ゲノムDNAを抽出し，CYP1A1プロモーター領域のメチル化頻度を解析したところ，胎児期にTCDD曝露を受けていないマウスに比べて，胎児期にTCDD曝露を受けたマウスでは低メチル化を起こしていることがわかった．このメチル化頻度を胎児，新生児，成熟個体と比較すると，正常個体では胎児期に低メチルだったものが成熟するにつれて高くなるが，TCDDに胎児期曝露すると，生後8日目から低下し始め，生後120日目でも低メチル状態で持続することが明らかとなった．また，同じ成熟個体の肝臓を用いて修飾ヒストンの状態を調べたところ，ゲノム領域のヒストンのアセチル化が胎児期TCDD投与群で有意に増加していた．生後120日目において胎児期TCDD投与群のCYP1A1発現レベルが対照群と同様に検出されなかったことから，このエピゲノムの差異は，胎生期TCDD曝露によって起こった変化が成熟後まで維持されたもの，いいかえるなら，胎児期に高誘導化のプログラムが行われた結果と考えられる．観察されたDNAメチル化とヒストン修飾の変化は，現在知られているエピゲノムの性質として，なぜCYP1A1遺伝子の転写がかかりやすいかを説明できるものである(図3)．

胎児期に化学物質で生じたエピゲノムというプログラムの変化が，その後成長してからの"病"につながる事例であり，新たなDOHaDモデルといえる．

プログラムには方向性がある？

成体／胎児でのダイオキシン曝露による変化のちがいは他の器官でも見られる．精巣から分泌されるテストステロンは，前立腺内において5α還元化酵素2型(SRD5A2)によって，より強力なアンドロゲンであるジヒドロテストステロン(DHT)に変換され，前立腺外分泌タンパクであるプロベイジンなどのアンドロゲン受容体(AR)依存性遺伝子の発現を増加する．これにより前立腺重量は増える．ところが成熟個体でTCDD投与すると，精巣内のステロイド産生が抑制され，テストステロンの産生が低下する．そしてテストステロンによって誘導されるSRD5A2の前立腺内の転写が抑えられ，結果として前立腺は小さくなる．興味深いことに胎児期ダイオキシン曝露実験で生まれたラットでは精巣のテストステロンの産生は正常であるが，SRD5A2の前立腺内レベルが高くなる．すなわち，同じダイオキシン曝露でも成熟期曝露と胎児期曝露では，SRD5A2遺伝子発現に対してまったく逆の作用を示すわけである[6][7]．前立腺におけるこの遺伝子のDNAメチル化を調べたところ，プロモーター領域の低メチル化が起きていることをここでも確認できた．胎児期にダイオキシン曝露された前立腺では，個体が成熟した後，ダイオキシン曝露を受けることをあたかも想定したかのようにSRD5A2遺伝子発現を上昇させている．なぜなら上述のように，SRD5A2は前立腺の重量増加に促進的に作用するからである．胎児期にあらかじめ成熟したときの環境に適応するようなプログラム変更が行われているように見える．

Barker仮説の低栄養胎児の場合を考えてみる．人類は数万年にわたり，過酷な環境下における食料供給の変化への適応を迫られてきたため，胎生期に低栄養環境に曝された成人は，少ないエネルギーを有効に利用できるよう形質転換され，出生後の将来の飢餓状態に適応するのだという考え方(thrifty phenotype hypothesis: 倹約表現型仮説)がある[8]．低栄養状態で生まれた子が肥満傾向になるのは，低栄養環境を生き抜いてもらいたいとする，胎生プログラムの方向性を物語っていると推察できる．ダイオキシン曝露で示されたCYPの強誘導性プログラム化の場合は，変異原という危険物質が体内に入ると発ガンリスクを高めてしまい，結果的に好ましくない事態になるわけだが，CYPは前述したように生体防御にとってきわめて重要な遺伝子で，その強誘導化は，変異原以外の他の環境中化学物質，たとえばフラボン類などの植物性化学物質の体外排出には効果的に効くはずである．母胎からのシグナルでCYPの強誘導化を起こすことは，個体にとっては食料の少ない

環境でもさまざまに栄養源を捕食する際には有利な変化となりうるだろう．これらはすべて，胎児期にあらかじめ，成熟したときの環境に適応するようなプログラムの方向性(適応プログラム化)があることを示唆しているように思える．

* *

Barker仮説でいう「次世代」というのはなにも胎児期環境が悪かった子のことばかりではなく，そのまた子(孫)，さらにはその子孫にまで影響するという意味らしい．本稿で紹介したようなエピゲノムの変化は，生殖細胞系列を介して後世代にも伝達されるかもしれないといわれている．適応プログラム化があるとするなら，種の環境への適応という観点から，生物進化を考えるうえでも興味深く思われる．

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50年前には

宇宙科学の振興と学術会議

富山小太郎

宇宙科学というのは新しい言葉である．(中略)宇宙旅行とか宇宙時代とかいう言葉が結構適用しているところをみると，宇宙科学も流行語となる資格は十分にある．最近新聞の伝えるところによると，科学技術庁がこの宇宙科学の振興にのりだすという．まことに抜け目のない出足である．

いったいこの振興案のほんとうの狙いがどこにあるのか，いっこうにわからない．それだけに却って，少数のグループの間で具体案をつくり，急いで予算化するとか，アメリカと協定してロケット利用の方策を立てるなどという，あまりにも颯爽としたその動きに不安を感ずるのである．この方面の研究は別に新しいものではない．(中略)しかしいずれも'じみ'な研究部門であった．それだけに，学術会議などが，この日の当らない部門を推進する運動を起したというのなら，結構なことに相違ないが，どうもこんどの動きはあわただしすぎる．目的はロケット技術にあり，ミサイル研究に連なる線を狙っているなどという噂もある．もちろん，当局はそれを否定するであろうが，このごろの防衛庁の動きなどからすると，一片の声明などでこの不安が消え去るものではない．当局者にもうすこし慎重な態度をとることをのぞむわけである．

こんどの問題にかぎらないが，政府が科学技術の方面で新しいことを始めるとき，常道というものが忘れられているような気がしてならない．政治家が科学や技術に積極的な関心をもつことは喜ばしいことであるが，これを具体的に進める際には一定の方式を守らなければならない．政治家は科学技術政策として基本的な第一段階において正当な見解をもてばよい．つぎの何をいかにして研究するかという問題は専門家の討議の結果に待つという常識が守られるべきであろう．(中略)

政府は学術会議を信用していない．これまでの学術会議が国民から期待されているような機能を果していないということは，ある程度事実であろう．しかしその責任の一半は政府の態度にある．政府が，ときには政府の意に反する場合でも，学術会議の審議の結果をうけ入れる良識を示すならば，事態はまったく変わるであろう．その審議の態度も変わるであろうし，建設的な意見をだす責任も感ずるにちがいない．現在では政府は学術会議をなるべく避けて通ろうとする．それで政策を審議するためでなく，それを認めさせるに都合のよいいろんな会議のようなものを次々につくろうとする．このような官僚の常套手段を見せつけられると，積極的な意見はださず，反対ばかりしているような学術会議の存在が逆に光ってみえるから皮肉である．

(後略)

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Long-term physical, hormonal, and sexual outcome of males with disorders of sex development

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Penis

Abstract

Purpose: We investigated the long-term physical, hormonal, and sexual outcomes of males with disorders of sex development (DSD) and discussed the necessity of long-term follow-up for these patients after surgery.

Patients and Method: Twelve DSD patients (average age, 21.0 ± 3.6 years old) who had been designated as male in childhood (3 ovotesticular DSD, four 45,XO/46,XY mixed gonadal dysgenesis, four 46,XX testicular DSD, and one 46,XY DSD; androgen insensitivity syndrome) were enrolled. For these patients, height, penile length, and testicular volume were evaluated in adulthood. Serum levels of luteinizing hormone, follicle-stimulating hormone, and testosterone were also measured during follow-up. In addition, sexual function and romantic relationships were evaluated.

Results: Development of the penis and testes was poor. According to the hormonal study, these patients were diagnosed with hypergonadotropic hypogonadism or normogonadism; 90% patients had experienced penile erection and masturbation at the time of participation, and 70% and 40% patients had experienced ejaculation and sexual intercourse with female partners, respectively. No patients preferred to avoid sexual contact with women.

Conclusion: Although DSD males had an undeveloped penis and testis and had hypergonadotropic hypogonadism or normogonadism, most had male sexual potential and male sex identity as long as testicular tissues were preserved.

Disorders of sex development (DSD) are congenital conditions with atypical development of chromosomal, gonadal, or anatomical sex [1]. The appearance of the external genitalia is ambiguous, often with hypospadias or clitoromegaly, with an undeveloped or ectopic testis or inadequate vagina.

Patients reared as males with ambiguous genitalia, especially proximal hypospadias, are commonly referred to a urologic clinic to undergo hypospadias repair, in which DSD patients are included. Generally, these patients need not only reconstruction surgery for ambiguous external genitalia but also surgical exploration of gonads and internal genitalia. The aim of reconstruction surgery in DSD is to create "functional" external genitalia consistent with the sex assigned to the patient; therefore, one of the most important issues is whether

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they can achieve sexual potential as male or female in the future; however, information about the long-term physical, hormonal, and sexual outcome of DSD patients cannot be easily determined until decades after surgery because many patients are lost to follow-up [2]. As a result, there are extremely few reports of the outcome after surgery of DSD patients, especially those raised as males [3-5].

In our urologic clinic, we usually attempt to observe DSD males for as long as possible. In this study, we investigated the long-term physical, hormonal, and sexual outcomes of DSD patients raised as males, including ovotesticular DSD, mixed gonadal dysgenesis (MGD), 46,XX testicular DSD, and 46,XY DSD, who had undergone hypospadias repair previously at our institute and with whom we had discussed the necessity of long-term follow-up after surgery.

1. Materials and methods

We reviewed the medical records of DSD patients who had been designated as male by both the medical team and family at our institution from 1975 to 1991 and observed at least to 15 years old. Twelve patients raised as males (3 ovotesticular DSD, 4 MGD, four 46,XX testicular DSD, and one 46,XY DSD; androgen insensitivity syndrome), who were 16 years or older at the time of the last visit (average age, 21.0 ± 3.6 years old), were enrolled in this study. Clinical record files of these patients detailed the initial clinical presentation, diagnostic investigations, and therapeutic surgical intervention. The diagnostic study between the first visit and operation had included a physical examination; chromosomal analysis; hormonal evaluation, including human chorionic gonadotropin (hCG) stimulation test (2400 IU/d for 3 days) and human menopausal gonadotropin (hMG) stimulation (112.5 IU/d for 3 days); the presence of the sex-determining region Y (SRY) sequence; magnetic resonance imaging; cystoscopy; vaginoscopy; laparoscopy; and gonadal biopsy. After 1993, the presence of the SRY sequence was examined by polymerase chain reaction method for most patients during follow-up.

The clinical descriptions of 12 patients in childhood are presented in Table 1. All patients presented with proximal hypospadias and had undergone hypospadias repair in childhood. All patients had both Wolffian (epidydimis and/or vas deferens) and Mullerian (fallopian tube and/or prostatic utricle) structures. All patients had a prostatic utricle, which opened into the central area of the verumontanum in the prostatic urethra.

Three ovotesticular DSD had several complicated clinical characteristics. Patient 1 with 46,XY had a right ovary with a fallopian tube and a uterus-like structure in the abdomen and a left testis with an epididymis and vas deferens in the scrotum. He had undergone right gonadectomy and resection of the uterus-like structure. Patient 2 with 46,XX had a right ovotestis in the abdomen and left ovotestis in the scrotum and had undergone bilateral gonadectomy. Patient 3 with 46, XX/46,XY/47,XX10+ had a right testis with an epididymis and vas deferens in the scrotum and a left ovary with an epididymis-like structure in the abdomen. He had undergone left gonadectomy. The hCG response was normal in all 3 patients, whereas the hMG response was present in 2 patients (patient 2). Patients 1 and 3 had an SRY sequence but patient 2 did not.

Four MGD patients had both a testis and streak gonad and underwent gonadectomy for the streak gonad. Although two had undergone orchiopexy for undescended testis in another clinic before the first visit, the other two (patients 4 and 6) had no testis in the scrotum at the first visit. All patients had a uterus and an epididymis or fallopian tube. The hCG and hMG stimulation test was performed for 2 patients (patients 4 and 7); the hCG response was normal, whereas the hMG response was absent. Patients 5, 6, and 7 were confirmed as having the SRY sequence.

Four 46,XX testicular DSD patients (patients 7-11) had not only an epididymis and vas deferens but also a Mullerian remnant. One patient (patient 10) had gynecomastia with age. All 46,XX testicular DSD patients had undergone open bilateral gonad biopsy for their diagnoses, and histologic examination showed bilateral testis. The left testis of one patient (patient 8) disappeared during his follow-up period. The enlarged Mullerian structure (prostatic utricle) was resected in one patient (patient 10) because of urinary tract infection. The hCG response was normal, whereas the hMG response was absent in all patients, showing that gonad tissues could potentially develop as testes without ovarian tissue. All 46,XX testicular DSD patients had no SRY sequence. One patient (patient 9) had a neurogenic bladder caused by myelomeningocele and underwent cystectomy and neobladder replacement at the age of 25.

One 46,XY DSD patient (patient 12) had epididymis and vas deferens. He had undergone open bilateral gonad biopsy, and histologic examination showed bilateral testis.

For these patients, their height, penile length, and testicular volume in adulthood were evaluated. Serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone were also measured by radioimmunoassay (RIA) during follow-up. Radioimmunoassays for LH, FSH, and testosterone were performed using RIA kits (LH and FSH, Japan DPC, Tokyo, Japan; testosterone, Amersham Biosciences, Tokyo, Japan).

In addition, sexual function, such as their experiences with penile erection, libido, orgasm, ejaculation, masturbation, and sexual intercourse, was evaluated. Their romantic relationships and marriage were also detailed.

2. Results

Patients were observed for a median 19.8 ± 3.3 years (range, 16-28 years) after diagnosis. Clinical features in

Table 1 Clinical characteristics of males with disorders of sexual development in childhood

	Diagnosis	Age at first visit	External genitalia	Internal genitalia		Gonad		Gonadectomy	Chromosome	HCG Stimulation test	HMG Stimulation test	SRY
				Wolffian structure	Mullerian structure							
1	Ovotesticular DSD	3yo	hypospadias (penoscrotal type)	+	+	Rt) ovary Lt) testis	Rt) abdomen Lt) srotum	Rt) ovarectomy	46,XY	+	+	+
2	Ovotesticular DSD	1mo	hypospadias (penoscrotal type)	+	+	Rt) ovotestis Lt) ovotestis	Rt) abdomen Lt) inguinal region	Bil) gonadectomy	46,XX	+	+	-
3	Ovotesticular DSD	2mo	hypospadias (penoscrotal type)	+	+	Rt) testis Lt) ovary	Rt) scrotum Lt) abdomen	Lt) ovarectomy	46,XX/46,XY/47, XX10	+	-	+
4	MGD	9mo	hypospadias (scrotal type)	+	+	Rt) streak gonad Lt) testis	Rt) inguinal region Lt) scrotum	Rt) gonadectomy	45,XO/46,XY	+	-	NA
5	MGD	0mo	hypospadias (scrotal type)	+	+	Rt) streak gonad Lt) testis	Rt) abdomen Lt) scrotum	Rt) gonadectomy	45,XO/46,XY	NA	NA	+
6	MGD	4yo	hypospadias (scrotal type)	+	+	Rt) testis Lt) streak gonad	Rt) scrotum Lt) abdomen	Bil) gonadectomy	45,XO/46,XY	NA	NA	+
7	MGD	10yo	hypospadias (penoscrotal type)	+	+	Rt) testis Lt) streak gonad	Rt) inguinal region Lt) abdomen	Lt) gonadectomy	45,XO/46,XY	+	-	+
8	46,XX testicular DSD	2 mo	hypospadias (scrotal type)	+	+	Bil) testis	Bil) scrotum	-	46,XX	+	-	-
9	46,XX testicular DSD	8 mo	hypospadias (scrotal type)	+	+	Bil) testis	Bil) scrotum	-	46,XX	+	-	-
10	46,XX testicular DSD	9 mo	hypospadias (scrotal type)	+	+	Bil) testis	Bil) scrotum	-	46,XX	+	-	-
11	46,XX testicular DSD	5 mo	hypospadias (scrotal type)	+	+	Bil) testis	Bil) scrotum	-	46,XX	+	-	-
12	46,XY DSD	4 mo	hypospadia (scrotal type)	+	+	Bil) testis	Rt) scrotum Lt) inguinal region	-	46,XY	+	-	+

yo: years old.
mo: months old.
NA: not assessed.
Rt: right.
Lt: left.
Bil: bilateral.
HCG stimulation test +; response by serum testosterone after hCG stimulation, -; response by serum testosterone after hCG stimulation.
HMG stimulation test +; response of urinary estradiol after hMG stimulation, -; no response of urinary estradiol after hMG stimulation.

adulthood are described in Table 2. The mean final height of the 12 patients was 154.5 ± 3.8 cm, and most were under the 10th percentile of the range of normal Japanese adult males. Development of the penis and testes was poor. Average penile size was 4.1 ± 0.7 cm. No gonadal tumors were detected during follow-up. They also had poor pubic hair.

Serum levels of LH, FSH, and testosterone were examined from infancy to adulthood in 10 patients by RIA (Fig. 1). As in normal males, the concentrations of these hormones in 10 of 12 patients, except patients 2 and 6, elevated spontaneously during the pubertal period. Testosterone levels were low normal or slightly decreased, and LH and FSH were elevated in these patients. These high levels of LH and FSH began to be observed in the peripubertal stage. Follicle-stimulating hormone was more frequently elevated than LH. According to the concentrations of these hormones, these patients were diagnosed with hypergonadotropic hypogonadism or normogonadism. Two patients (patients 2 and 6), who had undergone bilateral gonadectomy, received testosterone therapy (intramuscular depot injections of testosterone esters) immediately before puberty. One patient (patient 9), with mental retardation and neurogenic bladder caused by myelomeningocele, also received testosterone therapy for deficiency of sexual function at the age of 27, but he discontinued the therapy because of adverse effects.

Of 10 DSD males, 9 (90%) had experienced penile erection and masturbation at the time of participation. Of 10 patients, 7 (70%) had experienced ejaculation, and 5 of these 7 patients had ejaculation problems, including dribbling, retained, or delayed ejaculation. No patient complained of problems with libido and orgasms. Of 10 patients, 4 (40%) had experienced sexual intercourse with female sex partners in the past year. Only one patient (patient 11), a 46,XX testicular DSD, was married at the time of participation, and two 46,XX testicular DSD had a stable female partner and expressed a desire for marriage in the future. No patients preferred to avoid sexual contact with women. There were no cases of fertility, and the only semen analysis showed azoospermia (patient 11).

3. Discussion

There are several previous reports about the long-term physical, hormonal, and sexual outcomes of 46,XY DSD and 46,XX DSD, such as complete androgen insensitivity syndrome (AIS) and congenital adrenal hyperplasia, which are usually raised as female [6,7]; however, there are very few reports on the long-term outcome of DSD males. In this

Table 2 Physical and sexual outcome of males with disorders of sexual development

	Diagnosis	Present age	height	Testicular volume	Penile size	Erection	Masturbation	Ejaculation	Sexual intercourse	Married
1	Ovotesticular DSD	18 yo	151 cm	Rt) – Lt) 9.5 ml	4.5 cm	+	+	+	-	-
2	Ovotesticular DSD	16 yo	156 cm	Rt) – Lt) -	3.5 cm	NA	NA	NA	NA	NA
3	Ovotesticular DSD	19 yo	161 cm	Rt) 8.0 ml Lt) -	5.2 cm	+	+	+	-	-
4	MGD	22 yo	153 cm	Rt) – Lt) 2.9 ml	3.0 cm	NA	NA	NA	NA	-
5	MGD	19 yo	150 cm	Rt) – Lt) 2.8 ml	5.0 cm	+	+	+	NA	-
6	MGD	21 yo	155 cm	Rt) – Lt) -	3.8 cm	+	+	+	-	-
7	MGD	27 yo	150 cm	Rt) 3 ml Lt) -	4.0 cm	+	+	+	+	-
8	46,XX testicular DSD	23 yo	157 cm	NA	4.5 cm	+	+	+	+	-
9	46,XX testicular DSD	28 yo	150 cm	Rt) 5.1 ml Lt) -	5.0 cm	-	-	-	-	-
10	46,XX testicular DSD	21 yo	156 cm	Rt) 3.3 ml Lt) 3.2 ml	3.6 cm	+	+	+	+	-
11	46,XX testicular DSD	20 yo	160 cm	Rt) 4.2 ml Lt) 3.8 ml	3.6 cm	+	+	+	+	+
12	46,XY DSD	18 yo	155 cm	Rt) 4.5 ml Lt) 4.2 ml	4.0 cm	+	+	-	-	-

yo: years old.
NA: not assessed.
Rt: right.
Lt: left.
Bil: bilateral.

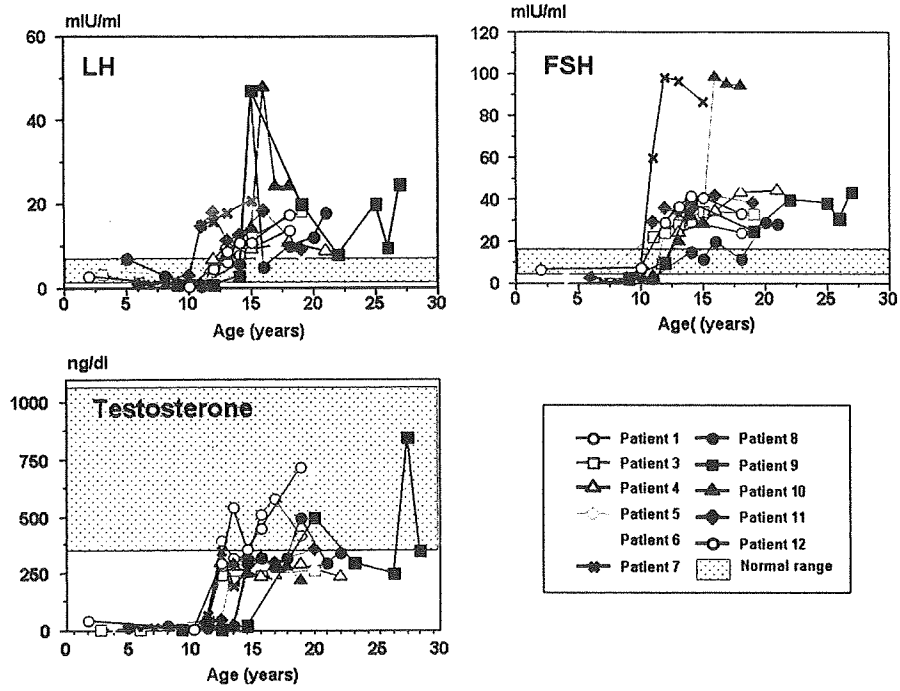

Fig. 1 Hormonal evaluation of DSD patients from childhood to adulthood after diagnosis and operation. Serum concentrations of LH, FSH, and testosterone of DSD patients were determined by RIA at the ages indicated.

study, we investigated the long-term physical, hormonal, and sexual outcomes of 12 DSD males, including ovotesticular DSD, MGD, 46,XX testicular DSD, and 46,XY DSD.

Consistent with previous reports, 46,XX testicular DSD and MGD were shorter than normal males [8,9]. Although their reduced height might be attributed to genes on the Y chromosome that control height [10], not only 46,XX testicular DSD but also ovotesticular DSD and 46,XY testicular DSD with a complete Y chromosome were also short.

Recent remarkable advances in hypospadias repair have provided satisfactory penile reconstruction, not only cosmetically but also functionally; however, female sex assignment has been considered optimal and common in severely undermasculinized cases because feminization with surgical and endocrine treatment was considered more successful than masculinization [11]. The size of the penis and its potential to develop at puberty into a sexually functional penis are one of the important concerns. There are several reports about penile development in sporadic hypospadias patients [12,13]. Mureau et al [12] reported that penile underdevelopment was noted about 2 times more often in hypospadias patients than among age-matched normal boys. Bracka et al [13] reported a clear correlation between the severity of hypospadias and penile length. On the other hand, there are few reports about penile development in DSD males. In 46,XX testicular DSD with genital abnormalities, penile size and testicular volume, measured directly or after surgery in the case of cryptorchid patients, were below the normal values obtained in controls [14]. The average penile size of Japanese men is approximately 8 cm, and the critical testicular volume indicating normal testicular function of Japanese men is approximately 30 mL [15]. In our study, all DSD males had proximal hypospadias and had an underdeveloped penis after puberty. This insufficient virilization of the external genitalia may result from hypogonadism. In the previous 46,XX testicular DSD studied, hypogonadism started in the peripubertal stage, similar to the pattern in Klinefelter's syndrome where high FSH levels are associated with normal or low testosterone levels [16]. Conversely, in a study of ovotesticular DSD, peripubertal testosterone levels were lower than in 46,XX testicular DSD [16]. In our study, 10 patients were diagnosed with hypergonadotropic hypogonadism or normogonadism, and the concentration of testosterone in these patients was low or low normal in young adults, concomitant with a rise in FSH, reflecting the degeneration of spermatogenesis and Leydig cell function. The 46,XX testicular DSD is generally characterized by azoospermia [8]. On the other hand, phenotypic males with normal spermatogenesis and phenotypic females with normal conception and delivery have been reported in ovotesticular DSD [17,18]. Although testicular functions of DSD in adulthood are unclear, complete testicular function may not be expected.

In our study, most patients showed potential for erection, ejaculation, and masturbation; however, they had some problems with ejaculation, such as dribbling, retained, or delayed ejaculation or the absence of ejaculation, which have been reported after hypospadias repair in patients even without DSD [19]. This may be secondary to dilation of the reconstructed urethra or the presence of a Mullerian remnant

[19]. On the other hand, no patient complained of problems with libido and orgasms. Patients with DSD had been considered to have an increased incidence of sexual dysfunction as a result of their condition and psychologic factors that impact on sexual function [20]. Children with DSD, their parents, and we always face the difficulty of dealing with the diagnosis and accepting reconstructive surgery of the genitalia to avoid psychosexual disturbance. Sex dissatisfaction may occur more frequently in individuals with DSD [3]. Fortunately, however, no patient had serious psychosexual problems. All patients were interested in women and desired to have contact and to fall in love with women, and interestingly, three 46,XX testicular DSD patients and 2 MGD patients had experienced sexual intercourse with a female sex partner, and one 46,XX testicular DSD patient was married. Both biologic and social factors seem responsible for the development of sex identity [3]. Because the DSD males in our study had received prenatal androgen exposure, although insufficient, and had been raised as male, as a result, they may have had male sex identity; however, more systemic psychologic assessments will be needed to conclude that these patients had no psychologic problems.

The necessity for testosterone therapy for all DSD males is controversial. Testosterone therapy may be expected to induce puberty, growth, sexual function, and support for psychosexual maturation [1]; however, we performed testosterone therapy for only 3 males—2 who underwent bilateral gonadectomy and 1 who complained of deficiency of sexual function. However, most patients with testis underwent no hormonal therapy because they did not desire additional therapy, and puberty and the potential for sexual function developed without hormonal therapy. Because routine hormonal therapy for a long period may affect the quality of life, with a greater risk of developing prostate cancer in the future [21], careful consideration may be required.

In conclusion, although DSD males usually have an undeveloped penis and testis and have hypergonadotropic hypogonadism or normogonadism, most may have male sexual potential and male sex identity without hormonal therapy, if testicular tissues are preserved. This means that testicular function in DSD males may be insufficient for masculinization of the fetal external genitalia but sufficient to achieve male sexual function and develop male sex identity; however, our study showed no difference in sexual potential and sex identity between DSD males and age-matched normal males because a limitation of this study is the small sample size. A large multicenter study is required to obtain more detailed information on the long-term physical, hormonal, and sexual outcomes of DSD males.

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Laparoscopic Orchiectomy and Subsequent Internal Ring Closure for Extra-abdominal Testicular Nubbin in Children

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OBJECTIVES To present our preliminary experience with laparoscopic groin exploration and subsequent laparoscopic orchiectomy and internal ring closure for testicular nubbin in children and discuss the usefulness of our new treatment strategy. The advantages of laparoscopic orchiopexy for intra-abdominal testis are the ability to start treatment as soon as a diagnosis has been made and to permit minimally invasive surgery. These advantages can apply to laparoscopic orchiectomy for a testicular nubbin.

METHODS A total of 6 boys with a testicular nubbin (age range 14-76 months, mean age 27.3 months) underwent laparoscopic orchiectomy at our institution from June 2007 to June 2008. We opened the posterior parietal peritoneum by incising the peritoneum lateral to the spermatic vessel, distal to the patent processus vaginalis and medial and distal to the vas deferens (laparoscopic groin exploration). Next, the testicular nubbin was pulled out into the abdomen and was then removed laparoscopically after identification and division of the gubernaculums. Finally, laparoscopic complete internal ring and peritoneal defect closure was performed with 5-0 Vicryl suture.

RESULTS The average operative time, including the diagnostic time, was 64 minutes. No intraoperative or postoperative complications developed. In 1 boy with a testicular nubbin, an open processus vaginalis was present, and simultaneous laparoscopic transection of the processus vaginalis and subsequent internal ring closure were performed.

CONCLUSIONS All patients with a testicular nubbin could be treated as soon as the diagnosis was made using only laparoscopic management, with minimal morbidity and good short-term results. UROLOGY 73: 515–519, 2009. © 2009 Elsevier Inc.

Nonpalpable testes can include testicular agenesis, an intra-abdominal testicular nubbin, an intra-abdominal testis, a vanishing testis, an extra-abdominal testicular nubbin, or an intracanalicular testis that is not palpable despite careful physical examination. Nonpalpable testes comprise about 20% of undescended testes.[1] Despite many reports, the optimal initial approach remains controversial. Laparoscopic surgery in pediatric urology has continued to gain acceptance and, in experienced hands, is safe in this population. It provides several advantages compared with standard open surgery, including a more rapid recovery, improved cosmetic outcome, less postoperative pain, and as a consequence, lower analgesic requirements. It has also been advocated at various stages in the management of nonpalpable testis and has been recommended as the initial approach.[2,3] The advantages of laparoscopic orchiopexy for intra-abdominal testes are the ability to start treatment as soon as the diagnosis has been made and to permit minimally invasive surgery. These advantages can apply to laparoscopic orchiectomy for a testicular nubbin. Surgery for a nonpalpable testis is intended to determine whether a testis is present and, if found, to either place it in the scrotum or remove it. Although many surgeons perform an inguinal incision as their procedure of choice for a nonpalpable testis, various approaches for the management of nonpalpable testes have been advocated. In particular, several reports have been published on the initial approach for a nonpalpable testis. Recently, laparoscopy has been advocated at various stages in the management of a nonpalpable testis.[3,4] When laparoscopy is used as the initial approach for nonpalpable testis, it is usually followed by laparoscopic orchiopexy when the testis is intra-abdominal or by laparoscopic orchiectomy when the testicular nubbin is intra-abdominal. However, a potential dilemma arises when laparoscopy is performed initially, and the vas and vessels are seen exiting the internal ring, because either a testis or nubbin

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could be found distally. These patients undergo open inguinal incision.[5,6] In contrast, O'Hali et al.[7] recommended initial open inguinal incision for the management of nonpalpable testis. When the inguinal incision findings are negative, the presence of an ipsilateral hernia sac requires additional exploration, including laparoscopic management. From 1993 to 2005, before the strategies in this study were in use, we performed initial inguinal incision. When we identified neither a normal testis nor a spermatic cord at inguinal incision, we subsequently performed laparoscopic observation through the internal inguinal ring.[8,9] The advantage of this procedure is to avoid extending the incision into an infraumbilical incision in cases of a blind-ending vas and vessels in the peritoneum with transinguinal laparoscopy. However, these previously reported procedures cannot necessarily avoid at least a 2.0- or 3.0-cm inguinal incision. The avoidance of an inguinal incision is considered superior for managing a nonpalpable testis. From November 2006, we therefore changed our strategy for nonpalpable testis treatment. We present our preliminary experience with laparoscopic groin exploration and subsequent laparoscopic orchiectomy and internal ring closure for a testicular nubbin in children and discuss the usefulness of our new treatment strategy.

MATERIAL AND METHODS

A total of 6 boys with a testicular nubbin (age range 14-76 months, mean age 27.3) underwent laparoscopic orchiectomy at our institution from June 2007 to June 2008. Of these 6 boys, the vanishing testis was on the right side in 2 and on the left in 4.

Laparoscopic Diagnosis

Laparotomy was performed with the patient under general anesthesia in the supine position.[4] The bladder was emptied with a 6F or 8F catheter. A 1-cm 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 a pneumoperitoneum was conducted at 8-10 mm Hg to observe the inside of the abdominal cavity clearly using a 5-mm, 30° laparoscope. Two additional trocars (3 and 5 mm) were inserted at the level caudal to the umbilicus in the mid-clavicular line bilaterally when additional laparoscopic maneuvers, including laparoscopic orchiopexy or orchiectomy, were needed. If the testis was intra-abdominal, we performed laparoscopic orchiopexy for an intra-abdominal testis. If the vas and vessels exited from the internal ring on the side of the nonpalpable testis, despite the presence or absence of a patent processus vaginalis, we diagnosed a testicular nubbin or intracanalicular undescended testis. We could distinguish to some extent between a testicular nubbin and an intracanalicular undescended testis by the thickness of the vessels (hypoplastic or normal size).

Laparoscopic Orchiectomy for Vanishing Testis

First, we opened the posterior parietal peritoneum by incising the peritoneum lateral to the spermatic vessel, distal to the patent processus vaginalis and medial and distal to the vas deferens (laparoscopic groin exploration). With careful traction of the whole cord using cloth tape from within the abdomen and pressure over the internal ring, the gonad was pulled out into the abdomen. If the cord structure terminated in a testicular nubbin, this was removed laparoscopically after identification and division of the gubernaculums. The vas and vessels were separated and divided, respectively, with electrocautery. Laparoscopic complete internal ring and peritoneal defect closure was performed with 5-0 Vicryl suture (Fig. 1). If the testis was found (intracanalicular undescended testis) after laparoscopic groin exploration, we performed laparoscopic orchiopexy.

RESULTS

All boys with a testicular nubbin underwent laparoscopic groin exploration and subsequent laparoscopic orchiectomy and internal ring closure. The average operative time, including the diagnostic time, was 64 minutes. In 1 of the 6 boys with a testicular nubbin, an open processus vaginalis was associated, and the boy underwent simultaneous laparoscopic transection of the processus vaginalis and subsequent internal ring closure (Fig. 2). No intraoperative or postoperative complications, including ileus and serious subcutaneous emphysema, developed. A little blood loss occurred. Mobilization and normal oral intake were allowed the next day in all boys. They had all resumed full activity at follow-up 3 days later. The follow-up period ranged from 6 to 12 months (mean 8.5). The wound was smaller than 5 mm in all and the cosmetic appearance of wounds after operation was good in all cases (Fig. 3). Pathologic analysis of the testicular nubbin revealed that all had a vas deferens, connective tissue, hemosiderin, and dystrophic calcification without seminiferous tubules.

COMMENT

Although the laparoscopic procedure was originally performed for diagnostic purposes, it has become more feasible in pediatric patients with urologic disease. It allows not only the diagnosis, but also adequate therapy and has become the reference standard for managing a nonpalpable testis.[2,3] In this study, we treated 6 boys with a testicular nubbin laparoscopically without an open inguinal incision. We used an initial laparoscopic approach, with subsequent laparoscopic orchiopexy for an intra-abdominal testis and intracanalicular testis and laparoscopic orchiectomy for a testicular nubbin. This strategy enabled us to achieve all nonpalpable testis treatment with laparoscopy without an open inguinal incision.

The usefulness of a scrotal incision for the management of a unilateral nonpalpable testis has been reported, because most testicular nubbins are easily removed using this approach and the cosmetic outcome has been excellent.[10-13] Van Savage[10] reported that when spermatic vessels are visualized exiting the internal inguinal ring on laparoscopy in the setting of a nonpalpable testis, a median raphe scrotal incision can be made to remove the testicular nubbin. However, several reports have been

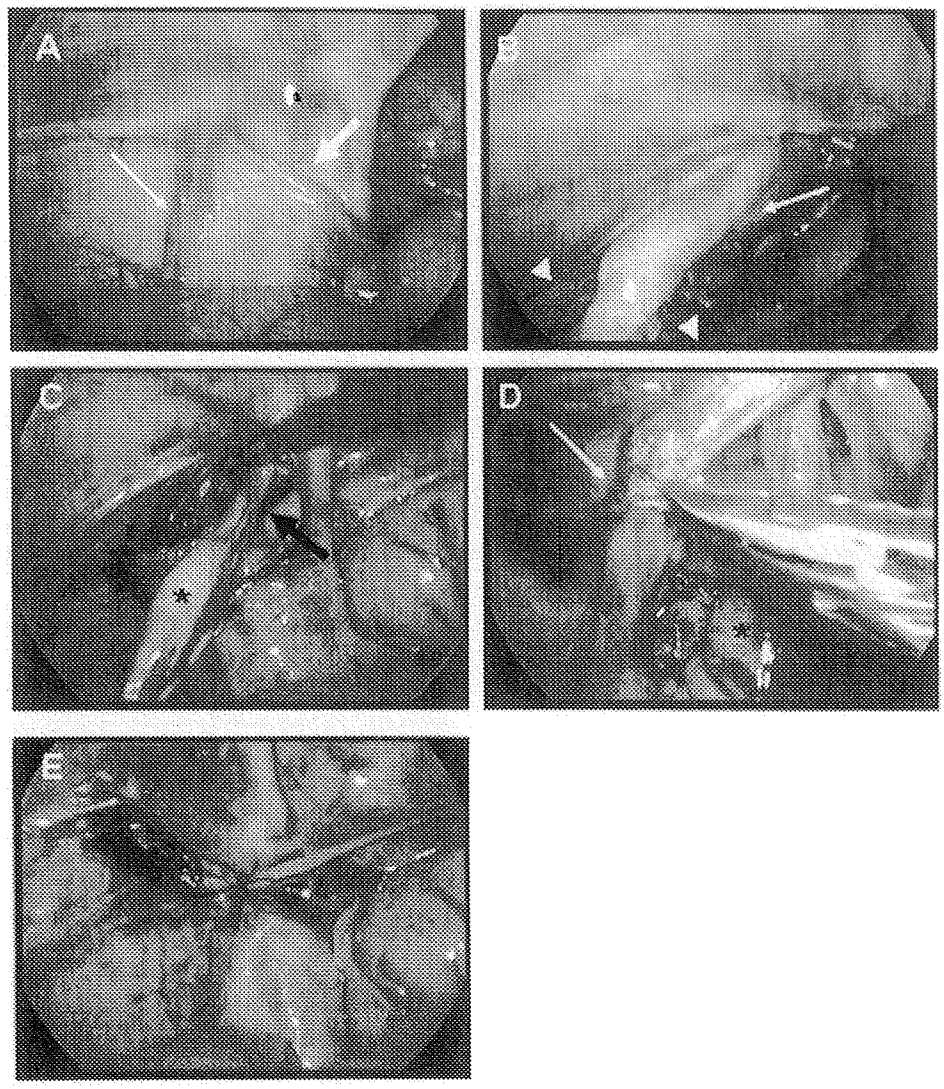

Figure 1. Laparoscopic orchiectomy for testicular nubbin. **(A)** Appearance of internal ring with vas deferens (large arrow) and hypoplastic vessels (small arrow). **(B)** Careful traction of whole cord using cloth tape (white arrowheads) from within abdomen and pressure over internal ring, after opening posterior parietal peritoneum by incising peritoneum lateral to spermatic vessel, distal to the patent processus vaginalis and medial and distal to the vas deferens (laparoscopic groin exploration). **(C)** Cord structure ended in testicular nubbin and epididymis (asterisk). **(D)** Vas and vessels were separated and divided, respectively, with electrocautery. **(E)** Laparoscopic complete internal ring and peritoneal defect closure with 5-0 Vicryl suture.

published on an initial scrotal incision for a unilateral nonpalpable testis, which could be definitive when a nubbin is identified, and laparoscopy should be performed to rule out an intra-abdominal testis only if the scrotal findings do not demonstrate a nubbin or seem inconclusive.[11-13] Laparoscopy is necessary after an initial scrotal incision in several cases, including scrotal nubbins with a patent processus vaginalis, a questionable nubbin, a definitive nubbin but with preoperative magnetic resonance imaging findings suggesting a testis and empty scrotum (intra-abdominal vanished testis and inguinal nubbins).[13] The advantage of laparoscopic groin exploration and subsequent orchiectomy for a vanishing testis might be the avoidance of "inconclusive" cases of nonpalpable testis, to distinguish between the testis and testicular nubbin more easily, and to remove the nubbin more completely, because the antegrade approach can provide good anatomic orientation for observation and removal. Additionally, in our case of a vanishing testis, an open processus vaginalis was associated; therefore, simultaneous laparoscopic transection of the processus vaginalis and subsequent internal ring closure were performed. Laparoscopic transection of the processus vaginalis can be performed without disturbing the inguinal canal.[14] Because the incidence of a contralateral patent processus vaginalis is considerable in patients presenting with a unilateral nonpalpable testis, the additional benefit of laparoscopy for nonpalpable testes is to identify a contralateral patent processus.[15]

The recent consensus has been to leave the internal ring open after the laparoscopic approach because the possibility of subsequent inguinal hernia is low even

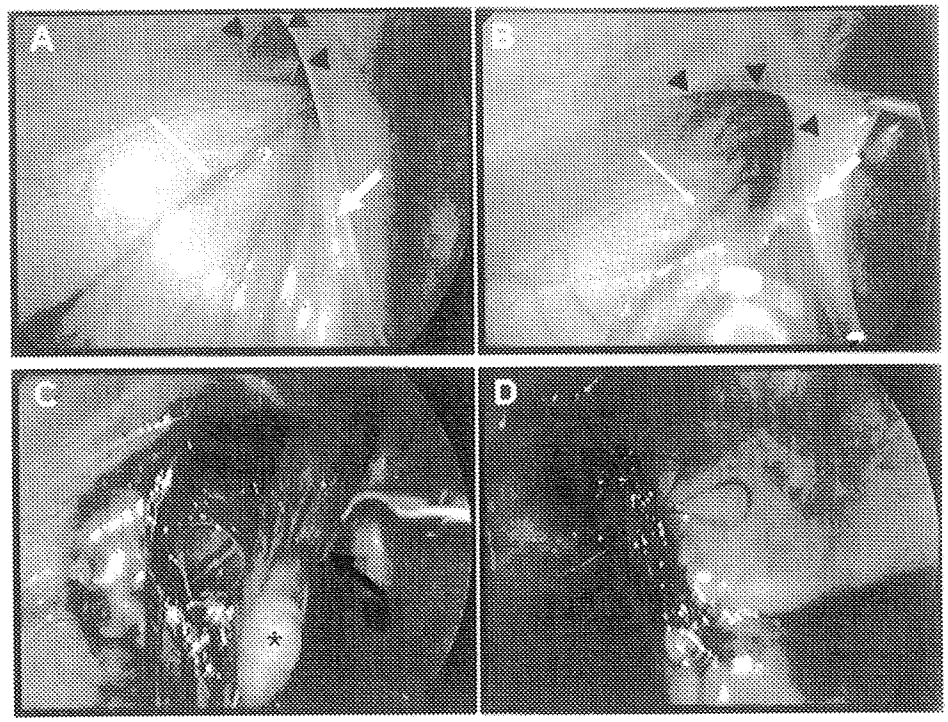

Figure 2. Laparoscopic orchiopexy for testicular nubbin with opened processus vaginalis. **(A,B)** Opened processus vaginalis (black arrowheads) with vas deferens (large arrow) and vessels (small arrow). **(C)** Careful traction of whole cord from within abdomen and pressure over internal ring after laparoscopic transection of processus vaginalis, and pulling cord structure ended in testicular nubbin and epididymis (asterisk). **(D)** Laparoscopic complete internal ring closure with 5-0 Vicryl suture.

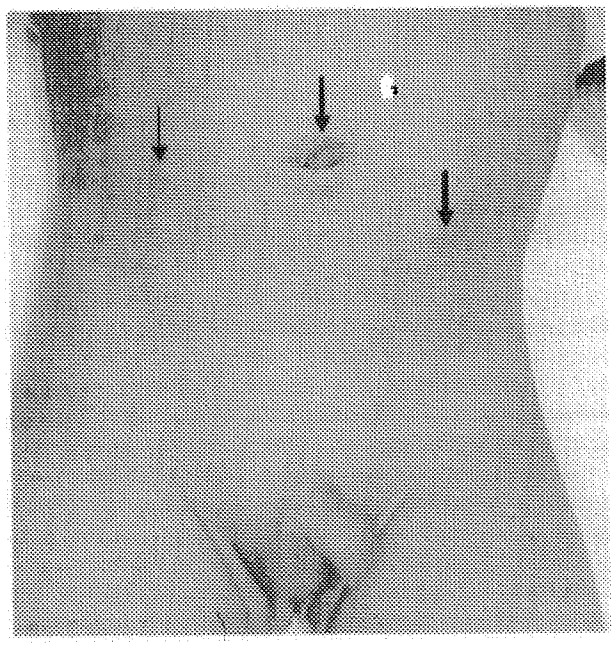

Figure 3. Postoperative appearance after 1 month in representative case. Wound measured 5 mm (large arrows) and 3 mm (small arrow).

when the internal ring is not closed; however, Metwalli and Cheng[16] reported a case of inguinal hernia formation after the laparoscopic approach to the internal inguinal ring, leaving the internal ring open. Baker et al.[17] also reported in their multi-institutional analysis a case of a hydrocele testis after laparoscopic orchiopexy. These reports imply the possibility of inguinal hernia formation after laparoscopic groin exploration. We believe that complete laparoscopic internal ring closure after laparoscopic orchiopexy and orchiectomy is better to prevent postoperative inguinal hernia or hydrocele testis formation, because this technique is not so difficult.

CONCLUSIONS

Four surgical findings are possible with diagnostic laparoscopy for a nonpalpable testis. The first is an intra-abdominal blind-ending vas and vessels, testicular agenesis, indicating that additional exploration is generally unnecessary. The second is an intra-abdominal testicular nubbin, for which laparoscopic orchiectomy is generally indicated. The third is an intra-abdominal testis, for which laparoscopic orchiopexy is generally indicated. The fourth is the vas and vessels exiting the internal ring, diagnosed as an extra-abdominal testicular nubbin or intracanalicular undescended testis, for which we performed laparoscopic groin exploration and subsequent laparoscopic orchiectomy for extra-abdominal testicular nubbin or laparoscopic orchiopexy for intracanalicular undescended testis. We believe all boys with a nonpalpable testis can be treated as soon as the diagnosis has been made using only laparoscopic management, with minimal morbidity and good short-term results. In addition, the other advantages of our technique are the ability to avoid inguinal incision, to detect an open

processus vaginalis, and to prevent a postoperative inguinal hernia or hydrocele testis for patients with a nonpalpable testis. However, additional evaluation of this strategy is needed.

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EDITORIAL COMMENT

The authors present their initial positive experience with a laparoscopic approach to removing a testicular nubbin located distally to the internal ring. Although their technique provides a complete laparoscopic approach to a problem that is typically approached through a single laparoscopic port for diagnosis and a small incision (scrotal vs inguinal) for orchiectomy, several primary questions remain, some of which have yet to be answered. The first question is whether these nubbins should be removed at all. Clearly, the debate continues and is not the intent of this report and so will remain a mystery. If one were to agree that orchiectomy eliminates any concern for malignancy (however, valid that argument is), the second question regards the value of this surgical approach. My personal approach has been to make a midline raphe incision through which I am able to identify and remove the nubbin in the vast majority of cases and am then able to fix the contralateral testis as added security against future torsion and possible anorchia. The cosmetic closure of this incision leads to a near natural appearance. The value of the authors' approach is dubious, other than as a laparoscopic exercise, because they do not address any management issues regarding the contralateral testis that might make this approach unnecessary. Also, it is not necessary to close the ipsilateral internal ring in these cases, just as it is not really needed even after laparoscopic orchidopexy.

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REPLY

We greatly appreciate the editorial comments on our report because we have wanted more debate about our strategy for treating a testicular nubbin. These comments will be very useful and important for us, as well as to readers.

First, whether the excision of the testicular nubbin is necessary in children with a nonpalpable testis remains controversial, because it has not yet been established whether testicular nubbins carry a greater risk of malignancy. Some investigators have claimed that testicular nubbins result in no greater risk of malignancy because most nubbins do not contain viable testicular tissue.[1,2] Recently, however, Storm et al.[3] presented a retrospective review demonstrating that testicular remnants carry a potential for malignant transformation because a significant number of testicular remnants associated with a vanishing testis harbored viable germ cell elements or seminiferous tubules (a total of 21%), although the exact fate of these residual elements remained unknown. In our unpublished data, the expression of several genes implicated in gonadal and testicular development was observed in the testicular nubbin of most cases, even if we could not identify viable germ cells or seminiferous tubules on histologic examination. To determine the malignancy risk of testicular nubbins, however, requires randomized, controlled, long-term follow-up studies.

Second, as indicated in the editorial comments, scrotal exploration for a testicular nubbin might be a good approach from the perspective of minimally invasive surgery. We agree in principle, but we wonder whether this approach would always allow the testicular nubbin to be found and the internal ring to be completely closed in children with a patent processus vaginalis, which is necessary to prevent potential inguinal hernia formation. Laparoscopic groin exploration and subsequent orchiectomy for a testicular nubbin might facilitate these procedures. Additionally, our approach facilitates contralateral orchiopexy and patent processus closure after orchiectomy for an extra-abdominal nubbin without placement of an extra trocar. Furthermore, our approach is simple, and we do not need a special procedure. The evidence to support the necessity of internal ring closure after laparoscopic orchiopexy and orchiectomy might be limited. However, as we described in our report, a case of inguinal hernia formation after a laparoscopic approach to the internal inguinal ring that left the internal ring open has been reported.[4] We do not know what percentage of children with the internal ring left open will develop an inguinal hernia in the future or during old age. In addition, because we encountered a patient with a testicular nubbin and an ipsilateral open processus vaginalis, we consider it appropriate to perform internal ring closure after laparoscopic orchiopexy and orchiec-

tomy. However, additional study is needed to definitively answer this question.

We believe that, until definitive evidence is available that children with a testicular nubbin have no greater risk of malignancy and inguinal hernia formation in the future, and as long as there is room for doubt about these potential complications, we should make every effort during the initial surgery to prevent these complications, because we have a responsibility to protect the patient's future health.

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Identification of Differentially Expressed Genes in Human Cryptorchid Testes Using Suppression Subtractive Hybridization

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Abbreviations and Acronyms

Ad = adult dark

EEF1A1 = eukaryotic translation elongation factor 1 alpha1

NuMA1 = nuclear mitotic apparatus protein 1

PCR = polymerase chain reaction

PLZF = promyelocytic leukemia zinc finger

RT = reverse transcriptase

TPT1 = tumor protein, translationally controlled 1

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Purpose: To restore fertility the current consensus suggests early orchiopexy for cryptorchidism. However, despite early orchiopexy it is reported that transformation of gonocytes into adult dark spermatogonia is already impaired at the time of surgery and consequently affects future fertility. To elucidate the biological processes occurring during germ cell maturation in the cryptorchid testis, we identified the genes affected by testicular maldescent using polymerase chain reaction based suppression subtractive hybridization, and investigated differentially expressed genes to determine whether they are related to cell differentiation and spermatogenesis.

Materials and Methods: Testicular tissues were excised from 24 boys 12 to 59 months old who underwent orchiopexy or hydrocelectomy at our hospital. Two-way subtraction was performed to compare their tissue samples and those from age matched boys with ipsilateral cryptorchidism and a descended testis. Differential expression was validated by real-time reverse transcriptase polymerase chain reaction. To clarify the distribution of candidate genes, immunohistochemical and western blot analysis was performed.

Results: We obtained 84 clones corresponding to transcripts representing differential expression. Basic local alignment search tool searches revealed 32 different known genes with 98% to 100% similarity. Among these genes we further investigated 3 genes, *TPT1*, *EEF1A1* and *NuMA1*, which were significantly more highly expressed in cryptorchidism than in descended testes and were detected in the spermatogonia from immature to adult testes.

Conclusions: *TPT1*, *EEF1A1* and *NuMA1* have cell growth related functions, suggesting that they have certain roles in germ cell differentiation and maintenance of stem cell potential. Changes in the expression levels of these genes in the testes might enable novel evaluation of spermatogenic failure caused by cryptorchidism.

Key Words: cell differentiation, cryptorchidism, spermatogenesis

CRYPTORCHIDISM is recognized in 2% to 4% of mature infants, and is the most common congenital abnormality among urological diseases. Male infertility is one of the serious complications of cryptorchidism, and based on paternity data a large cohort study demonstrated that 10% to 38% of the patients were infertile.[1]

To restore fertility, the current consensus suggests early orchiopexy (before age 1 year) on the basis of the histological evaluation compared to descended testes.[2] However, Hadziselimovic and Herzog have reported

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that a third of males with cryptorchidism have an abnormal sperm count 20 years later, even when orchiopexy was performed before age 1 year.[3] These authors focused on the histological findings of cryptorchid testes and stated that transformation of gonocytes into Ad spermatogonia is crucial for future fertility. Currently, it is believed that spermatogenic failure in patients with cryptorchidism is mainly caused by heat stress. From animal experiments it is clear that a high temperature around the testes induces germ cell apoptosis and testicular atrophy.[4] However, the issue of whether undescended testes exhibit intrinsic spermatogenic failure remains unclear.

Recently comprehensive studies have investigated differential gene expression using molecular biological techniques such as microarray.[5] However, the cryptorchid models may not be entirely reflective of clinical conditions. Furthermore, an appropriate culture system for germ cell lines has not been established, and germ cell differentiation and inducement in vitro are arduous. Consequently it is difficult to elucidate the molecular biological basis of germ cell differentiation and maturation.

To elucidate the biological processes that occur during germ cell maturation in cryptorchid testes, we performed PCR based suppression subtractive hybridization using a testicular biopsy specimen obtained at orchiopexy and investigated the differentially expressed genes to determine whether they are related to cell differentiation or degeneration. We detected 18 up-regulated genes and 16 down-regulated genes, some of which were strongly expressed in germ cells or Sertoli cells. It is likely that the impairment of differentiation potential is related to changes in the expression of these genes. Furthermore, they are potentially available as objective indices to assess the compromised spermatogenesis in cryptorchid testes and are also helpful in predicting future fertility.

MATERIALS AND METHODS

Sample Preparation

We used specimens from testicular biopsy taken during hydrocele and orchiopexy surgery to determine their histopathological characteristics. Approximately 1 to 3 mm^3 specimens were excised at the same time for RNA and protein extraction. These tissues were frozen immediately in liquid nitrogen and then preserved at −80C. For the subtraction experiment we used testicular tissue excised from 2 age matched boys with ipsilateral cryptorchidism (canalicular type) and hydrocele testis because we estimated that the difference in gene expression between both testes might be subtle. Both patients were 1 year and 9 months old, and neither had a complicated deformity. Since hydrocele alone appears to have no direct effect on later fertility, we regarded the hydrocele testes as controls.[6] We used another 13 specimens of cryptorchid testes and 7 specimens of hydroceles for real-time RT-PCR, immunohistochemical analysis and western blot analysis. Patients were 1 year to 4 years and 11 months old. Studies using human testicular tissue were performed after receiving informed consent from the families of the patients and approval of the institutional review board of Nagoya City University Hospital.

PCR Based Suppression Subtractive Hybridization

Suppression subtractive hybridization was performed as previously described.[7] First, we set up cDNA derived from cryptorchid testes as a tester and those from hydrocele testes as a driver. Next, we performed reverse subtraction and attempted to clarify the genes associated with differential expression in cryptorchidism.

Validation of Differential Expression

To validate the differential expression of the subtracted library, real-time RT-PCR was carried out with single strand cDNA. PCR reactions were performed with sequence specific primers designed for 12 identified genes (table 1). A G3PDH primer was used as an internal control. Each real-time PCR, comprising 40 cycles of 95C for 10 seconds and 60C for 1 minute, was run in duplicate in the same run. Production of the expected amplification fragments without unanticipated products and primer

Table 1. *Primer set used for RT-PCR*

Gene Symbol	Primers		Amplicon Length (bp)
	Forward	Reverse	
GAPDH	5′-CAGCCTCAAGATCATCAGCA	5′-TGTGGTCATGAGTCCTTCCA	106
TPT1	5′-TCAGCCACGATGAGATGTTCTC	5′-TTCCACCAATGAGCGAGTCA	129
EEF1A1	5′-CTGTATTGGATTGCCACACG	5′-GCAGCATCACCAGACTTCAA	124
ZSCAN2	5′-CATTGGTGTGGTTCCTGGTTG	5′-CCCTCCATCTTGACCTGAAA	163
FTH1	5′-GACCCCCATTTGTGTGACTT	5′-CAGGGTGTGCTTGTCAAAGA	150
NUMA1	5′-CACTGAAGAGGGACAGCAAA	5′-GGACTTTTGGAGCTCATGGT	204
IFITM3	5′-CGTGAAGTCTAGGGACAGGAAGAT	5′-CGATGAGCAGAATGGTCATGAG	125
NRPA2B1	5′-GACTGTGTGGTAATGAGGGATCCT	5′-TGGCTCAACTACTCTCCCATCA	135
SOD1	5′-AAAACACGGTGGGCCAAA	5′-TGGTCTCCTGAGAGTGAGATCA	123
CLU	5′-GAGCAGCTGAACGAGCAGTTTA	5′-AAGGAACGTCCGAGTCAGAAGT	121
C3orf28	5′-GGCTTACCAGAAGCTCTGAT	5′-TGAAGCGACCTGACCATATGAG	153
MYL6	5′-ACTTCGGGTGTTTGACAAGG	5′-CGACAGGATATGCCTCACAA	175
PTGDS	5′-GTGCATGACGGAACAATAGG	5′-CTGACTTGCTTCCGGAGTTT	181

dimers was confirmed by melting curve analysis and gel electrophoresis. To determine the relative amounts of the products we used the comparative (threshold cycle) method according to the manufacturer instructions.

Statistical Analysis

The expression value of G3PDH gene was used to calculate an accurate normalization factor and the mean quantity of transcript (raw data) was divided by the respective normalization factor to obtain a normalized value for each transcript. The normalized target values (undescended testes) were divided by the calibrator normalized target values (hydrocele testes) to generate the relative expression levels. Next, the mean gene expression levels were calculated and analyzed by Mann-Whitney U test with 95% confidence intervals. All values are expressed as mean ± SD, as calculated using StatView® for Windows, version 4.5.

Immunohistochemical Analysis

Human, mouse and rat testicular tissues were fixed with 4% paraformaldehyde or Bouin's solution and embedded in paraffin. All animal experimental procedures were performed in accordance with protocols approved by the Animal Care Committee of Nagoya City University Graduate School of Medical Sciences. Among the candidate genes we performed immunohistochemical analysis on human testes using anti-TPT1 antibody 1:200 (SC-20426, Santa Cruz Biotechnology® Inc, Santa Cruz, California), anti-EEF1A1 antibody 1:100 (#11402-1-AP, Proteintech Group Inc, Chicago, Illinois) and anti-NuMA1 antibody 1:400 (#LS-B60, LifeSpan Biosciences Inc, Seattle, Washington).

Western Blot Analysis

For the extraction of whole protein from human testicular specimens we used a cell lysis reagent following the manufacturer instructions. Samples containing 30 μg total protein were separated by running on 10% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were subsequently transferred onto polyvinylidene difluoride membranes. The membranes were then blocked for 1 hour using 5% skim milk in tris buffered saline (pH 7.5) polysorbate 20 at room temperature and sequentially incubated with polyclonal antibodies of TPT1, 1:200, EEF1A1, 1:250 or NuMA1, 1:1,000 overnight at 4C. Protein bands were visualized with enhanced chemiluminescence western blotting analysis according to the manufacturer instructions.

RESULTS

By PCR subtraction cloning we obtained 54 and 30 clones corresponding to transcripts that were presumed to be expressed at considerably higher and lower levels, respectively, in cryptorchidism compared to descended testes. To further characterize the library, we sequenced their inserts. All 84 clones contained an insert and their characteristics are outlined in table 2. They represented 45 and 21 identified sequences in the higher and lower expression groups, respectively. A total of 18 nonredundant clones exhibited homology with only unannotated expressed sequence tag or genomic sequences. A total of 66 identified sequences were compared with known sequences in GenBank® by basic local alignment search tool analysis against the mammal nr database. Basic local alignment search tool searches showed that the 66 sequences were homologous to 32 different known genes with 98% to 100% similarity. Of the identified genes 12 coded for ribosomal proteins. The gene symbols, names, functions and accession numbers are outlined in the Appendix.

Table 2. *Characterization of estimated sequence transcripts represented in subtracted library*

	High Expression (%)	Low Expression (%)
Identified sequences	45 (83.3)	21 (70.0)
No significant match	4 (7.4)	2 (6.7)
Genomic sequences	5 (9.3)	7 (23.3)
Totals	54 (100)	30 (100)

Based on sequence analysis of 84 inserts.

To compare the relative quantification of the candidate gene expression between undescended and descended testes, real-time RT-PCR was performed for 12 genes. Mean relative quantification of the gene transcripts studied is illustrated in figure 1. Among the higher expression genes *EEF1A1*, *TPT1* and *NuMA1* had significantly higher RNA levels in undescended testes compared to descended testes. *NuMA1* exhibited the largest difference and the p value of *EEF1A1* was the lowest (0.0034). Conversely, the relative expression values of *CLU*, *C3ORF28* and *NRPA2B1* were lower in undescended testes but not significantly so. Contrary to our expectation, 4 genes, *SOD1*, *PTGDS*, *MYL6* and *IFITM3*, tended to be expressed at higher levels in undescended testes. On the basis of the frequency of their clones and the difference in their expression patterns, we further investigated 3 genes, *EEF1A1*, *TPT1* and *NuMA1*. Results of immunohistochemical analysis to clarify the association between these genes and spermatogenesis in the rodent testis are illustrated in figures 2 and 3.

TPT1 was initially described as a growth related protein in mouse ascites and erythroleukemia cells.[8] Guillaume et al detected TPT1 from a proteome analysis of rat spermatogonial proteins based on 2-dimensional gel electrophoresis.[9] In the present study TPT1 protein was strongly represented in the spermatogonia and elongated spermatids (steps 9 to 12) in a stage specific manner. TPT1 was not detectable in Sertoli cells or Leydig cells. In human testes TPT1 was detected in spermatogonia (fig. 3, *A*). In undescended testes seminiferous tubules were slightly smaller than in descended testes and hypertrophy of interstitial tissue was recognized, although the TPT1 signals were stronger.

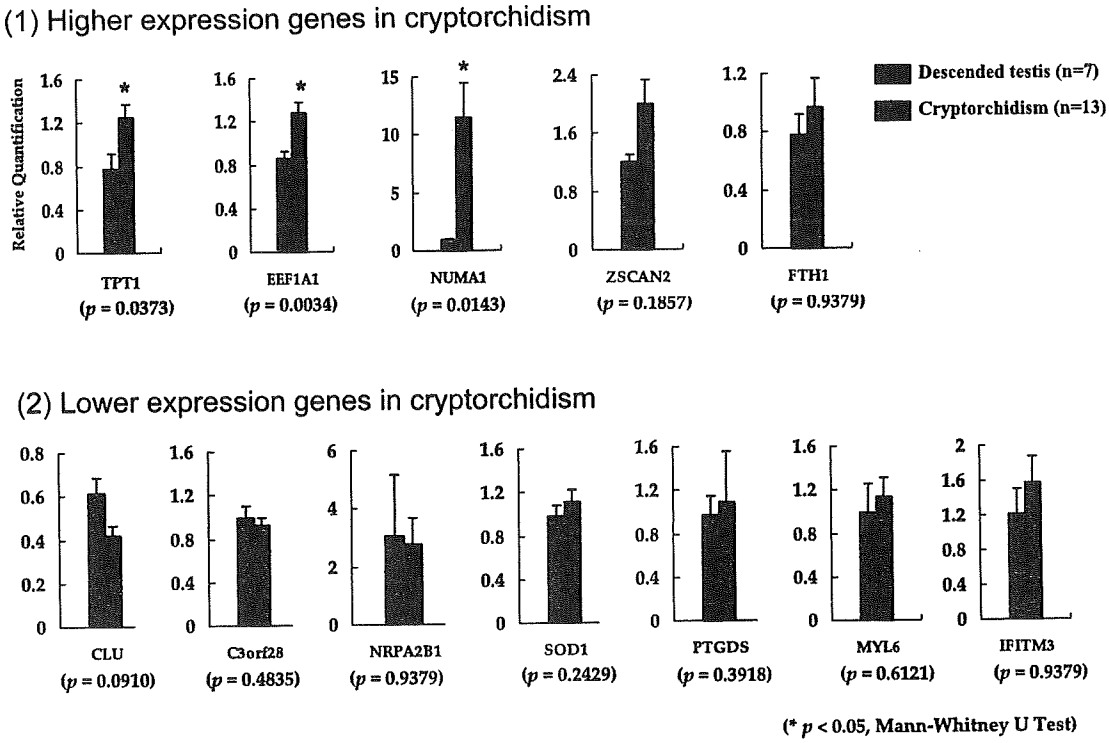

Figure 1. Quantification of specific transcripts by real-time RT-PCR. Values of relative quantification in 5 genes with higher expression as determined by subtraction (*EEF1A1, TPT1, NUMA1, ZSCAN2* and *FTH1*) and 7 genes with lower expression (*CLU, C3ORF28, NRPA2B1, SOD1, PTGDS, MYL6* and *IFITM3*) were determined by real-time RT-PCR. Relative expression values were calculated using comparative (threshold cycle) method and *GAPDH* levels as internal control. All values represent mean ± standard deviation.

EEF1A1 is a guanosine triphosphate binding protein catalyzing the binding of charged aminoacyl-tRNA to the A-site of the ribosome.[10] In the present study the expression pattern of EEF1A1 protein was represented in the cytoplasm of spermatogonia, spermatocytes and elongated spermatids in a stage specific manner. From an examination of the testes of patients with unilateral cryptorchidism the number of EEF1A1 positive spermatogonia in the undescended testis was greater than in the contralateral scrotal testis.

NUMA1 was the first described nuclear protein that has a functional role in mitotic spindle assembly. Recently, it was reported that NuMA1 has a role in meiotic cell division.[11] In our study NuMA1 was expressed in the nucleus of Sertoli cells, spermatogonia, spermatocytes and a part of the spermatids in rat testis. Its expression pattern was in accordance with seminiferous tubule stage. In cryptorchid testes the number of NuMA1 positive cells was markedly increased, and their intensity was also higher than in descended testes. Most of the NuMA1 positive cells were Sertoli cells morphologically.

Western blotting also revealed that TPT1, EEF1A1 and NuMA1 proteins were detected more intensely in cryptorchid testes (fig. 3, *B*). This finding is consistent with the results obtained by immunohistochemical analysis.

DISCUSSION

In this study we detected differentially expressed genes in human testes between cryptorchid and descended testes using PCR based suppression subtractive hybridization. From 84 randomly selected clones we obtained 66 identified sequences representing 32 known genes. Of these samples we validated the expression of 12 genes by real-time RT-PCR, and further investigated the *TPT1*, *EEF1A1* and *NuMA1* genes, which were significantly more highly expressed in cryptorchid testes than in descended testes.

TPT1 is a highly conserved protein, and is widely expressed in many tissues and cell types. Homozygous mutants of *TPT1* are embryonic lethal,[12] which suggests that this protein has an essential role in development. In our study *TPT1* was detected not only in the spermatogonia, but also in elongated spermatids in a stage specific manner. This finding suggests that *TPT1* might have certain roles in spermatogenesis. Recently it was reported that *Tpt1* activates the transcription of *Oct4* and *Nanog*, which

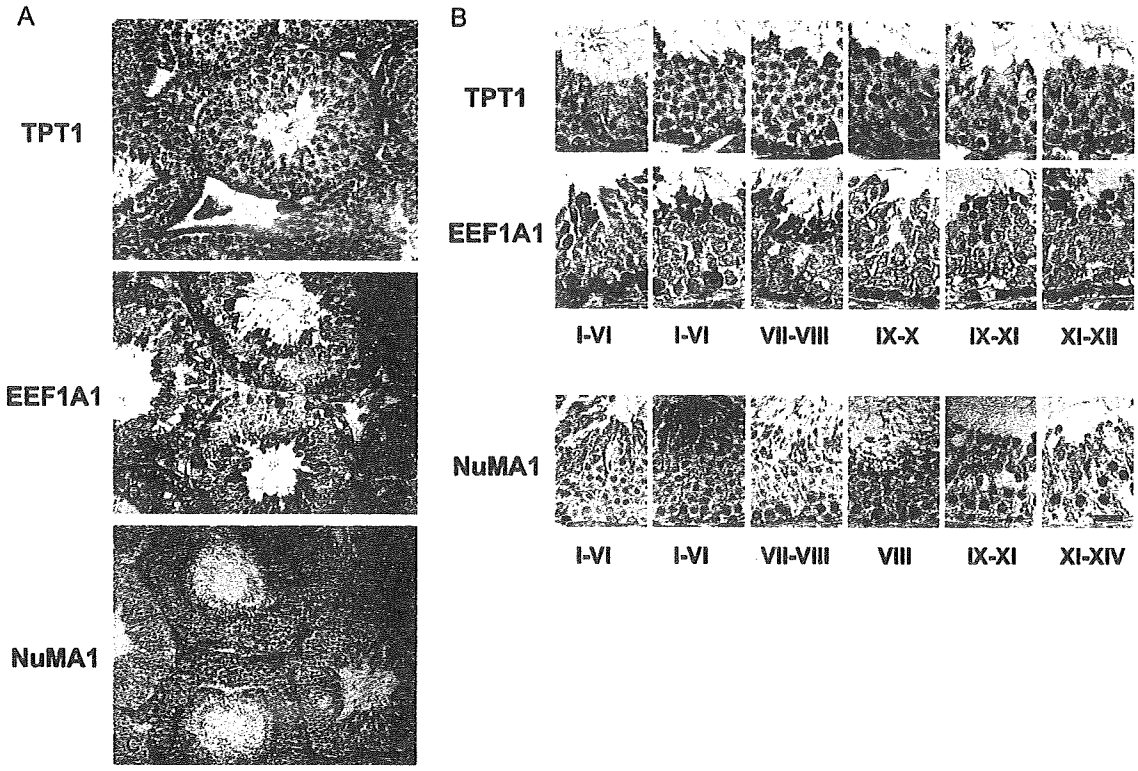

Figure 2. Immunohistochemical staining. *A*, sections of adult mouse testes were stained with anti-TPT1 (*a*) or anti-EEF1A1 antibody (*b*). Adult rat testis was used for staining with anti-NuMA1 antibody (*c*). Scale bar indicates 50 μm. *B*, high magnification views of seminiferous tubule stained with anti-TPT1, anti-EEF1A1 or anti-NuMA1 antibody. For TPT1 and EEF1A1 figures are classified according to mouse spermatogenic stage (I to XII), and for NuMA1 according to rat spermatogenic stage (I to XIV). Scale bar indicates 10 μm.

have a crucial role in maintaining pluripotency.[13] *Oct4* undergoes down-regulation during oogenesis and spermatogenesis,[14] and is considered a marker of undifferentiated cells, including spermatogonia.

EEF1A1 was first described as aminoacyl transferase 1 and is involved in protein synthesis.[15] *EEF1A1* was observed to have similarities to *CCS-3* (cervical cancer suppressor 3), which interacts with the BTB domain of *PLZF* and prevents nuclear translocation.[16] Because *PLZF*, as a spermatogonia specific transcription factor, is required to regulate self-renewal and maintenance of the stem cell pool,[17] *EEF1A1* might have a role in regulating the differentiation and maintenance of spermatogonia by interacting with *PLZF*.

Of the other genes of the subtracted library *NuMA1* exhibits the greatest difference in mRNA expression. NUMA1 is a large nuclear protein that accumulates at the spindle poles in mitosis[11] and may have an important role in meiotic cell division. Of the lower expressed genes *Clusterin* was first characterized in the male reproductive system, and Plotton et al reported that *Clusterin* mRNA levels are decreased in the human cryptorchid testis, suggesting that alteration of Sertoli cell function may be involved in constitutive or idiopathic spermatogenic failures.[18] This finding is consistent with our data and, hence, *Clusterin* is potentially useful as a specific marker of Sertoli cell function.

This study demonstrates that diverse genetic changes occur in human testes in cases of cryptorchidism, and it is suggested that these changes are associated with histological variations between cryptorchid and descended testes. Generally in cryptorchidism a decrease in the number of Leydig cells can be observed, and the delay of differentiation from gonocytes to spermatogonia, the decrease in seminiferous tubule diameter, and the proliferation of collagen around the tubules and vessels are remarkable. Hadziselimovic et al reported that the proportion of spermatogonia in undescended testes is significantly smaller than in normal testes, although the total number of germ cells is not affected.[19] Since the expression of genes associated with the cell cycle or cell proliferation, such as *TPT1*, *EEF1A1* and *NuMA1*, were high in the present study, it is suggested that the majority of germ cells in cryptorchidism consist of more proliferative and undifferentiated spermatogonia compared with those in age matched descended testes. Although patient age ranged widely in our study (12 to 59 months), we did not observe a correlation between gene expres-

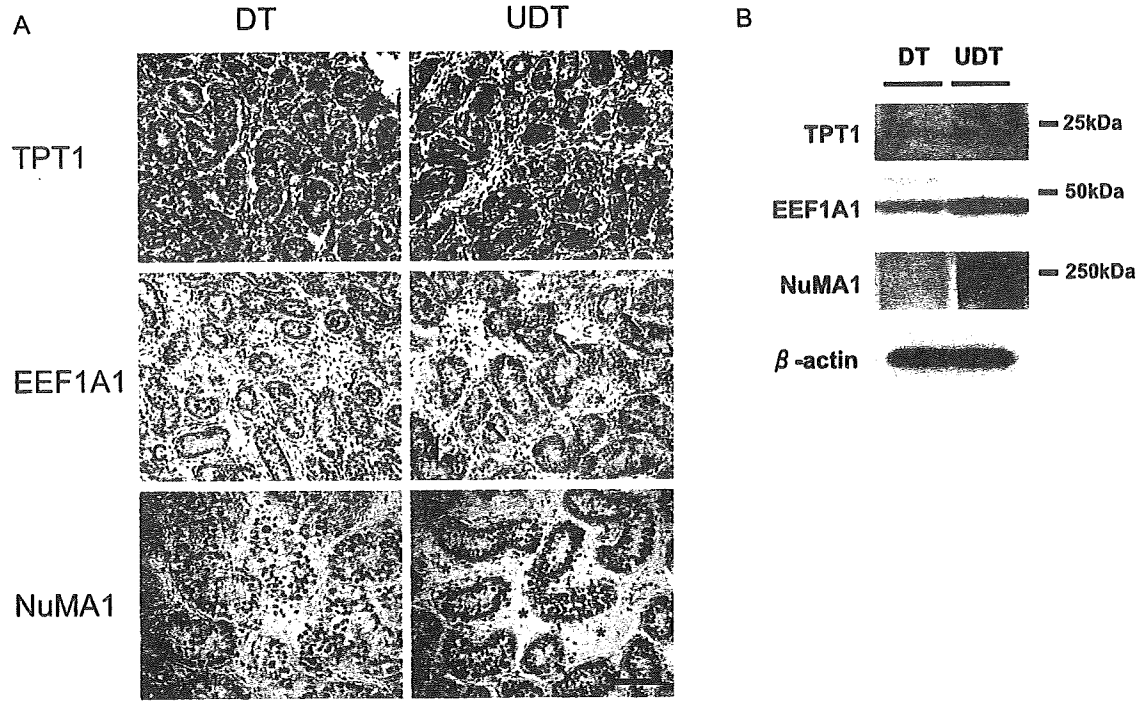

Figure 3. Immunohistochemical staining. *A*, sections of testes from individual patients with descended and undescended testes were stained with anti-TPT1 (*a* and *b*), anti-EEF1A1 (*c* and *d*) or anti-NuMA1 antibody (*e* and *f*). Scale bar indicates 50 μm. In undescended testes seminiferous tubules were slightly smaller (arrows) and hypertrophy of interstitial tissue was recognized (asterisks). *B*, western blot analysis with anti-TPT1 (23 kDa), anti-EEF1A1 (50 kDa) and anti-NuMA1 antibody (238 kDa). Whole proteins were extracted from individual cases of descended and undescended testes. *DT*, descended testes. *UDT*, undescended testes.

sion and age (data not shown). Thus, the present study led us to conjecture that the expression pattern of these genes reflects the delay of germ cell maturation, which is compatible with the findings of previous studies.

Cryptorchidism is a multifactorial disease and exhibits heterogeneous phenotypes. The etiology of spermatogenic failure in cryptorchidism has not been identified. However, we consider that the undescended status of the testes has direct or indirect influence on testicular development. The former concerns hereditary disorders such a chromosome aberration or gene mutation, whereas the latter is related to hormonal dysfunction and environmental effects such as endocrine disruptors and high temperature surrounding the testes. Although we have not clarified whether the undescended testes themselves have an intrinsic disorder, the associated gene expression pattern might be characteristic of the degree of germ cell maturation.

Since hypoplasia of Leydig cells has been observed in undescended testes, Hadziselimovic et al have speculated that failure of the testosterone surge turns off the switch controlling the transformation of gonocytes into Ad spermatogonia.[19] Moreover, recent prospective randomized trials of hormonal therapy for cryptorchidism have been reported in which neoadjuvant gonadotropin-releasing hormone therapy before orchiopexy improved histological findings of treated testes and increased the number of Ad spermatogonia per seminiferous tubule.[20] However, few reports have elucidated the biomolecular cascade between hormonal stimulation and germ cell maturation, and thus further investigation is needed. Although the number of Ad spermatogonia at orchiopexy is used to predict the future fertility of patients with cryptorchidism,[19] it is feasible that the genes detected in the present study are more suitable as objective markers of germ cell maturation or predictors of future fertility. Nevertheless, further studies will be required to gain additional insight into the process of germ cell maturation and the involvement of these genes.

CONCLUSIONS

We investigated genes that exhibited differential expression between cryptorchid and descended testes to explicate genetic changes during germ cell maturation. Among 32 identified genes we focused on 3, *TPT1*, *EEF1A1* and *NuMA1*, which were more highly expressed in cryptorchid than in descended testes. Each of these genes was detected in the sper-