improve the fertility index in undescended testes: a prospective randomized trial. J Urol 2005; **173:** 974.

EDITORIAL COMMENT

The authors describe their findings from applying the suppression subtractive hybridization technique to a small series of boys with cryptorchidism. This method enabled the authors to identify a small set of up-regulated and down-regulated genes in the testicular tissue of these subjects compared to control subjects with normally descended testes. Differential expression of genes was corroborated with other techniques, namely RT-PCR, western blotting and immunohistochemical analysis.

Although the reported data are promising, they are scant and preliminary. The cohort consisted of merely 24 boys of varying ages (1 to 5 years). While we applaud the efforts of the authors, more studies are required to confirm their findings and to dissect the pathways of cryptorchidism associated spermatogenic defects proposed by their work.

Michael H. Hsieh
Division of Pediatric Urology
Baylor College of Medicine
Houston, Texas

REPLY BY AUTHORS

We agree that this is a small series. However, the study is important because we could detect that *TPT1, EEF1A1* and *NuMA1* genes showed differential expressions specific to cryptorchid testes in humans. We assume that the characteristics of germ cells change in cryptorchidism owing to genetic, hormonal or microenvironmental conditions. Further studies are needed to elucidate the roles of the aforementioned genes in germ cell maturation. Long-term followup studies on the relationship of gene expression to fertility and paternity are also required.

Advances in Molecular Genetics of Cryptorchidism

Yoshiyuki Kojima, Kentaro Mizuno, Kenjiro Kohri, and Yutaro Hayashi

Cryptorchidism is the most common congenital disorder in boys; one major complication of this disorder is male infertility. Androgens are key hormones to complete testicular descent; therefore, impaired fetal androgen action can result in this anomaly; its molecular etiology, however, remains unknown. Recent molecular approaches might provide an opportunity to identify not only candidate genes but also several predictive markers of future fertility. The purpose of this review is to summarize the recent insight into the genetic pathway of testicular descent and the molecular etiology of isolated cryptorchidism, and discuss the prospects of treatment to achieve future fertility in such patients. UROLOGY 74: 571–578, 2009. © 2009 Elsevier Inc.

Cryptorchidism (undescended testis) is the most common congenital malformation in newborn boys. An undescended testis can be located anywhere between the abdominal cavity and immediately external to the scrotum, unilaterally or bilaterally. About 3% of full-term infants are affected by this anomaly; of these, most have testes that descend normally within a few months. The remaining 1% with a persisting cryptorchid condition should be considered for medical or surgical intervention.[1] Cryptorchidism is usually an isolated anomaly and a multifactorial disease.[2] Androgens are key hormones to complete testicular descent; therefore, impaired fetal androgen action can result in this anomaly. A recent report showed that androgen-driven masculinization was programmed by androgen action early in fetal life, before morphologic differentiation occurs, and deficient androgen action only within this early programming window could induce cyptorchidism[3]; however, the underlying mechanism by which impaired androgen action produces "isolated" cryptorchidism is not yet clear. Androgen-independent events may also be responsible for testicular descent.

Recent molecular approaches, including gene targeting approaches in mice, microarray-based expression profiling, polymerase chain reaction (PCR)–based suppressive subtractive hybridization, and single nucleotide polymorphism (SNP) genotyping, might provide an opportunity to identify not only candidate genes of these anomalies but also several predictive markers of future fertility, as well as new strategies to improve future spermatogenesis in cryptorchidism. *Homeobox A10* (*HOXA10*), *insulin-like factor 3* (*INSL3*), and *INSL3 receptor* (*LGR8/GREAT*) have been suggested to be possible regulators of testicular descent that may be responsible for isolated cryptorchidism.[2] In addition, these anomalies may be associated with a specific haplotype of the gene for *estrogen receptor alpha* (ESR1), which mediates the estrogenic effects of environmental endocrine disrupters (EEDs), and the effects of EEDs on testicular descent might depend on individual genetic susceptibility. This review summarizes the recent insight into the genetic pathway of testicular descent from animal models, including gene-targeting approaches, and to discuss the molecular etiology of patients with isolated cryptorchidism.

Male infertility is a serious complication of cryptorchidism, particularly bilateral cases. Orchiopexy is the most frequently used surgical procedure for cryptorchidism, and has been observed to have a beneficial effect on fertility.[4] Patients with untreated bilateral cryptorchidism have a very high risk of infertility; paternity studies have shown decrease in risk if the condition has been corrected, although age at treatment has not been investigated in these studies.[5] Lee and Coughlin[6] reported that paternity rates are significantly lower in men with previous bilateral cryptorchidism who have attempted to father a child (65.3%), compared with men with previous unilateral cryptorchidism (89.7%) and controls (93.2%). Some previous bilaterally cryptorchid patients are predicted to have lost their paternity potential.[7] Because these studies mean that orchiopexy does not always guarantee subsequent fertility and paternity, additional therapeutic options should be considered to improve spermatogenesis and achieve fertility and paternity for such patients.[7] The molecular approach can provide important clues to find new strategies in cryptorchidism to

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From the Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Reprint requests: Yutaro Hayashi, M.D., Ph.D., Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467–8601, Japan. E-mail: yutaro@med.nagoya-cu.ac.jp

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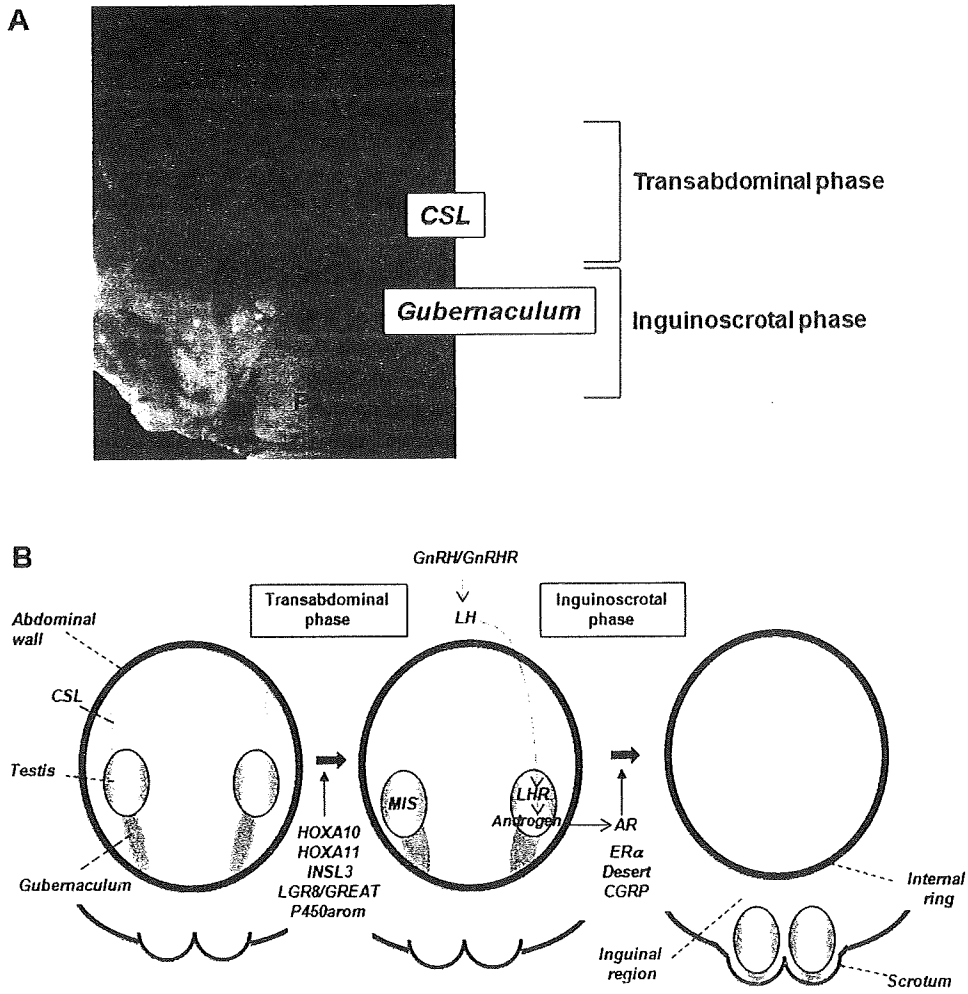

Figure 1. Two-phase theory, the transabdominal and inguinoscrotal phases in testicular descent and associated genes. **(A)** Intra-abdominal appearance at the testicular descended stage in an 18-day rat embryo. T, testis; Ad, adrenal; K, kidney; Ao, aorta; B, urinary bladder; P, penis; S, scrotum. **(B)** Schematic illustration of each phase of testicular descent and associated genes and hormones. The transabdominal phase is controlled by enlargement of the gubernaculum and regression of the cranial suspensory ligament (CSL). The inguinoscrotal phase requires migration of the gubernaculum from the groin to the scrotum. Disruption of each phase can induce cryptorchidism.

improve spermatogenesis and achieve fertility and paternity for these patients; therefore, we will also discuss the future prospects of treatment to achieve future fertility in patients with cryptorchidism.

MOLECULAR AND GENETIC REGULATION OF TESTICULAR DESCENT AND CANDIDATE GENES OF CRYPTORCHIDISM

Testicular descent is a complex, multistage process requiring the interaction of anatomic and hormonal factors. Generally, the two-phase theory, which includes transabdominal and the inguinoscrotal phases, has been accepted in descent of the testis (Fig. 1). The transabdominal phase (8-15 weeks) is controlled by enlargement of the caudal genitoinguinal ligament (gubernaculum) and regression of the cranial suspensory ligament (CSL). The inguinoscrotal phase (25-35 weeks) requires migration of the gubernaculum from the groin to the scrotum. Animal models have provided extensive knowledge of the mechanism regulating testicular descent, some of which suggested candidate genes for patients with cryptorchidism (Table 1).

Although cryptorchidism may be associated with other congenital disorders, including many syndromes for which the corresponding candidate genes are known,[2] most cases show spontaneous occurrence and the obvious cause remains unknown. Recently, however, several researches have examined the association between cryptorchidism and genetic alterations of *homeobox A10* (*HOXA10*), *insulin-like factor 3* (*INSL3*), *INSL3 receptor* (*LGR8/GREAT*), *androgen receptor* (*AR*), *estrogen receptor α* (*ESR1*), *Ad4BP/SF-1* genes, and suggested that some of these genes may be responsible for some cases of cryptorchidism (Table 1). The most common genetic variation, SNP, is a single-base DNA polymorphism at a specific location in the genome that is by definition found

Table 1. Animal model of cryptorchidism and candidate genes of human "isolated" cryptorchidism

Gene	Animal			Human		
	Model	Species	Testicular Location	Chromosome	Genetic Variant	Outcome
HOXA10	KO	Mouse	Intra-abdominal	7p15-p14	—	NS
HOXA11	KO	Mouse	Intra-abdominal	7p15-p14	—	NA
INSL3	KO	Mouse	Intra-abdominal	19p13.2-p12	C-19G, V18N, A24G, V43L, P49S, A60T, W69R, R73X, P93L, R102C, R102H, N105H, N110K	S
LGR8/GREAT	KO	Mouse	Intra-abdominal	13q13.1	T222P, R223K, I604V	S
P450arom	TG (overexpression)	Mouse	Intra-abdominal	15q21.1	—	NA
GnRH	KO	Mouse	Inguinal	8p21-p11.2	—	NA
GnRH receptor	KO	Mouse	Inguinal	4q21.2	—	NA
LH receptor	KO	Mouse	Inguinal	2p21	—	NA
AR	KO	Mouse	Inguinal	Xq11.2-q12	CAG/GGN repeat length polymorphism	S
	Anti-androgen exposure	Rat	Inguinal			
Erα	E2 exposure to KO	Mouse	Inguinal	6q25.1	Specific "AGATC" haplotype	S
Desrt	KO	Mouse	Inguinal	10q21.2	—	NA
CGRP	TS-rat	Rat	Inguinal	11p15.2-p15.1	—	NS
Ad4BP/SF-1	KO	Mouse	(Gonadal dysgenesis)	9q33	G146A	S

KO = knockout mouse; TG = transgenic mouse; S = significant; NS = nonsignificant; NA = not assessed; TS = trans-scrotal.

in >1% of the population. The total number of SNPs reported in public SNP databases exceeds 9 million. SNP analysis has been developed to promote research on various diseases. SNP genotyping and haplotyping (combinations of SNPs within a contiguous segment of DNA) provide valuable information in the study of cryptorchidism.

Transabdominal Phase

***Hoxa10* and *Hoxa11*.** *Hox* genes, which are evolutionarily related to *Drosophila* homeotic genes, play a key role in the morphogenesis of the segmented structure along the primary body axis, such as the branchial arches, vertebrae, cranial nerves, and ganglia.[8] Satokata et al[9] reported that *Hoxa10* knockout mice showed intra-abdominal bilateral cryptorchidism. Hox10 mRNA expression appeared predominantly in the gubernaculums.

The testes of *Hoxa11* knockout mice showed incomplete descent into the scrotal sac, but the degree of maldescent was variable; however, some animals had intra-abdominal testes.[10] Because *Hoxa11* knockout mice did not attain the fertility rates observed in wild-type animals after orchiopexy, other factors may be necessary for normal spermatogenesis.[11]

Although *Hoxa10* knockout mice exhibit cryptorchidism, there is no evidence of a relationship between *HOXA10* variants and cryptorchidism in humans. Kolon et al[12] reported that altered *HOXA10* genes were present in some children with cryptorchidism and *HOXA10* polymorphisms exist in normal control subjects as well as in cryptorchid patients. Bertini et al[13] also reported that 1 silent polymorphism of *HOXA10* was detected in both cryptorchidism and the control in about 10% of patients.

Insl/Insl3 Receptor. Insl3 is expressed in Leydig cells of the testis and theca cells of the ovary. This peptide affects testicular descent by acting on the gubernaculum via its specific receptor leucine-rich repeat-containing G protein–coupled receptor 8 (Lgr8), also known as G protein–coupled receptor affecting testis descent (GREAT), and therefore an apparent regulator of testicular descent. Insl3 production is also related to luteinizing hormone (LH),[14] and reduced Insl3 action is a possible cause of cryptorchidism because *Insl3* knockout mice showed isolated bilateral cryptorchidism because of developmental abnormalities of the gubernaculum.[15,16] In double-mutant male mice with *Insl3* and *AR* genes, testes are positioned adjacent to the kidneys and held in the abdomen by the CSL. These findings demonstrate that the Insl3 induces gubernaculum development in an androgen-independent manner, whereas androgen-mediated regression of the CSL occurs independently of Insl3. In addition, overexpression of transgenic *Insl3* causes male-like gubernaculum differentiation, ovarian descent into the lower abdominal position.[17] Intrascrotal orchiopexy can rescue these congenitally cryptorchid *Insl3* knockout testes from their intra-abdominal fate of Sertoli cells only, leading to fertility.[18] Overexpression of *Insl3* does not prevent cryptorchidism in *GnRH* receptor (Gnrhr) or

Hoxa10 knockout mice, and Insl3 is sufficient to direct the first transabdominal phase of testicular descent in the absence of HPG (hypothalamic-pituitary-gonadal) axis signaling or Hoxa10, but its presence is important for inguinoscrotal testicular descent.[19] A mutation of the *LGR8/GREAT* gene causes failure of the transabdominal phase of descent, identical to that seen in *Insl3* deficient mutants, suggesting that *Insl3/Insl3* receptor signaling regulates gubernacular development.[17]

Most reports have focused on sequence analysis of the human *INSL3* and *LGR8/GREAT* genes, which identified several allelic variants. Several *INSL3* mutations, including C-19G, V18N, A24G, V43L, P49S, A60T, W69R, R73X, P93L, R102C, R102H, N105H and N110K, and *LGR8/GREAT* mutations, including T222P, R223K, and I604V, were previously reported to be associated with cryptorchidism; however, the relevance of *INSL3* and *LGR8/GREAT* mutations to cryptorchidism remains minor (1.5%-2.0%); no relationship has been established between mutation and the severity of cryptorchidism, and most of these mutations were heterozygous.[2,20-22] Definitive in vitro proof of the causative role of many of these mutations is still lacking. Feng et al found in vitro that the I604V *LGR8/GREAT* variant receptor responds to INSL3 stimulation similar to the wild-type receptor.[20] El Houate et al[21] reported that the V18M mutation in the INSL3 signal peptide had a significant deleterious effect on activating *LGR8/GREAT* in ex vivo studies. Bogatcheva et al[22] demonstrated that T222P *LGR8/GREAT* mutation, which was present only in affected patients, and induced structural alterations of the ligand-binding part of *LGR8/GREAT*, resulted in the inability of the receptor to be expressed on the cell-surface membrane and might have been responsible for the abnormal testicular phenotype in patients.

Recently, Ferlin et al[23] have determined the frequency of genetic alterations such as karyotype anomalies and *INSL3*, *LGR8/GREAT*, and *AR* gene mutations in cryptorchidism in their large study. They reported that the overall frequency of genetic alterations was significantly higher in boys with cryptorchidism (5.3%) than in controls (0.3%). As a result, the odds ratio for the association of cryptorchidism with genetic alterations was 16.7, indicating a significant association between cryptorchidism and genetic alterations.

P450aromatase. The androgen/estrogen ratio may be more important than only one hormone action per se in both sexes. This ratio is controlled by aromatase. P450aromatase (*Cyp19a1*)-overexpressed transgenic male mice are characterized by an imbalance in sex hormone metabolism, resulting in elevated serum E2 concentrations, combined with significantly reduced testosterone and follicle-stimulating hormone (FSH) levels. These mice also have a multitude of severe structural and functional alterations in the reproductive organs, such as cryptorchidism (intra-abdominal testis) associated with Leydig cell hyperplasia, dysmorphic seminiferous tubules, and disrupted spermatogenesis.[24]

Inguinoscrotal Phase

GnRH/GnRH Receptor and LH Receptor. The HPG axis regulates the development and the endocrine and reproductive functions of the gonads throughout all phases of life. Hypogonadotropic hypogonadism, which is induced by HPG axis dysfunction, is a common cause of cryptorchidism. Several animal models have hypogonadotropic hypogonadism, which shows cryptorchidism. Hypogonadal (HPG) male mice lacking *GnRH* are cryptorchid, but have a normal gubernaculum, and their testes develop and descend normally if treated with gonadotropins.[25] Pask et al[26] also reported *GnRH receptor* gene (Gnrhr) loss-of-function mutation in male mice that caused hypogonadotropic hypogonadism during *N*-ethyl-*N*-nitrosourea mutagenesis screening. Affected males had a micropenis and small, undescended testes with spermatogenesis arrested in the pachytene stage of meiosis, leading to male infertility. *LH receptor* (*LHR*) knockout male mice also showed undescended testes, small testis, underdeveloped scrotum, small penis, and arrested spermatogenesis.[27] Cryptorchidism in *LHR* knockout was caused by defects in gubernacular development because of testosterone deficiency, and testosterone-replacement therapy reversed all morphologic and gene expression changes except Insl3, suggesting that testosterone secreted by Leydig cells facilitates the completion of testicular descent.[28] These knockout male mice had inguinoscrotal testes, suggesting that the HPG axis is distributed in the inguinoscrotal phase of testis descent.

Androgen/Androgen Receptor (AR). Two androgens, namely testosterone and dihydrotestosterone (DHT), are crucial for the development, function, and pathologic status of the testis and male internal and external genitalia. Administration of an antiandrogen agent to pregnant rats causes cryptorchidism.[29] Histologically, apoptotic cells were markedly increased, the seminiferous tubules were degenerated, and disturbance in spermatid differentiation was observed in the undescended testes. The activation of a transcription factor, namely, nuclear factor-kappa B (NF-κB), was associated with germ cell apoptosis in these cryptorchid testes.[30] Early orchiopexy improved subsequent testicular development and spermatogenesis in this cryptorchid rat.[31] By contrast, the testicular feminized (Tfm) mouse lacks functional *AR*s and develops with a female external phenotype. The testes had descended normally to the internal ring by the time of birth but further descent was absent.[32] Because these cryptorchid model male mice had inguinoscrotal testes, androgens are also a major mediator of the inguinoscrotal phase of testis descent, although it is accepted that androgens are present in both the gubernaculum and the CSL.

Because androgen is a key factor in not only testicular descent but also sexual development, *AR* gene mutations result in remarkable changes in the internal and external

genitalia in humans. Androgen insensitivity syndrome (AIS) is an X-linked inherited disorder caused by *AR* gene mutations. Partial androgen insensitivity syndrome shows a disorder including variable development of the Wolffian ducts, cryptorchidism, and hypospadias; therefore, *AR* mutations are not a frequent cause of isolated cryptorchidism. This gene exhibits 2 polymorphic sites in exon 1, characterized by different numbers of CAG and GGN repeats. Recent reports have shown an association between CAG/GGN repeat-length polymorphisms and cryptorchidism.[33,34]

Estrogen Receptor (ER). The *ER* knockout mouse showed small cremaster sacs and testes retracted inside the abdominal cavity[35]; however, the role of estrogen and *ER* has remained unclear. The *ESRα* knockout mice had descended testis with defects in cremaster muscle development; by contrast, *ESRβ* knockout mice had no abnormal fertility. In mice, prenatal exposure to 17β-estradiol (E2) and the nonsteroidal synthetic estrogen diethylstilbestrol (DES) disturbs the endocrine balance, causing cryptorchidism, and these exposures downregulate Insl3 expression in embryonic Leydig cells.[36] Cederroth et al[37] reported that *ESRα* was a major contributor to estrogen-mediated fetal testis dysgenesis and cryptorchidism in their E2 and DES exposure study of *ERα* knockout mice. These animal studies imply that estrogen and ER may play a role in testicular descent.

Several researchers have examined the association between the SNPs or specific *estrogen receptor α* (*ESR1*) haplotype, and the risk for cryptorchidism in humans. Wang et al[38] reported that SNP12 (rs 6932902) in the 3′-terminal region of ESR1 was not associated with the occurrence of cryptorchidism but was associated with the severity of cryptorchidism. They also reported that the 4 estimated haplotypes in the 3′ region of the *ESR1* gene were not associated with the occurrence of cryptorchidism, but the haplotype AGATC was associated with the severity of cryptorchidism.

Desrt. *Desrt* is the A-T-rich interaction domain (ARID) family of transcription factors that is widely expressed and encodes a DNA-binding protein. *Desrt* knockout males were found to be either unilaterally or bilaterally cryptorchid, with undescended testes generally located in the inguinal region.[39]

Calcitonin Gene-Related Peptide. Anatomic studies of the genitofemoral nerve in neonatal rodents identified calcitonin gene-related peptide (CGRP). Specific binding sites for CGRP have been found on developing cremaster muscle fibers within the gubernaculums. CGRP may stimulate gubernacular migration during testicular descent by release from the genitofemoral nerve. The natural mutant trans-scrotal (TS) rat strain has a unilateral or bilateral cryptorchidism caused by a decreased number of CGRP binding sites in gubernaculums.[40] Although this neuropeptide was discovered several decades ago, molecular biological data are lacking. Mutation screening of the coding regions and intron-exon boundaries of *CGRP* revealed polymorphic variants but no pathogenic sequence changes in the 90 selected cases of idiopathic unilateral or bilateral cryptorchidism.[41]

Müllerian-Inhibiting Substance. Although persistent Müllerian duct syndrome in humans, which results in genetic defects in the Müllerian-inhibiting substance (*MIS*) and *MIS receptor*, shows cryptorchid testes, MIS had been considered responsible for not only regression of the Müllerian duct but also testicular descent; however, both *MIS* and *MIS receptor* knockout mice and *MIS/MIS receptor* double knockout male mice had testes that were fully descended and produced functional sperm.[42] XY *Tfm/MIS* double mutants developed as females, with a uterus, coiled oviducts, and no male reproductive organs, except undescended dysfunctional testes.[42] Whether MIS plays an important factor in testicular descent has remained controversial.

Others

Ad4BP/SF-1. Ad4BP/SF-1 is an orphan member of the nuclear hormone receptor superfamily of transcription factors. Ad4BP/SF-1 is important for the maintenance of steroidogenesis in the human testis.[43] Although *Ad4BP/SF-1* knockout mice have a complex phenotype that includes adrenal and gonadal agenesis, impaired function of pituitary gonadotropes, abnormalities of the ventromedial hypothalamic nucleus, and variable loss of *Ad4BP/SF-1* function can be associated with a wide range of reproductive phenotypes in humans.[44] Genotyping analysis was performed for G146A polymorphism in the *Ad4BP/SF-1* gene, which is known to reduce transactivation function by approximately 20%, revealing that the Ala allele, the Ala/Gly genotype, and Ala/Ala plus Ala/Gly genotype frequencies were significantly higher in patients than in control males.[45]

ENVIRONMENTAL ENDOCRINE DISRUPTERS AND GENETIC SUSCEPTIBILITY IN CRYPTORCHIDISM

Exposure to xenoestrogens during pregnancy may disrupt the development and function of male sexual organs and may induce isolated cryptorchidism.[46] EEDs are primarily modulated by ER. Certain phthalate esters have been shown to produce reproductive toxicity in male rodents, and produce a syndrome of reproductive abnormalities, including cryptorchidism.

Although mutations of several genes explain a minority of cases of cryptorchidism, research into genetic polymorphisms that may also influence susceptibility to EEDs is shedding light on this field. Cryptorchidism may be associated with a specific haplotype of the *ESR1* gene that mediates the estrogenic effects of EEDs. A set of 15 SNPs, SNP1-SNP15, along the genomic region of the *ESR1* gene, suggested that homozygosity for a specific

"AGATA" haplotype within an approximately 50-kb linkage disequilibrium block of *ESR1* gene may increase susceptibility to cryptorchidism by enhancing estrogenic effects of EEDs.[47] The effects of EEDs on testicular descent might depend on individual genetic susceptibility.

PREDICTION OF FERTILITY IN CRYPTORCHIDISM BY MOLECULAR APPROACH

One of the major complications of cryptorchidism is male infertility. The main goal of treatment for cryptorchid boys is to achieve fertility and paternity. Early orchiopexy is becoming the standard practice according to histologic examination.[48] Although early orchiopexy improves subsequent testicular development and spermatogenesis in the experimental cryptorchid rat model,[31] few detailed studies to predict future fertility after early orchiopexy in humans have been reported. Genetic markers may have the potential of becoming useful parameters to predict the future fertility of cryptorchidism patients. Comprehensive studies that have investigated differential gene expression using molecular biological techniques, such as microarrays and PCR-based suppression subtractive hybridization, have been reported to predict the fertility potential of patients with cryptorchidism.

Nguyen et al[49] have recently examined differential spermatozoal gene expression patterns using microarray. Several transcriptional factors (CUL3, PRM1, HSPCD35) and a testis-specific cell-adhesion gene (*TPX-1*) involved in germ cell were underexpressed and an antiapoptotic gene (TNFAIP3) was highly overexpressed in cryptorchid samples. Changes in the spermatozoal expression of transcriptional and antiapoptotic genes may result in poor seminal parameters in previously cryptorchid males.

Hadziselimovic et al[48] reported decreased maturation in the cryptorchid testes of gonocytes to adult dark (Ad) spermatogonia, which form the adult stem cell reservoir, and the presence of Ad spermatogonia at surgery is an excellent prognostic parameter for future fertility. Because hypoplasia of Leydig cells has been observed in undescended testes, it is speculated that failure of the testosterone surge turns off the switch that controls the transformation of gonocytes into Ad spermatogonia in cryptorchidism. Recently, we have detected differentially expressed genes in human testes between cryptorchid and descended testes using PCR-based suppression subtractive hybridization, and found that *TPT1*, *EEF1A1*, and *NuMA1* genes, which are expressed in spermatogonia, were significantly more highly expressed in cryptorchid testes.[50] *TPT1*, *EEF1A1*, and *NuMA1* have cell growth–related functions, suggesting that they play certain roles in germ cell differentiation and the maintenance of stem cell potential (Fig. 2).

Spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis. They can self-renew and generate a large number of differentiated germ cells. The balance between SSC renewal and differentiation

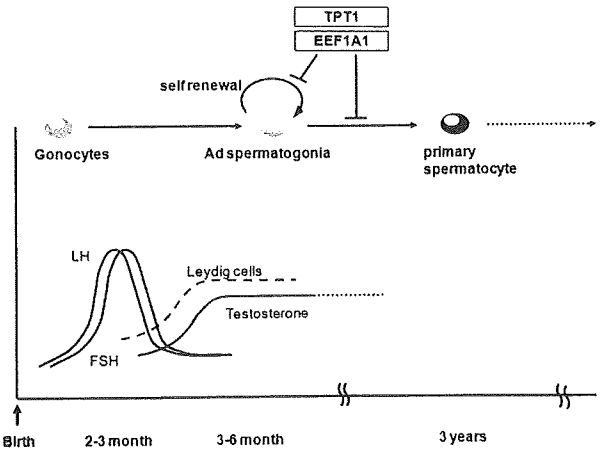

Figure 2. A model of TPT1 and EEF1A1 control of germ cell differentiation and the maintenance of stem cell potential. Decreased maturation in the cryptorchid testes of gonocytes to adult dark (Ad) spermatogonia, which form the adult stem cell reservoir, and the presence of Ad spermatogonia at surgery is an excellent prognostic parameter for future fertility in cryptorchidism.[48] These genes are potentially available as objective indexes to assess compromised spermatogenesis in cryptorchid testes, and may also help predict future fertility.[50]

in the adult testis is essential to maintain normal spermatogenesis and fertility.[51] Recently, Orwig et al[52] reported that genes involved in post-transcriptional regulation are over-represented in stem/progenitor spermatogonia of cryptorchid mouse testes that are enriched with spermatogonial stem cells. Stem cell research in the testis may provide new insight into the prediction of fertility in patients with cryptorchidism in the future.

FUTURE TREATMENT STRATEGIES FOR CRYPTORCHIDISM TO ACHIEVE FERTILITY

As described previously, orchiopexy is one of the most frequent surgical procedures for cryptorchidism, and has been shown to have a beneficial effect on future fertility; however, orchiopexy, especially for bilateral cryptorchidism, does not always guarantee subsequent fertility and paternity.[7] Histologically, tubular degenerative change, especially the loss of germ cells in seminiferous tubules, starts as early as 6 months of age.[53] Although patients have received orchiopexy at <6 months of age, one third were reported to have an abnormal sperm count later in life.[48] There is doubt about whether orchiopexy alone is sufficient for the complete restoration of spermatogenesis.[48] GnRH agonist treatment after successful orchiopexy may have a positive effect on germ cells, but there is insufficient evidence to support the efficacy of this treatment. Recent techniques of assisted reproductive technology, especially testicular sperm extraction with

intracytoplasmic sperm injection (TESE-ICSI), have brought about revolutionary changes in clinical therapy for infertility. TESE-ICSI is sometimes a treatment option and is successful in male fertility patients associated with cryptorchidism. Assessment of paternity potential based on the presence of spermatozoa in the testes and correlation of the serum FSH level and testicular volume in adults with nonobstructive infertility is useful for predicting the paternity potential of previous cryptorchid pubertal boys who had undergone orchiopexy in their childhood.[7] On the basis of this assessment study, we previously analyzed the correlation between serum FSH level and testicular volume in pubertal boys with a history of bilateral orchiopexy in their childhood, and predicted their future paternity potential. This study demonstrated that 19% of previously bilaterally cryptorchid pubertal boys were predicted to lose their future paternity potential even if TESE-ICSI had been conducted, because they were predicted to have no spermatozoa in the testis.[7] To prevent or reverse impaired spermatogenesis before or at puberty, additional therapeutic options may be required for pubertal boys with a history of bilateral orchiopexy, especially when serum FSH levels are increased and testicular volume is decreased, before paternity potential is lost.

We previously investigated the effect of epidermal growth factor in combination with orchiopexy on the cryptorchid rat testis in which tubular deterioration had become partially irreversible.[54] We demonstrated in this animal study that orchiopexy alone might not be sufficient for complete recovery of spermatogenesis, but spermatogenesis maturity was significantly higher and the number of apoptotic germ cells tended to be smaller in orchiopexy with epidermal growth factor administration to seminiferous tubules. This study suggests that epidermal growth factor administered with orchiopexy may be more effective in restoring spermatogenesis than orchiopexy alone.

Gene therapy is a new and promising approach, which opens a new door to the treatment of human diseases. There have been many reports about gene transfer to sperm and testis, the original purpose of which was to develop more effective and simple methods to obtain transgenic animals.[55] In the future, however, this technique has the potential to be the most useful approach for the treatment of male infertility.[55] Several researchers have attempted gene transfer into animal testis using several vectors. There are 2 main types of gene delivery vector: nonviral (eg, liposome or electroporation) and viral (eg, adenovirus, retrovirus, lentivirus, or baculovirus). The choice of vectors is one of the most important issues in clinical trials of gene therapy for male infertility. In the future, the promise of gene therapy as a useful strategy to improve spermatogenesis of the patients with cryptorchidism may become credible and warrants further investigation.

CONCLUSIONS

Because various mutations and polymorphisms have been reported previously, they may not constitute major susceptibility factors for isolated cryptorchidism, suggesting that other genetic factors might also affect these anomalies. Further advances in the molecular approach will refine our knowledge of the genetic mechanisms involved in testicular descent, reducing the incidence of cryptorchidism and leading to new strategies for clinical management of this anomaly.

References

1. Trussell JC, Lee PA. The relationship of cryptorchidism to fertility. *Curr Urol Rep.* 2004;5:142-148.
2. Foresta C, Zuccarello D, Garolla A, et al. Role of hormones, genes, and environment in human cryptorchidism. *Endocr Rev.* 2008;29: 560-580.
3. Welsh M, Saunders PT, Fisken M, et al. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest.* 2008;118:1479-1490.
4. Engeler DS, Hösli PO, John H, et al. Early orchiopexy: prepubertal intratubular germ cell neoplasia and fertility outcome. *Urology.* 2000;56:144-148.
5. Gapany C, Frey P, Cachat F, et al. Management of cryptorchidism in children: guidelines. *Swiss Med Wkly.* 2008;138:492-498.
6. Lee PA, Coughlin MT. Fertility after bilateral cryptorchidism. Evaluation by paternity, hormone, and semen data. *Horm Res.* 2001;55:28-32.
7. Kojima Y, Hayashi Y, Mizuno K, et al. Assessment of serum follicle-stimulating hormone level and testicular volume for prediction of paternity potential in pubertal boys who underwent bilateral orchiopexy in childhood. *J Urol.* 2006;175:2290-2294.
8. Krumlauf R. Hox genes in *vertebrate* development. *Cell.* 1994;78: 191-201.
9. Satokata I, Benson G, Maas R. Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. *Nature.* 1995;374:460-463.
10. Hsieh-Li HM, Witte DP, Weinstein M, et al. Hoxa 11 structure, extensive antisense transcription, and function in male and female fertility. *Development.* 1995;121:1373-1385.
11. Lewis AG, Pecha BR, Smith EP, et al. Early orchiopexy restores fertility in the Hoxa 11 gene knockout mouse. *J Urol.* 2003;170: 302-305.
12. Kolon TF, Wiener JS, Lewitton M, et al. Analysis of homeobox gene HOXA10 mutations in cryptorchidism. *J Urol.* 1999;161:275-280.
13. Bertini V, Bertelloni S, Valetto A, et al. Homeobox HOXA10 gene analysis in cryptorchidism. *J Pediatr Endocrinol Metab.* 2004;17: 41-45.
14. Ferlin A, Foresta C. Insulin-like factor 3: a novel circulating hormone of testicular origin in humans. *Ann N Y Acad Sci.* 2005; 1041:497-505.
15. Nef S, Parada LF. Cryptorchidism in mice mutant for Insl3. *Nat Genet.* 1999;22:295-299.
16. Zimmermann S, Steding G, Emmen JM, et al. Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. *Mol Endocrinol.* 1999;13:681-691.
17. Bogatcheva NV, Truong A, Feng S, et al. GREAT/LGR8 is the only receptor for insulin-like 3 peptide. *Mol Endocrinol.* 2003;17: 2639-2646.
18. Nguyen MT, Showalter PR, Timmons CF, et al. Effects of orchiopexy on congenitally cryptorchid insulin-3 knockout mice. *J Urol.* 2002;168:1779-1783.
19. Feng S, Bogatcheva NV, Truong A, et al. Over expression of insulin-like 3 does not prevent cryptorchidism in GNRHR or HOXA10 deficient mice. *J Urol.* 2006;176:399-404.

20. Feng S, Cortessis VK, Hwang A, et al. Mutation analysis of INSL3 and GREAT/LGR8 genes in familial cryptorchidism. *Urology*. 2004;64:1032-1036.
21. El Houate B, Rouba H, Sibai H, et al. Novel mutations involving the INSL3 gene associated with cryptorchidism. *J Urol*. 2007;177: 1947-1951.
22. Bogatcheva NV, Agoulnik AI. INSL3/LGR8 role in testicular descent and cryptorchidism. *Reprod Biomed Online*. 2005;10:49-54.
23. Ferlin A, Zuccarello D, Zuccarello B, et al. Genetic alterations associated with cryptorchidism. *J Am Med Assoc*. 2008;300:2271-2276.
24. Li X, Nokkala E, Yan W, et al. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. *Endocrinology*. 2001;142:2435-2442.
25. Charlton HM, Halpin DM, Iddon C, et al. The effects of daily administration of single and multiple injections of gonadotropin-releasing hormone on pituitary and gonadal function in the hypogonadal (hpg) mouse. *Endocrinology*. 1983;113:535-544.
26. Pask AJ, Kanasaki H, Kaiser UB, et al. A novel mouse model of hypogonadotrophic hypogonadism: N-ethyl-N-nitrosourea-induced gonadotropin-releasing hormone receptor gene mutation. *Mol Endocrinol*. 2005;19:972-981.
27. Zhang FP, Poutanen M, Wilbertz J, et al. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol Endocrinol*. 2001;15:172-183.
28. Yuan FP, Lin DX, Rao CV, et al. Cryptorchidism in LhrKO animals and the effect of testosterone-replacement therapy. *Hum Reprod*. 2006;21:936-942.
29. Mizuno K, Hayashi Y, Kojima Y, et al. Influence for testicular development and histological peculiarity in the testes of flutamide-induced cryptorchid rat model. *Int J Urol*. 2007;14:67-72.
30. Mizuno K, Hayashi Y, Kojima Y, et al. Activation of NF-kappaB associated with germ cell apoptosis in testes of experimentally induced cryptorchid rat model. *Urology*. 2009;73:389-393.
31. Mizuno K, Hayashi Y, Kojima Y, et al. Early orchiopexy improves subsequent testicular development and spermatogenesis in the experimental cryptorchid rat model. *J Urol*. 2008;179:1195-1199.
32. Blackburn WR, Chung KW, Bullock L, et al. Testicular feminization in the mouse: studies of Leydig cell structure and function. *Biol Reprod*. 1973;9:9-23.
33. Ferlin A, Garolla A, Bettella A, et al. Androgen receptor gene CAG and GGC repeat lengths in cryptorchidism. *Eur J Endocrinol*. 2005;152:419-425.
34. Radpour R, Rezaee M, Tavasoly A, et al. Association of long polyglycine tracts (GGN repeats) in exon 1 of the androgen receptor gene with cryptorchidism and penile hypospadias in Iranian patients. *J Androl*. 2007;28:164-169.
35. Donaldson KM, Tong SY, Washburn T, et al. Morphometric study of the gubernaculum in male estrogen receptor mutant mice. *J Androl*. 1996;17:91-95.
36. Emmen JM, McLuskey A, Adham IM, et al. Involvement of insulin-like factor 3 (Insl3) in diethylstilbestrol-induced cryptorchidism. *Endocrinology*. 2000;141:846-849.
37. Cederroth CR, Schaad O, Descombes P, et al. Estrogen receptor alpha is a major contributor to estrogen-mediated fetal testis dysgenesis and cryptorchidism. *Endocrinology*. 2007;148:5507-5519.
38. Wang Y, Barthold J, Figueroa E, et al. Analysis of five single nucleotide polymorphisms in the ESR1 gene in cryptorchidism. *Birth Defects Res A Clin Mol Teratol*. 2008;82:482-485.
39. Lahoud MH, Ristevski S, Venter DJ, et al. Gene targeting of Desrt, a novel ARID class DNA-binding protein, causes growth retardation and abnormal development of reproductive organs. *Genome Res*. 2001;11:1327-1334.
40. Ikadai H, Ajisawa C, Taya K, et al. Suprainguinal ectopic scrota of TS inbred rats. *J Reprod Fertil*. 1988;84:701-707.
41. Zuccarello D, Morini E, Douzgou S, et al. Preliminary data suggest that mutations in the CgRP pathway are not involved in human sporadic cryptorchidism. *J Endocrinol Invest*. 2004;27:760-764.
42. Behringer RR, Finegold MJ, Cate RL. Müllerian-inhibiting substance function during mammalian sexual development. *Cell*. 1994; 79:415-425.
43. Kojima Y, Sasaki S, Hayashi Y, et al. Role of transcription factors Ad4BP/SF-1 and DAX-1 in steroidogenesis and spermatogenesis in human testicular development and idiopathic azoospermia. *Int J Urol*. 2006;13:785-793.
44. Lin L, Achermann JC. Steroidogenic factor-1 (SF-1, Ad4BP, NR5A1) and disorders of testis development. *Sex Dev*. 2008;2:200-209.
45. Wada Y, Okada M, Fukami M, et al. Association of cryptorchidism with Gly146Ala polymorphism in the gene for steroidogenic factor-1. *Fertil Steril*. 2006;85:787-790.
46. Olesen IA, Sonne SB, Hoei-Hansen CE, et al. Environment, testicular dysgenesis and carcinoma in situ testis. *Best Pract Res Clin Endocrinol Metab*. 2007;21:462-478.
47. Yoshida R, Fukami M, Sasagawa I, et al. Association of cryptorchidism with a specific haplotype of the estrogen receptor alpha gene: implication for the susceptibility to estrogenic environmental endocrine disruptors. *J Clin Endocrinol Metab*. 2005;90:4716-4721.
48. Hadziselimovic F, Herzog B. The importance of both an early orchidopexy and germ cell maturation for fertility. *Lancet*. 2001; 358:1156-1157.
49. Nguyen MT, Delaney DP, Kolon TF. Gene expression alterations in cryptorchid males using spermatozoal microarray analysis. *Fertil Steril. In press*.
50. Mizuno K, Kojima Y, Kurokawa S, et al. Identification of differentially expressed genes in human cryptorchid testes using suppression subtractive hybridization. *J Urol*. 2009;181:1330-1337.
51. Dadoune JP. New insights into male gametogenesis: what about the spermatogonial stem cell niche? *Folia Histochem Cytobiol*. 2007;45: 141-147.
52. Orwig KE, Ryu BY, Master SR, et al. Genes involved in post-transcriptional regulation are overrepresented in stem/progenitor spermatogonia of cryptorchid mouse testes. *Stem Cells*. 2008;26: 927-938.
53. Wilkerson ML, Bartone FF, Fox L, et al. Fertility potential: a comparison of intra-abdominal and intracanalicular testes by age groups in children. *Horm Res*. 2001;55:18-20.
54. Kurokawa S, Kojima Y, Mizuno K, et al. Effect of epidermal growth factor on spermatogenesis in the cryptorchid rat. *J Urol*. 2005;174: 2415-2419.
55. Kojima Y, Kurokawa S, Mizuno K, et al. Gene transfer to sperm and testis: future prospects of gene therapy for male infertility. *Curr Gene Ther*. 2008;8:121-134.

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Original Article: Clinical Investigation

Laparoscopic dismembered pyeloplasty for ureteropelvic junction obstruction in children

Yoshiyuki Kojima, Shoichi Sasaki, Kentaro Mizuno, Keiichi Tozawa, Yutaro Hayashi and Kenjiro Kohri

Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan

Objectives: To present our initial experience with laparoscopic pyeloplasty and to evaluate the safety and short-term outcome of this technique in children.

Methods: Thirteen kidney units in twelve children underwent laparoscopic dismembered pyeloplasty for the management of ureteropelvic junction obstruction (UPJO) at our institution between 2005 and 2008. Patient age at surgery was 18–177 months (mean 89.8 months). There were six boys and six girls. Ten had unilateral UPJO with a normal contralateral kidney, one had bilateral UPJO and one had UPJO of a solitary kidney. We used 3- and 5-mm instruments for grasping, blunt dissection, incising and suturing to facilitate safe and precise surgery. The outcome was measured by the operative time and resolution of obstruction and symptoms.

Results: Median operative time was 275 min (range 154–420). There was a slight relationship between age and operative time. No major perioperative complications occurred in any cases. Median renal pelvic anterior–posterior diameter at ultrasonography significantly decreased from 8.6 cm (range 3.8–22.0) preoperatively to 3.9 cm (1.0–8.9) postoperatively ($P < 0.05$). The median pre- and postoperative split renal function on diuretic renography in unilateral cases was 37.3% (range 29.7–46.4) and 39.5% (27.8–48.0), respectively. Overall, successful resolution of UPJO was observed in 12 of 13 kidneys (92.3%).

Conclusions: Laparoscopic pyeloplasty represents a safe and effective option in the surgical treatment of children with UPJO.

Key words: children, laparoscopy, pyeloplasty, ureteropelvic junction obstruction.

Introduction

Ureteropelvic junction obstruction (UPJO), which is defined as the restricted flow of urine from the renal pelvis to the ureter, remains the most common obstructive uropathy in children. Although various surgical procedures have been described for repairing UPJO, open pyeloplasty is still the gold standard with a success rate exceeding 90%.[1] Recently, laparoscopic pyeloplasty has gradually gained acceptance as a feasible and reliable treatment associated with minimal morbidity in the pediatric population of Western countries since its first report for children in 1995.[2] A recent report obtained from the pediatric health information system in the USA showed that 6.2% of procedures were performed laparoscopically from 2002 to 2007.[3]

The low risk of complications demonstrated in the large series confirms that pediatric laparoscopic procedures are safe, although there possibly remains a risk of significant injury.[4] Although various laparoscopic techniques through transperitoneal and retroperitoneal approaches have been reported,[5,6] laparoscopic pyeloplasty for children has not been accepted yet in Japan and there are very few reports.

In this study, we present our initial experience of laparoscopic pyeloplasty for children at our institution and evaluate the safety and short-term outcome of this technique.

Methods

Thirteen kidneys of 12 children underwent laparoscopic dismembered pyeloplasty for the management of UPJO at our institution between 2005 and 2008. Patient age at surgery was 18–177 months (mean 89.8 months), and there were 6 boys and 6 girls. Ten had unilateral UPJO with a normal contralateral kidney, one had bilateral UPJO and one had UPJO of a solitary kidney; 11 were on the left and two on the right. Nine children had symptomatic UPJO. All children underwent preoperative radiological imaging, including ultrasonography and diuretic renography, for the diagnosis of UPJO. The indications of surgery included an increasing degree of hydronephrosis, a low split renal function (<40%) and/or an obstructive pattern on diuretic renography and/or symptoms such as pain, urinary tract infection or constipation. Three patients had undergone percutaneous nephrostomy before surgery because of acute renal failure, abdominal pain and large hydronephrosis.

Laparoscopic dismembered pyeloplasty was carried out in the lateral position under general anesthesia. Cystoscopy and retrograde pyelography were performed at the beginning of the procedure to identify the UPJO and other anomalies of the urinary tract system. A small skin incision was made at a level just cephalad to the umbilicus, and the peritoneum was dissected under direct vision. A 5-mm trocar was inserted intraperitoneally and pneumoperitoneum was conducted at 8 to 10 mmHg to observe the inside of the abdominal cavity clearly using a 5-mm, 30-degree scope. Two additional trocars (3 mm and 5 mm) were inserted (Fig. 1). Occasionally, an extra 3-mm trocar was placed for retraction purposes. We used 3- and 5-mm curve dissectors for grasping and blunt dissection, 3-mm curve scissors for incising and a 3-mm needle driver for suturing and tissue anastomosis to facilitate safe and precise surgery.

The peritoneum overlying the kidney was incised to expose the UPJO with medial mobilization of the colon. A percutaneous hitch stitch was placed in the pelvis to facilitate exposure. The stenotic segment was excised and the ureter spatulated (Fig. 2). The lower corner of the ureter was sutured to the lower edge of the pelvis with an everting 5-0 Vicryl suture. A 4.7 Fr double-J catheter was inserted in an antegrade fashion over a guidewire. Ureteropelvic anastomosis was

Correspondence: Shoichi Sasaki MD, Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, 1, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. Email: ssk@med.nagoya-cu.ac.jp

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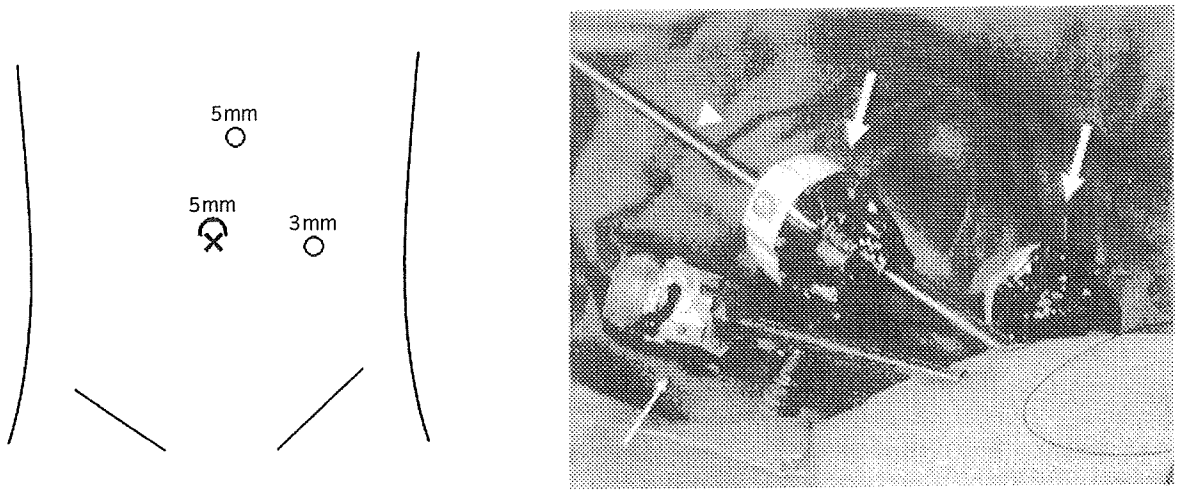

Fig. 1 Trocar placement for left pyeloplasty. A 5-mm trocar was inserted intraperitoneally and pneumoperitoneum was conducted at 8 to 10 mmHg to observe the inside of the abdominal cavity clearly using a 5-mm, 30-degree scope. Two additional trocars (3 mm and 5 mm) were inserted Arrowhead: 5-mm, 30-degree scope. Large arrows: 5-mm trocar. Small arrow: 3-mm trocar.

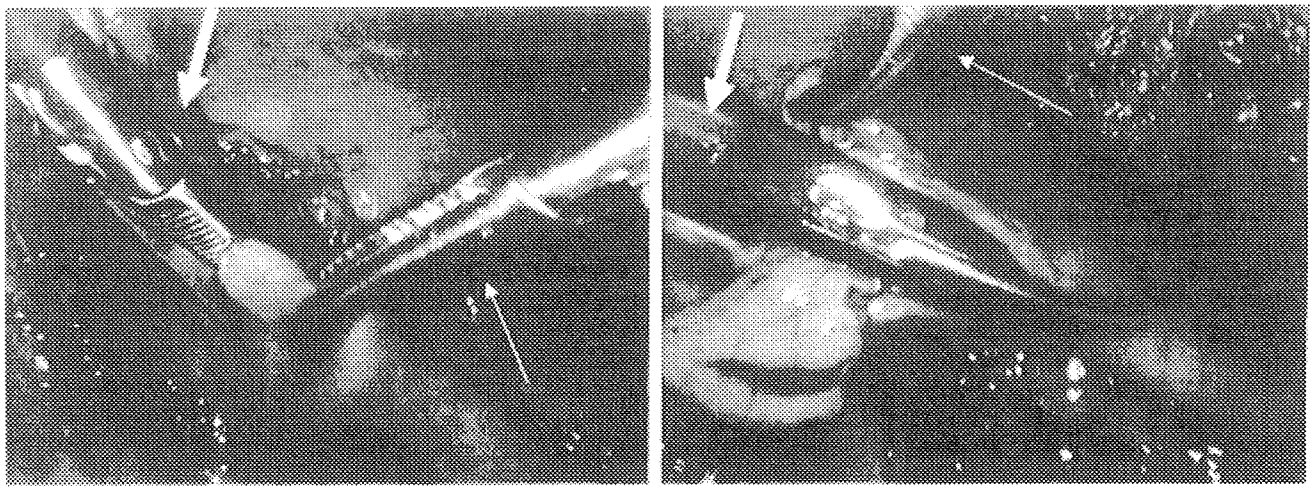

Fig. 2 Laparoscopic pyeloplasty for children with ureteropelvic junction obstruction. The stenotic segment is excised and the ureter was spatulated. Large arrows: 3-mm curve scissors. Small arrow: 3-mm curve dissector.

basically performed with interrupted 5-0 Vicryl sutures. The renal pelvis was then closed with a running 5-0 Vicryl suture. Modest reduction of the renal pelvis is routinely performed. An intra-abdominal Penrose drain was left in all patients through a port site.

The double-J catheter was removed after six to eight weeks under general anesthesia. Postoperative ultrasonography was performed monthly, and a diuretic renogram was performed at 6 and 12 months, and annually thereafter. The criteria for short-term success were a marked reduction of hydronephrosis on ultrasonography, preservation of split renal function and improvement in the drainage curve on diuretic renography, and symptom resolution at 6 months.

The correlation between patient age and the operation time was evaluated with Pearson correlation tests using StatView 4.5 software (Abacus Concept, Inc., Cary, NC, USA). The significance of differences in the renal pelvic anterior–posterior diameter on ultrasonography and split renal function on diuretic renography between pre- and post-operation was determined by the unpaired *t*-test using the same software. Significance was defined as $P < 0.05$.

Results

All operations were completed by laparoscopic dismembered pyeloplasty following the principles of the open Anderson-Hynes procedure. One child with bilateral UPJO underwent right laparoscopic pyeloplasty 6 months after left laparoscopic pyeloplasty.

Median operative time was 275 min (range 154–420). There was a slight relationship between age and operative time (Fig. 3a; $r = 0.43$). The time required for exposure of UPJ was recorded at a median of 78.7 min (range 40–140), which had a slight relationship with age (Fig. 3b; $r = 0.40$). The time required for ureteropelvic anastomosis was recorded at a median of 156.9 min (range 84–260), including the time needed for placement of the Double-J catheter (Fig. 3c; $r = 0.26$). One girl had a huge pelvis of 3 L capacity, requiring an extensive reduction, which would have extended the operative time. There was no difference in the operating time between right- and left-sided procedures. No aberrant crossing vessel was observed in any cases. No patient required open conversion. In principle, a liquid diet was started 6 h after opera-

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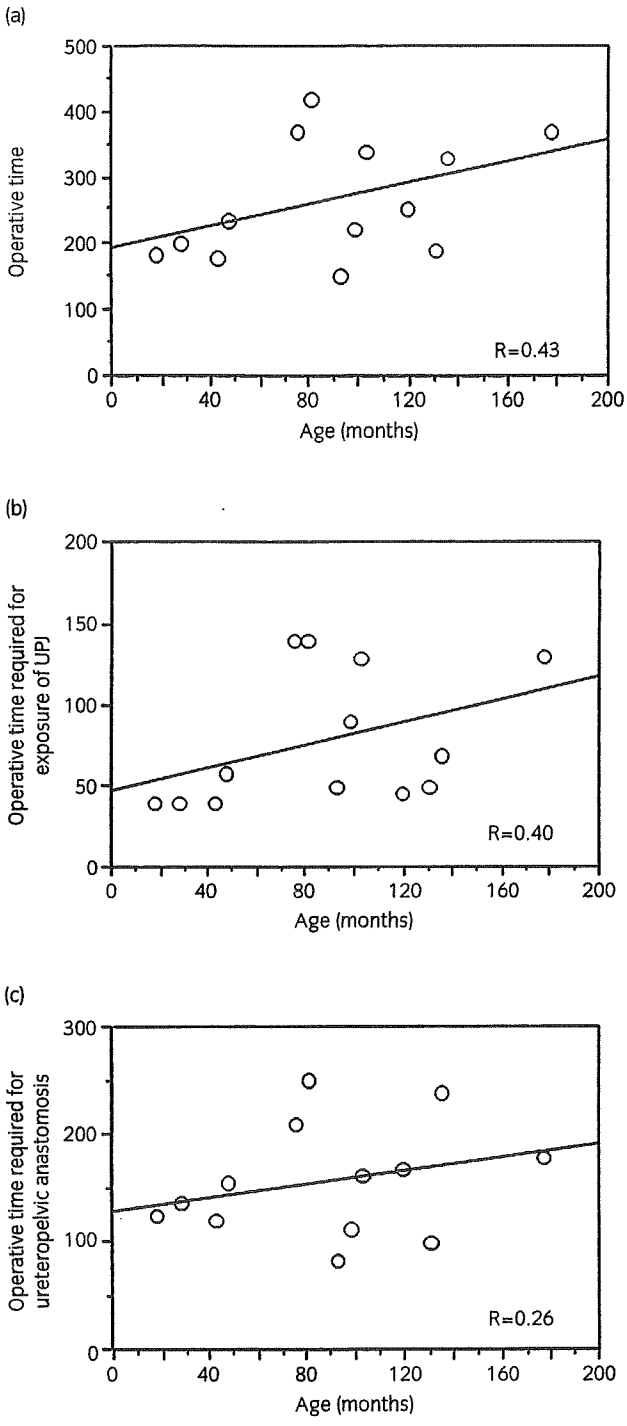

Fig. 3 (a) Correlation between patient age and median total operative time, (b) median operative time required for exposure of ureteropelvic junction (UPJ) or (c) median operative time required for ureteropelvic anastomosis.

tion and was rapidly increased if tolerated by the patients. Feeding began after a mean of 1.5 (0–3) days. The urethral catheter was removed after a mean of 3.6 (3–7) days. Children passed stools after a mean of 2.1 (1–5) days. No major perioperative complications occurred in any cases. One patient had postoperative urine leakage and subileus, which resolved spontaneously after a short period. No patients required perioperative percutaneous nephrostomy. Postoperative pain management was optimal using only nonsteroidal anti-inflammatory drugs for a few days.

No patient required treatment for urinary tract infection with oral antibiotics while the Double-J catheter was indwelling.

Median follow-up months were 16.4 months (range 6–40). Median renal pelvic anterior–posterior diameter on ultrasonography was statistically significantly decreased from 8.6 cm (range 3.8–22.0) preoperatively by 3.9 cm (1.0–10.2) postoperatively (Fig. 4a; $P < 0.05$). The median pre- and postoperative split renal function on diuretic renography in unilateral cases was 37.3% (range 29.7–46.4) and 39.5% (27.8–48.0), respectively (Fig. 4b). One patient underwent repeat laparoscopic pyeloplasty after 11 months due to persistent hydronephrosis and decreasing split renal function. The child improved after the second operation. Ultimately, successful resolution of UPJO was observed in 12 of 13 kidneys (92.3%). The wound was smaller than 5 mm in all and the cosmetic appearance of wounds after the operation was good in all cases (Fig. 5).

Discussion

In this study, we demonstrated the short-term outcome of pediatric laparoscopic pyeloplasty at our institution. The success rate was 92.3%, similar to that of open and laparoscopic pyeloplasty reported previously. No open conversion or perioperative major complication was observed. We documented its efficacy and safety in children in our series, and whether it could become the minimal invasive treatment of choice.

There are several advantages of laparoscopic pyeloplasty in children as well as adults. First, laparoscopic pyeloplasty has an advantage with regard to pain and cosmetic value. Even a 3–5-cm posterior lumbodorsal incision for open pyeloplasty necessitates several weeks before a return to normal activity and a flank incision requires even more time,[2] because significant tissue retraction is needed to expose the field and the muscle incision and damage is often more than that anticipated. On the other hand, laparoscopic surgery needs only a 3–10-mm skin incision and less muscle damage corresponds to the skin incision and can be performed safely with good exposure. Additionally, we used 3- and 5-mm curve dissectors for grasping and blunt dissection, 3-mm curve scissors for incising and a 3-mm needle driver for suturing and tissue anastomosis to facilitate safe and precise surgery.

The second main advantage is that all medical staff, including the surgeon, assistant, anesthesiologist, nurses, residents and medical students, share the same real-time operative view through the monitor. This enables us not only to avoid complications and technical insecurity, but also better educates residents and medical students. At our institute, both pediatric urological specialists and laparoscopic specialists certificated by each association in Japan are involved in laparoscopic pyeloplasty for children, especially this year, to avoid perioperative complications.

On the other hand, several disadvantages of laparoscopic pyeloplasty have been pointed out in previous reports. The disadvantage of laparoscopic pyeloplasty is that operative times are significantly higher than open pyeloplasty.[7,8] In particular, laparoscopic suturing for children is challenging and time-consuming and requires a learning curve;[7] however, it significantly improved, even with pediatric laparoscopic pyeloplasty, with increased experience in previous reports.[7,9]

Open pyeloplasty is performed through the retroperitoneal approach, which has the advantage of less risk of intraperitoneal organ injury, postoperative ileus, and avoidance of potential deleterious effects of peritoneal exposure to blood and urine. Although adhesions may occur

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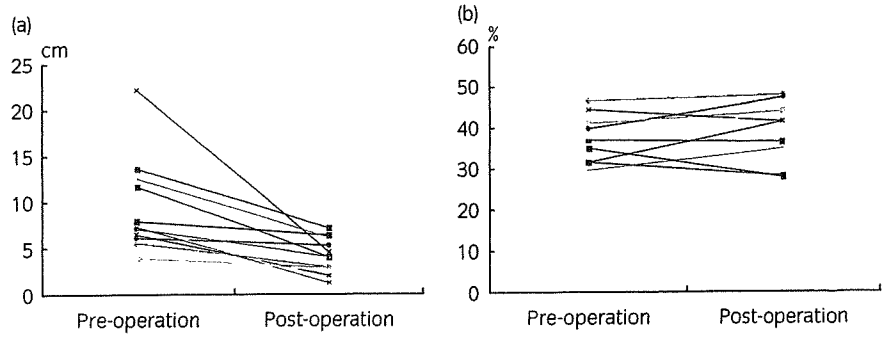

Fig. 4 (a) Median pre- and postoperative renal pelvic anterior–posterior diameter on ultrasonography. (b) Median pre- and postoperative split renal function on diuretic renography in unilateral cases.

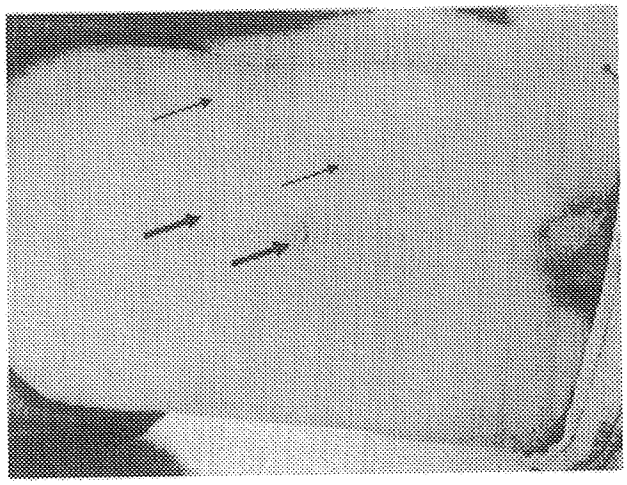

Fig. 5 Postoperative appearance after 6 months in a representative case. The wound measures 5 mm (large arrows) and 3 mm (small arrows).

with pediatric urological laparoscopic procedures, the incidence appears lower than would be expected with open exploration.[10] Recently, several reports have demonstrated the usefulness of retroperitoneoscopic pyeloplasty for not only adults but also children. Although there are some reports on retroperitoneoscopic pyeloplasty,[11–13] we used a transperitoneal approach because we find that the working space is not limiting and we are more comfortable with this approach. Canon *et al.* reported in their comparison study between retroperitoneoscopic and laparoscopic pyeloplasty in children that no major difference exists between the two approaches for correcting UPJO, although the average operative time for the retroperitoneoscopic approach was significantly longer than that for the laparoscopic approach because of the larger working space for suturing, the perceived ease of antegrade stent placement and the subjective improvement in cosmetic outcome.[12] Metzelder *et al.* concluded that no disadvantage was attributable to the transabdominal approach in children.[9] There have been no large-scale randomized and prospective studies comparing the approaches. Retroperitoneoscopic pyeloplasty remains a technically challenging procedure in children and further studies will be needed.

Laparoscopic dismembered pyeloplasty is an acceptable option for UPJO in infants and younger children.[9,10,14] A recent report has demonstrated that laparoscopic dismembered pyeloplasty is technically possible in infants younger than 6 months.[15] Our study included two children under 3 years old. Interestingly, a slight relationship between age and operative time was observed, despite the small series study. In addition, the time required for UPJ exposure had a slight relationship with age. In our impression, as younger children generally have little surrounding fatty tissue, it may be easier to expose the UPJ.

Conclusion

In conclusion, laparoscopic pyeloplasty is a safe and effective option in the surgical treatment of children with UPJO. Recently, several reports have shown that robot-assisted pyeloplasty was another safe and effective modality for treating children with UPJO,[16,17] having improved the surgical manipulation of laparoscopic surgery and provided short-term results similar to those of conventional laparoscopic pyeloplasty in Western countries. Because of the limitations of public medical insurance and cost performance, it is not yet acceptable in Japan. Pediatric laparoscopic pyeloplasty for children will gradually become a more acceptable procedure with the development and improvement of instruments and a better training system in Japan, as in Western countries.

References

1 Tan BJ, Smith AD. Ureteropelvic junction obstruction repair: When, how, what? *Curr. Opin. Urol.* 2004; **14**: 55–9.
2 Peters CA, Schlussel RN, Retik AB. Pediatric laparoscopic dismembered pyeloplasty. *J. Urol.* 1995; **153**: 1962–5.
3 Tanaka ST, Grantham JA, Thomas JC, Adams MC, Brock JW III, Pope JC IV. A comparison of open vs laparoscopic pediatric pyeloplasty using the pediatric health information system database: Do benefits of laparoscopic approach recede at younger ages? *J. Urol.* 2008; **180**: 1479–85.
4 Passerotti CC, Nguyen HT, Retik AB, Peters CA. Patterns and predictors of laparoscopic complications in pediatric urology: The role of ongoing surgical volume and access techniques. *J. Urol.* 2008; **180**: 681–5.
5 Tan HL. Laparoscopic Anderson-Hynes dismembered pyeloplasty in children. *J. Urol.* 1999; **162**: 1045–7.
6 Sweeney DD, Smaldone MC, Docimo SG. Minimally invasive surgery for urologic disease in children. *Nat. Clin. Pract. Urol.* 2007; **4**: 26–38.
7 Piaggio LA, Franc-Guimond J, Noh PH *et al.* Transperitoneal laparoscopic pyeloplasty for primary repair of ureteropelvic junction obstruction in infants and children: Comparison with open surgery. *J. Urol.* 2007; **178**: 1579–83.
8 Ravish IR, Nerli RB, Reddy MN, Amarkhed SS. Laparoscopic pyeloplasty compared with open pyeloplasty in children. *J. Endourol.* 2007; **21**: 897–902.
9 Metzelder ML, Schier F, Petersen C, Truss M, Ure BM. Laparoscopic transabdominal pyeloplasty in children is feasible irrespective of age. *J. Urol.* 2006; **175**: 688–91.
10 Moore RG, Kavoussi LR, Bloom DA *et al.* Postoperative adhesion formation after urological laparoscopy in the pediatric population. *J. Urol.* 1995; **153**: 792–5.

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11 Yeung CK, Tam YH, Sihoe JD, Lee KH, Liu KW. Retroperitoneoscopic dismembered pyeloplasty for pelvi-ureteric junction obstruction in infants and children. *BJU Int.* 2001; **87**: 509–13.
12 Canon SJ, Jayanthi VR, Lowe GJ. Which is better – retroperitoneoscopic or laparoscopic dismembered pyeloplasty in children? *J. Urol.* 2007; **178**: 1791–5.
13 Bonnard A, Fouquet V, Carricaburu E, Aigrain Y, El-Ghoneimi A. Retroperitoneal laparoscopic versus open pyeloplasty in children. *J. Urol.* 2005; **173**: 1710–13.
14 Cascio S, Tien A, Chee W, Tan HL. Laparoscopic dismembered pyeloplasty in children younger than 2 years. *J. Urol.* 2007; **177**: 335–8.
15 Kutikov A, Resnick M, Casale P. Laparoscopic pyeloplasty in the infant younger than 6 months: Is it technically possible? *J. Urol.* 2006; **175**: 1477–9.
16 Passerotti CC, Nguyen HT, Eisner BH, Lee RS, Peters CA. Laparoscopic reoperative pediatric pyeloplasty with robotic assistance. *J. Endourol.* 2007; **21**: 1137–40.
17 Atug F, Woods M, Burgess SV, Castle EP, Thomas R. Robotic assisted laparoscopic pyeloplasty in children. *J. Urol.* 2005; **174**: 1440–2.

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Activation of NF-κB Associated With Germ Cell Apoptosis in Testes of Experimentally Induced Cryptorchid Rat Model

Kentaro Mizuno, Yutaro Hayashi, Yoshiyuki Kojima, Akihiro Nakane, Keiichi Tozawa, and Kenjiro Kohri

OBJECTIVES To evaluate the behavior of NF-κB in the cryptorchid testes, we investigated the testes of an experimentally induced cryptorchid rat model. Cryptorchid testes exhibit degenerative changes in the germinal epithelium with germ cell apoptosis, which results in future infertility. However, the mechanisms of apoptosis in the germ cells are multifactorial and have not been completely elucidated. NF-κB is a transcription factor considered to play an important role in the apoptosis of various cells.

METHODS The cryptorchid rat model was generated by the administration of an antiandrogenic agent during the fetal period. The unilateral cryptorchid testes and contralateral descended testes were removed at 7-10 weeks after birth, and histopathologic examination and Western blot analysis were performed. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining, and the expression of NF-κB and IκB proteins was investigated using immunohistochemistry.

RESULTS In the present model, spermatogenesis was arrested at the spermatid differentiation stage, and elongated spermatids or spermatozoa were not detected. Apoptosis was noted predominantly in the primary spermatocytes, and Sertoli cells and Leydig cells did not undergo apoptosis. On immunohistochemistry, NF-κB signals were increased and focused on the nucleus of the germ cells in the cryptorchid testes, consistent with TUNEL-positive cells. On Western blot analysis, the NF-κB and IκB proteins were detected more intensely in the cryptorchid testes.

CONCLUSIONS The activation of NF-κB was associated with germ cell apoptosis in experimentally induced cryptorchid testes, suggesting that NF-κB has certain roles in germ cell apoptosis. The results of the present study may guide toward new therapeutic possibilities for the impairment of spermatogenesis in patients with cryptorchidism. UROLOGY 73: 389–393, 2009. © 2009 Elsevier Inc.

Cryptorchidism is the most common congenital abnormality among all urologic diseases, with an incidence of 2%-4% in mature infant boys.[1] Histopathologic investigations of undescended testes have revealed a decrease in the number of germ cells and Leydig cells by 18 months after birth.[2] The delay in differentiation from gonocytes to adult dark spermatogonia, decrease in seminiferous tubule diameter, and proliferation of collagen around the tubules and vessels have also been recognized.[3] These degenerative changes in the germinal epithelium cause male infertility, one of the serious complications of cryptorchidism. However, the molecular and cellular mechanisms of spermatogenic failure are not yet completely understood. Some investigators have proposed 2 possible reasons for the impairment of spermatogenesis in cryptorchid testes: decreased proliferation and increased germ cell apoptosis. Bernal-Mañas et al.[4] demonstrated that the proliferation rate of spermatogonia was lower in cryptorchid testes than in control testes. Several studies on germ cell apoptosis in cryptorchid testes have been reported. Germ cell apoptosis is multifactorial, and its mechanisms are as follows: heat stress,[5] increased oxidative stress,[6] loss of germ cell-specific glucose transporters,[7] and a differential response to gonadotropins.[8] In particular, it has been reported that heat stress induces germ cell apoptosis through p53-dependent, Fas-dependent,[5] and nitric oxide synthase-dependent[9] pathways; however, these findings are not sufficient to completely explain germ cell apoptosis in cryptorchid testes. NF-κB is a transcription factor that has been considered a major regulator of immune and stress responses, and it plays an important role in cell proliferation and apoptosis.[10] Despite the extensive study of NF-κB in the past several years, no reports have been published on the behavior of NF-κB in cryptorchid testes.

From the Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan

Reprint requests: Yutaro Hayashi, M.D., Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601 Japan. E-mail: yutaro@med.nagoya-cu.ac.jp

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To elucidate whether NF-κB is associated with germ cell apoptosis in cryptorchid testes, we investigated the testes of an experimentally induced cryptorchid rat model. Similar to our previous study,[11] the present study also demonstrated an increase in the rate of apoptosis in germ cells, particularly spermatocytes, in the cryptorchid rat model. In the present study, a coincidence between NF-κB-positive cells and apoptotic germ cells was observed, and the expression of the NF-κB inhibitory protein IκBα was investigated. The results of the present study support the evidence that germ cell apoptosis induced by cryptorchidism is associated with the activation of NF-κB in the rat testis.

MATERIAL AND METHODS

Rats

To generate cryptorchid rats, pregnant Sprague-Dawley rats were exposed to flutamide, as previously described.[11] In brief, each pregnant rat was administered 7.5 mg flutamide daily for 7 days from the 14th to 20th day of gestation. In the male offspring, we observed the location of the testes at 28 days after birth, which was when almost all testes should have descended to the scrotum in normal rats. The rats with unilateral cryptorchidism were defined as the model rats and were used in the following experiments. We considered the contralateral descended testes of the flutamide-induced unilateral cryptorchid model rats as the controls. The testes were removed and weighed at 7-10 weeks after birth; one half of the testes were fixed with 4% paraformaldehyde and then embedded in paraffin, and the remaining testes were cryopreserved at −80°C for protein analysis. All experimental procedures were performed in accordance with the protocols approved by the Animal Care Committee of Nagoya City University Graduate School of Medical Sciences.

Histologic Analysis and Immunohistochemistry

Spermatogenesis in the model rats was evaluated by hematoxylin-eosin staining. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining using an Apoptosis In Situ Detection Kit (Wako, Osaka, Japan). For examination of the NF-κB protein, we performed immunohistochemistry using an anti-p65/RelA antibody (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA) and an anti-p50 antibody (sc-114, Santa Cruz Biotechnology). Immunohistochemistry for IκBα, a marker of the NF-κB complex was also performed (anti-IκBα antibody, sc-371, Santa Cruz Biotechnology). In brief, after blocking with 5% skim milk in phosphate-buffered saline, 5-μm-thick serial sections were incubated overnight at 4°C with the anti-p65/RelA antibody (dilution 1:100), anti-p50 antibody (dilution 1:1000), and anti-IκBα antibody (dilution 1:500). Signals were detected with an avidin-biotinylated enzyme complex system using the VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA) and anti-rabbit IgG (PK-6101), according to the manufacturer's instructions. We defined the apoptosis cell index as the number of TUNEL-positive cells among 50 seminiferous tubules in the sections of cryptorchid and descended testes and used this index for the assessment of germ cell apoptosis. Furthermore, we observed whether TUNEL-positive cells were consistent with the signals of NF-κB proteins and IκBα protein using the serial sections of the testes. We also measured the seminiferous tubular diameter using a micrometer eyepiece and calculated the mean seminiferous tubular diameter by averaging the diameter of 50 round, randomly selected, seminiferous tubules in each tissue section. We compared the differences between the descended and cryptorchid testes using the Student *t* test. All values are expressed as the mean ± standard deviation using StatView, version 5.0 (SAS Institute, Cary, NC).

Table 1. Testicular weight, ACI, and STD

Testes	Weight (g)	ACI	STD (μm)
Descended (n = 11)	1.38 ± 0.23	0.60 ± 0.55	264.0 ± 17.8
Cryptorchid (n = 11)	0.53 ± 0.21*	51.8 ± 16.1	181.0 ± 11.0*

ACI, apoptosis cell index; STD, seminiferous tubular diameter.
* Statistically significant, $p < .001$ vs descended testes.

Western Blot Analysis

For protein extraction from the testes, we used the Cell Culture Lysis Reagent (Promega, Madison, WI) according to the manufacturer's instructions. In brief, frozen testicular tissue samples were immersed in 1× lysis buffer and sonicated on ice sufficiently. After incubation on ice for 15 minutes, the supernatants were collected by centrifugation, and the total protein concentration was spectrophotometrically quantified using the BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Samples containing 30 μg total protein were mixed with a loading buffer (Laemmli Sample Buffer; Bio-Rad Laboratories, Hercules, CA). After boiling at 100°C for 10 minutes, the protein was separated by running on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and was then transferred onto PDVF membranes (Immobilon, Millipore, Bedford, MA). Subsequently, the membranes were blocked for 1 hour using 5% skim milk in Tris-buffered saline (pH 7.5)-Tween 20 at room temperature and sequentially incubated with the polyclonal antibodies, namely p65/RelA (dilution 1:200), p50 (dilution 1:2000), and IκBα (dilution 1:500), overnight at 4°C. After washing with Tris-buffered saline-Tween 20, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibodies for 1 hour at room temperature and washed again with Tris-buffered saline-Tween 20. The protein bands were visualized using enhanced chemiluminescence Western blotting analysis kits (Pierce Biotechnology, Rockford, IL), according to the manufacturer's instructions. The final data were acquired by scanning the protein band density. As a loading control, the membranes were probed with an antibody to β-actin (A5316, Sigma-Aldrich, St. Louis, MO).

RESULTS

The cryptorchid testicular tissues were atrophic, and they exhibited hypospermatogenesis with germ cell apoptosis. The seminiferous tubules were degenerated and significantly smaller than those of the descended testes (Table 1). Spermatogenesis was arrested at the spermatid differentiation stage, and elongated spermatids or spermatozoa were not detected. Degenerative changes in the cryptorchid testes increase and the numbers of germ cells decrease the older the cryptorchid model rats; therefore, we performed the histologic examinations using the testes of 7-week-old model rats.

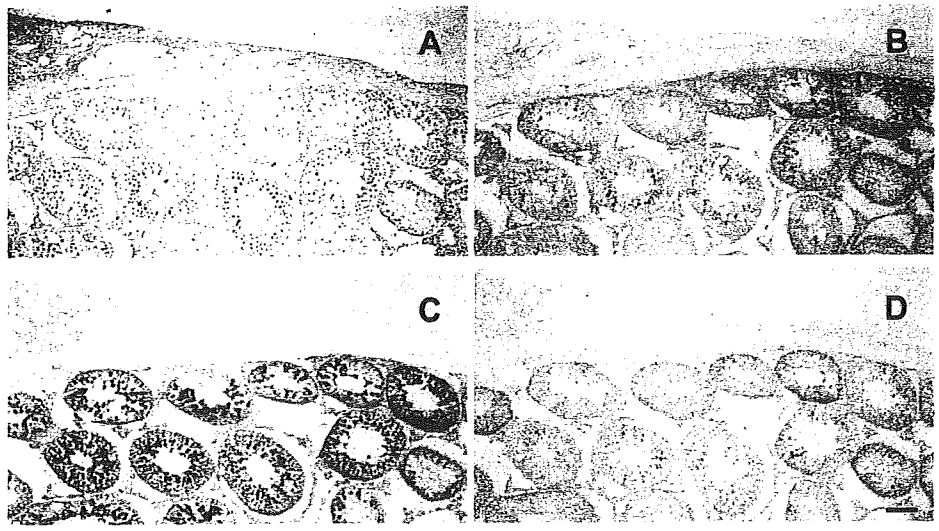

Figure 1. Serial section of cryptorchid rat testes at 7 weeks old. **(A)** Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining, and immunohistochemistry of **(B)** p65, **(C)** p50, and **(D)** IκBα (D) in low-power field. Scale bar = 50 μm.

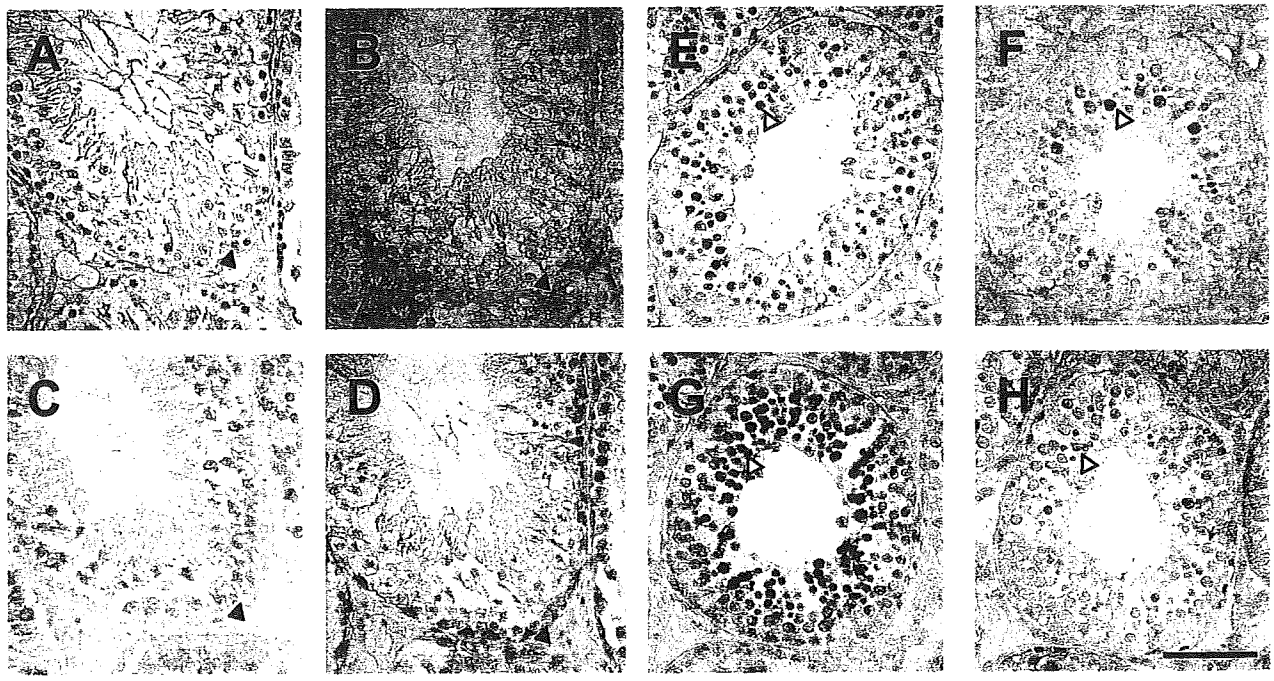

Figure 2. Histologic examinations of a rat testis obtained at 7 weeks. **(A-D)** Contralateral descended testes and **(E-H)** cryptorchid testes. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining **(A,E)** and immunohistochemistry for p65 **(B,F)**, p50 **(C,G)**, and IκBα **(D,H)** shown. Cells corresponding to TUNEL-positive cells in Figs. A and E shown in Figs. B-D (arrowheads) and F-H (white arrowheads), respectively. Scale bar = 50 μm.

The TUNEL staining and immunohistochemistry of NF-κB proteins and IκBα protein from the cryptorchid testis in serial sections are shown in Figure 1. The number of TUNEL-positive cells varied among the seminiferous tubules; nevertheless, the distinction of the stages of the tubules was unfeasible because of the degenerative changes in the testicular tissue. In the seminiferous tubules containing TUNEL-positive cells, NF-κB protein and IκBα protein were strongly expressed compared with those not containing TUNEL-positive cells. Conversely, in the descended testis, TUNEL staining revealed hardly any apoptotic germ cells and only a few spermatogonia (Fig. 2A). All TUNEL-positive cells were germ cells, and apoptosis of the Sertoli cells or Leydig cells was not detected (Fig. 2E). Apoptosis was predominantly detected in the spermatocytes. Immunohistochemistry revealed that the NF-κB protein was present in the cytoplasm of the spermatocytes and Sertoli cells in the

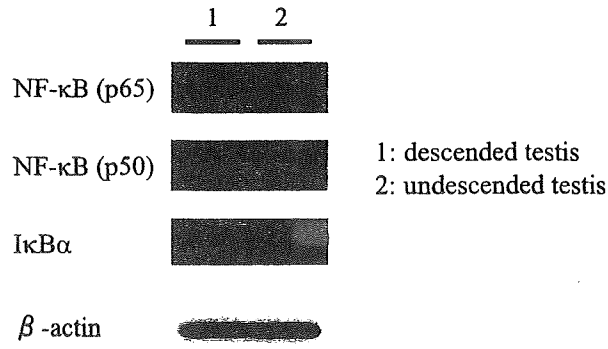

Figure 3. Western blot analysis for NF-κB and IκBα in model rat testes. Testicular proteins (30 μg each) were extracted from cryptorchid testes and contralateral descended testes. β-actin was used as loading control.

descended testes (Fig. 2B,C). In contrast, its signal was increased and focused on the nucleus of the germ cells, consistent with TUNEL-positive cells, in the cryptorchid testes (Fig. 2F,G). Compared with p65, intense nuclear staining of p50 was observed in the primary spermatocytes of the cryptorchid testes (Fig. 2G). The IκBα protein was also detected in the cytoplasm of the Sertoli cells and spermatocytes in the descended testes (Fig. 2D), although its signal pattern was altered in the cryptorchid testes. In the undescended testes, TUNEL staining and immunohistochemistry revealed that the signal pattern of IκBα was similar to that of NF-κB (Fig. 2H).

The findings of Western blot analysis are shown in Figure 3. The NF-κB protein was detected more intensely in the cryptorchid testes. Similarly, the amount of IκBα protein was also greater in the cryptorchid testes than in the contralateral descended testes. These data were in agreement with the results of immunohistochemistry.

COMMENT

Male infertility is one of the serious complications of cryptorchidism, and according to paternity data, a large cohort study revealed that 10%-38% of the patients were infertile.[12] Although the mechanisms underlying the inhibition of spermatogenesis in cryptorchidism have not been completely elucidated, it has been reported that the germ cell loss associated with apoptosis is induced by various types of stress.[5,9] Our primary aim was to clarify the mechanisms of germ cell apoptosis in cryptorchid testes. We have demonstrated that germ cell apoptosis is associated with the activation of NF-κB in the flutamide-induced cryptorchid rat model.

NF-κB consists of 2 family members of the 5 known subunits (p65/RelA, RelB, c-rel, p50, and p52) and functionally regulates the transcription of a variety of genes.[13] Each subunit is assembled to form either homodimers or heterodimers, and the p50-p65 heterodimer is the major complex in most cells. Under nonstimulation conditions, NF-κB is bound to IκB family proteins, which physically cover the nuclear localization sequence of NF-κB and sequester in the cytoplasm. Various extracellular stresses lead to IκB phosphorylation and, consequently, to the activation of NF-κB. Delfino and Walker[14] demonstrated that in rat testis, the NF-κB proteins localize in the Sertoli cells and germ cells specifically, depending on the stage of the seminiferous tubule. Spermatogenesis, a complicated process of male germ cell differentiation and proliferation, is required for the concordant expression of many genes. Some spermatogenesis-regulating genes such as Müllerian-inhibiting substance and stem cell factor were found to have a κB motif within their transcription initiation sites[15,16]; thus, it is likely that NF-κB plays some roles in the regulation of spermatogenesis.

In the present study, the NF-κB protein was detected in the cytoplasm of the spermatocytes and Sertoli cells in the descended testes. Additionally, in the descended testes, germ cell apoptosis was hardly detected, and the translocation of the NF-κB protein into the nucleus was not observed in the TUNEL-positive cells. These findings suggest that the activation of NF-κB is not necessary for germ cell apoptosis in the descended testes. We also investigated the behavior of NF-κB in the cryptorchid testes and found that the signals of the NF-κB protein were consistent with the pattern of positive TUNEL staining. Intense nuclear staining of p50 was observed in the cryptorchid testes, indicating that NF-κB is activated and translocated to the nucleus in cryptorchidism. Western blot analysis revealed an increase in the amount of the NF-κB protein in the cryptorchid testes compared with that in the contralateral undescended testes, suggesting that NF-κB expression is not affected by administration of flutamide, but by the location of the testes.

In normal testes, although the expression of NF-κB is most intense in pachytene spermatocytes at Stages VII through XI,[14] we could not distinguish the stages of the seminiferous tubules because of the degenerative changes in the cryptorchid testes. However, the number of TUNEL-positive cells varied among the seminiferous tubules, and most of the cells were spermatocytes, suggesting that specific germ cells are more susceptible to apoptosis in cryptorchid testes. Similarly, in a surgically induced cryptorchidism model, degenerative changes were first detected in the primary spermatocytes.[17] It is likely that primary spermatocytes are more sensitive than other cells to several stimuli.

The IκB proteins are considered a marker of NF-κB activation, and several members of the IκB protein family are found in mammalian cells, including IκBα, IκBβ, and IκBε. Thompson et al.[18] have demonstrated that the activation of NF-κB involves 2 overlapping phases—a transient phase mediated through IκBα and a persistent phase mediated through IκBβ.[18] In the present study, we demonstrated the localization of IκBα in both primary spermatocytes and Sertoli cells, suggesting that the transient activation of NF-κB is associated with germ cell apoptosis in cryptorchid testes. Lysiak et al.[19] demonstrated the presence of IκBα in the Sertoli cells of murine

testes after an ischemia-reperfusion injury. They found that germ cell apoptosis was induced by the activation of the NF-κB pathway in the Sertoli cells.[19] It is likely that an alternative pathway of NF-κB activation is associated with germ cell apoptosis in cryptorchidism. Thus, apoptosis might occur predominantly within the spermatocytes per se, not through signals from the Sertoli cells such as the Fas-FasL interaction.[5]

At present, whether NF-κB has a pro- or antiapoptotic function in cryptorchidism is not known; NF-κB upregulates various proapoptotic genes, including *Fas* and *FasL*, and also increases the expressions of antiapoptotic genes such as *Bcl-*X_L, *XIAP*, *TRAF1&2*, and *c-IAPA1&2*.[20] An in vitro study conducted with human testes demonstrated that the NF-κB proteins in the Sertoli cells exert proapoptotic effects on germ cells, but the role of NF-κB on germ cell apoptosis is potentially altered, depending on the type of extracellular stress.[21] In either case, it is reasonable to suppose that the activation of NF-κB is associated with germ cell apoptosis in cryptorchidism. Even in normal testes, germ cell apoptosis was spontaneously induced, and apoptosis might be a mechanism for controlling the number of germ cells.[22] The physiologic significance of apoptosis in germ cells is as yet unclear; however, the results of the present study suggest that the mechanisms of germ cell apoptosis in cryptorchid testes are different from those in normal testes.

The reduced fertility in cryptorchidism has been linked to the reduced number of germ cells. Recently, it has been reported that the transformation of gonocytes into adult dark spermatogonia is crucial for future fertility[3]; however, it is obvious that apoptosis is the one of the important causes of germ cell loss in cryptorchid testes. The association between NF-κB and germ cell apoptosis raises the possibility that pharmacologic intervention of NF-κB could be a therapeutic target in transient stress situations involving excessive germ cell death. Various pathways might be involved in the apoptosis of germ cells, and the results of the present study may bring new insights into the etiology of male infertility with cryptorchidism. Nevertheless, additional investigation regarding the apoptotic pathways in cryptorchid testes is required.

CONCLUSIONS

We have shown that the activation of NF-κB is associated with germ cell apoptosis in experimentally induced cryptorchid testes. Apoptosis was predominantly observed in the primary spermatocytes, and the IκBα protein was presented in the same cell type. It is likely that NF-κB has certain roles as a pro- or antiapoptotic agent in germ cells; thus, the present results may guide toward new therapeutic possibilities for the impairment of spermatogenesis in cryptorchidism.

References

1. Barthold JS, Gonzalez R. The epidemiology of congenital cryptorchidism, testicular ascent and orchiopexy. *J Urol.* 2003;170:2396-2401.
2. Huff DS, Hadziselimovic F, Snyder HM III, et al. Postnatal testicular maldevelopment in unilateral cryptorchidism. *J Urol.* 1989; 142:546-548.
3. Hadziselimovic F, Hocht B, Herzog B, et al. Infertility in cryptorchidism is linked to the stage of germ cell development at orchidopexy. *Horm Res.* 2007;68:46-52.
4. Bernal-Mañas CM, Morales E, Pastor LM, et al. Proliferation and apoptosis of spermatogonia in postpuberal boar (*Sus domesticus*) testes with spontaneous unilateral and bilateral abdominal cryptorchidism. *Acta Histochem.* 2005;107:365-372.
5. Yin Y, Stahl BC, DeWolf WC, et al. p53 and Fas are sequential mechanisms of testicular germ cell apoptosis. *J Androl.* 2002;23:64-70.
6. Peltola V, Huhtaniemi I, Ahotupa M, et al. Abdominal position of the rat testis is associated with high level of lipid peroxidation. *Biol Reprod.* 1995;53:1146-1150.
7. Farooqui SM, Al-Bagdadi F, O'Donnell JM, et al. Degenerative changes in spermatogonia are associated with loss of glucose transporter (Glut 3) in abdominal testis of surgically induced unilateral cryptorchidism in rats. *Biochem Biophys Res Commun.* 1997;236:407-412.
8. Iizuka A, Park MK, Mori T. Effects of unilateral cryptorchidism on the expression of gonadotropin receptor mRNA. *Biochem Biophys Res Commun.* 1996;221:290-294.
9. Zini A, Abitbol J, Schulsinger D, et al. Restoration of spermatogenesis after scrotal replacement of experimentally cryptorchid rat testis: assessment of germ cell apoptosis and eNOS expression. *Urology.* 1999;53:223-227.
10. Barkett M, Gilmore TD. Control of apoptosis by rel/NF-kappaB transcription factors. *Oncogene.* 1999;18:6910-6924.
11. Mizuno K, Hayashi Y, Kojima Y, et al. Influence for testicular development and histological peculiarity in the testes of flutamide-induced cryptorchid rat model. *Int J Urol.* 2007;14:67-72.
12. Lee PA, Coughlin MT, Bellinger MF. Inhibin B: comparison with indexes of fertility among formerly cryptorchid and control men. *J Clin Endocrinol Metab.* 2001;86:2576-2584.
13. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell.* 2002;109:S81-S96.
14. Delfino F, Walker WH. Stage-specific nuclear expression of NF-kappaB in mammalian testis. *Mol Endocrinol.* 1998;12:1696-1707.
15. Hoshiya Y, Gupta V, Segev DL, et al. Mullerian inhibiting substance induces NFkB signaling in breast and prostate cancer cells. *Mol Cell Endocrinol.* 2003;211:43-49.
16. Da Silva CA, Heilbock C, Kassel O, et al. Transcription of stem cell factor (SCF) is potentiated by glucocorticoids and interleukin-1beta through concerted regulation of a GRE-like and an NF-kappaB response element. *FASEB J.* 2003;17:2334-2336.
17. Farooqui SM, Al-Bagdadi F, Houslay MD, et al. Surgically induced cryptorchidism-related degenerative changes in spermatogonia are associated with loss of cyclic adenosine monophosphate-dependent phosphodiesterases type 4 in abdominal testes of rats. *Biol Reprod.* 2001;64:1583-1589.
18. Thompson JE, Phillips RJ, Erdjument-Bromage H, et al. I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell.* 1995;80:573-582.
19. Lysiak JJ, Bang HJ, Nguyen QA, et al. Activation of the nuclear factor kappa B pathway following ischemia-reperfusion of the murine testis. *J Androl.* 2005;26:129-135.
20. Rasoulpour RJ, Boekelheide K. NF-kappaB is activated in the rat testis following exposure to mono-(2-ethylhexyl) phthalate. *Biol Reprod.* 2005;72:479-486.
21. Pentikäinen V, Suomalainen L, Erkkilä K, et al. Nuclear factor-kappa B activation in human testicular apoptosis. *Am J Pathol.* 2002;160:205-218.
22. Dunkel L, Hirvonen V, Erkkilä K. Clinical aspects of male germ cell apoptosis during testis development and spermatogenesis. *Cell Death Differ.* 1997;4:171-179.