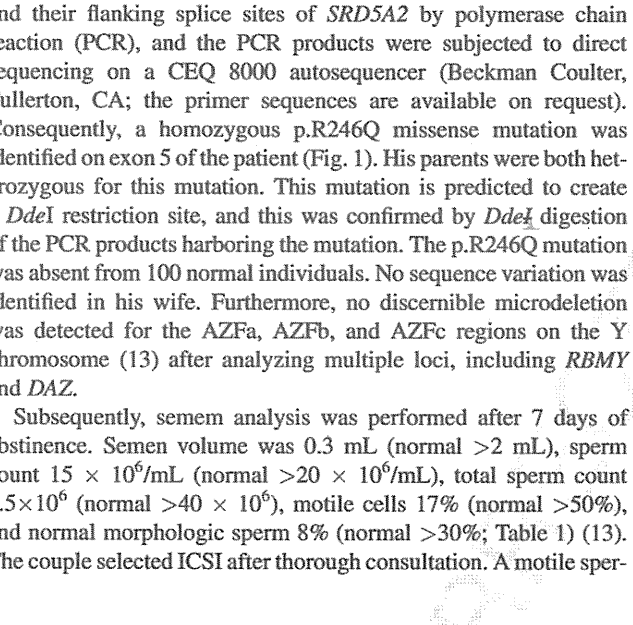
At 29 years of age, he came to us with his wife, because of infertility despite an ordinary conjugal life for 2 years. His penile length was 4.5 cm (age-matched normal: 9.0 ± 1.0 cm), pubic hair development at Tanner stage 4 (normal stage 4–5), and testis volume 10 mL bilaterally (normal 13–20 mL) (8–10). Wolffian structures (epididymides and vasa deferentia) were apparently normal, and there was no varicocele. Basal serum LH was 2.08 mIU/mL (normal 1.8–5.2 mIU/mL) (11), FSH 6.98 mIU/mL (normal 2.9–8.2 mIU/mL), T 6.56 ng/mL (normal 2.5–11.0 ng/mL), DHT 0.27 ng/mL (normal 0.23–0.85 ng/mL), and a T/DHT ratio 24.3 (normal 12 ± 3) (11, 12). His karyotype was 46,XY in all of the 50 lymphocytes examined. Therefore, he was diagnosed as having 5α-reductase-2 deficiency.

To confirm the diagnosis, mutation analysis was performed for *SRD5A2*, encoding steroid 5α-reductase-2. The present study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and was performed after obtaining written informed consent. In brief, leukocyte genomic DNA was amplified with the primers for all five exons and their flanking splice sites of *SRD5A2* by polymerase chain reaction (PCR), and the PCR products were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA; the primer sequences are available on request). Consequently, a homozygous p.R246Q missense mutation was identified on exon 5 of the patient (Fig. 1). His parents were both heterozygous for this mutation. This mutation is predicted to create a *Dde*I restriction site, and this was confirmed by *Dde*I digestion of the PCR products harboring the mutation. The p.R246Q mutation was absent from 100 normal individuals. No sequence variation was identified in his wife. Furthermore, no discernible microdeletion was detected for the AZFa, AZFb, and AZFc regions on the Y chromosome (13) after analyzing multiple loci, including *RBMY* and *DAZ*.

Subsequently, semem analysis was performed after 7 days of abstinence. Semen volume was 0.3 mL (normal >2 mL), sperm count 15 × 10^6/mL (normal >20 × 10^6/mL), total sperm count 4.5×10^6 (normal >40 × 10^6), motile cells 17% (normal >50%), and normal morphologic sperm 8% (normal >30%; Table 1) (13). The couple selected ICSI after thorough consultation. A motile spermatozoa was microinjected into each of 20 mature (metaphase II) oocytes that were obtained after ovarian stimulation with FSH. Ten oocytes were fertilized and a single healthy-looking embryo was transfered to the uterus on day 3. This resulted in a successful production of a healthy 3.32 kg and 52.0 cm male infant at 41 weeks of gestation.

FIGURE 1

Mutational analysis of *SRD5A2*. The patient is homozygous for the p.R246Q missense mutation, and the parents are heterozygous for the mutation.



Matsubara. ICSI and steroid 5α-reductase-2 deficiency. Fertil Steril 2010.

DISCUSSION

This Japanese patient with 5α-reductase-2 deficiency had oligozoospermia and achieved paternity by ICSI. Because the p.R246Q mutation identified in this patient has been shown to be a hypomorphic mutation by functional studies (14, 15), it would explain why he had relatively mild clinical features, such as undermasculinized but obviously male genital development, unequivocally male gender role behavior, and a relatively low T/DHT ratio. It is likely that the residual enzymatic activity led to oligozoospermia rather than azoospermia, thereby permitting successful paternity by ICSI.

To date, twelve 46,XY patients with 5α-reductase-2 deficiency, including the present case, have received semen analysis and/or achieved paternity (Table 1) (4–7). They are invariably homozygotes for hypomorphic missense mutation (cases 1–10) or compound heterozygotes for hypomorphic missense mutations (cases 11 and 12), and there is no report documenting semem findings or paternity in patients with more severe mutations (e.g., patients with nonsense mutations on both alleles). It is assumed, therefore, that patients with residual activities have usually been reared as male, whereas those with more severe mutations have usually been raised as female.

Spermatogenic function was variable among the 12 patients, although external genitalia were invariably undermasculinized. Indeed, even in the same pedigree, cases 3 and 5–10 had severely impaired spermatogenesis, whereas case 2 had small semen volume and high sperm concentration and fathered three children by intrauterine insemination and case 4 showed oligozoospermia (4, 5). In addition, cases 11 and 12 retained fertility, although semen analysis was not performed (6, 7). Furthermore, such variability in spermatogenic function is apparently independent of the presence or absence of cryptorchidism (Table 1). Such variability in spermatogenic function, however, would not be unexpected, because, in contrast to external genital formation that occurs in the fetal life, spermatogenic function is influenced by multiple genetic and environmental factors for a long time (>20 y). Therefore, although residual enzymatic activities would have a certain effect on spermatogenic function, prediction of spermatogenic function appears to be difficult in patients with 5α-reductase-2 deficiency.

Nevertheless, it is noteworthy that four patients with 5α-reductase-2 deficiency, including the present case, achieved paternity with and without artificial reproductive technology (5–7). Recently, male gender assignment has been recommended for 46,XY patients with 5α-reductase-2 deficiency, primarily because they tend to show male gender role behavior (16, 17). The reports of successful paternity provide additional support for male gender assignment in 46,XY patients with 5α-reductase-2 deficiency, especially those with residual enzymatic activities.

When performing artificial reproductive techniques, including ISCI, in patients with genetic disorders for male infertility, thorough genetic counseling is required regarding the transmission risk of genetic abnormalities. Indeed, vertical transmission from father to son has been reported for a Yq deletion and an autosomal dominant mutation affecting spermatogenesis (18, 19).

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TABLE 1

Summary of 46,XY patients with 5α-reductase-2 deficiency who received semem analysis and/or achieved paternity.

Patient			Genital findings		Endocrine data			Semen parameters					
Case no.	Age (y)	*SRD5A2* mutation	External genitalia	Testis vol., R, L (mL)	T (ng/mL)	DHT (ng/mL)	T/DHT ratio	Vol. (mL)	SC ($\times 10^6$/mL)	TSC ($\times 10^6$)	MC (%)	NM (%)	Paternity
1	29	p.R246Q (homo)	MP, HY, RT (B)	10, 10	6.56	27	24	0.3	15	4.5	17	8	ICSI
2	20	p.R246W (homo)	MP, HY	25, 20	11.00	0.29	38	0.15	321	48	56	71	
	34				6.81	0.12	57	0.5	165	83	33	ND	IUI
	36				6.69	0.10	67	0.5	65	33	66	ND	IUI
3	24	p.R246W (homo)	HY	14, 15	7.93	0.25	31	0.5	2	1	0	36	
4	21	p.R246W (homo)	HY	ND	11.06	0.18	61	0.4	12	4.8	10	59	
5	22	p.R246W (homo)	HY	18, 20	8.79	0.16	55	0.8	<1	<1	ND	ND	
6	24	p.R246W (homo)	HY	13, 13	8.18	0.17	48	0.1	<1	<1	0	<1	
7	22	p.R246W (homo)	HY	15, 12	10.4	0.51	20	1.0	0	0	0	0	
8	30	p.R246W (homo)	HY, CO (U)	ND	10.51	0.17	62	<0.05	<1	<1	0	4	
9	24	p.R246W (homo)	HY, CO (U)	15, 12	10.51	0.18	58	0.5	0	0	0	0	
10	20	p.R246W (homo)	HY, RT (B)	10, 8	8.61	0.23	37	0.2	0	0	0	0	
11	23[a]	p.H231R/p.G196S	MP, HY, CO	NA	8.93	0.19	47	ND	ND	ND	ND	ND	Natural
12	22[b]	p.H231R/p.G196S	MP, HY, CO	NA	2.7	ND	ND	ND	ND	ND	ND	ND	Natural
Normal value					2.5–11.0	0.23–0.85	12 ± 3	>2	>20	>40	>50	>30	
Reference					(11)	(11)	(12)	(13)	(13)	(13)	(13)	(13)	

Note: Case 1 is the present case, cases 2–10 are derived from a large pedigree (4, 5), and cases 11 and 12 are brothers (6, 7). All of the cases received surgical repair and/or testosterone treatment for undermasculinized external genitalia. B = bilateral; CO = cryptorchidism; DHT = dihydrotestosterone; HY = hypospadias; ICSI = intracytoplasmic sperm injection; IUI = intrauterine insemination; L = left; MC = motile cells; MP = micropenis; ND = not determined; NM = normal morphology; R = right; RT = retractile testis; SC = sperm count; T = testosterone; TSC = total sperm count; U = unilateral.

[a] Endocrine data were obtained at 16 years of age.

[b] Endocrine data were obtained at 14 years of age.

Matsubara. ICSI and steroid 5α-reductase-2 deficiency. Fertil Steril 2010.

For an autosomal recessive disease, if a spouse were heterozygous for the corresponding genes, half of children should have biallelic mutations for male infertility. Thus, we performed *SRD5A2* analysis in his wife to examine a possible transmission risk.

In summary, we observed successful paternity by ISCI in a male patient with molecularly confirmed 5α-reductase-2 deficiency and oligozoospermia. Further studies in various aspects, including spermatogenic function, will permit to set forth better management strategies in this condition.

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1 **Semen analysis and successful paternity by intracytoplasmic sperm injection in a man with steroid 5α-reductase-2 deficiency**

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Tokyo, Japan

Successful paternity was achieved by intracytoplasmic sperm injection in a male patient with molecularly confirmed 5α-reductase-2 deficiency and oligozoospermia.

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Mamld1 Knockdown Reduces Testosterone Production and *Cyp17a1* Expression in Mouse Leydig Tumor Cells

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Abstract

Background: *MAMLD1* is known to be a causative gene for hypospadias. Although previous studies have indicated that *MAMLD1* mutations result in hypospadias primarily because of compromised testosterone production around the critical period for fetal sex development, the underlying mechanism(s) remains to be clarified. Furthermore, although functional studies have indicated a transactivation function of *MAMLD1* for the non-canonical Notch target *Hes3*, its relevance to testosterone production remains unknown. To examine these matters, we performed *Mamld1* knockdown experiments.

Methodology/Principal Findings: *Mamld1* knockdown was performed with two siRNAs, using mouse Leydig tumor cells (MLTCs). *Mamld1* knockdown did not influence the concentrations of pregnenolone and progesterone but significantly reduced those of 17-OH pregnenolone, 17-OH progesterone, dehydroepiandrosterone, androstenedione, and testosterone in the culture media. Furthermore, *Mamld1* knockdown significantly decreased *Cyp17a1* expression, but did not affect expressions of other genes involved in testosterone biosynthesis as well as in insulin-like 3 production. *Hes3* expression was not significantly altered. In addition, while 47 genes were significantly up-regulated (fold change >2.0×) and 38 genes were significantly down-regulated (fold change <0.5×), none of them was known to be involved in testosterone production. Cell proliferation analysis revealed no evidence for compromised proliferation of siRNA-transfected MLTCs.

Conclusions/Significance: The results, in conjunction with the previous data, imply that *Mamld1* enhances *Cyp17a1* expression primarily in Leydig cells and permit to produce a sufficient amount of testosterone for male sex development, independently of the *Hes3*-related non-canonical Notch signaling.

Citation: Nakamura M, Fukami M, Sugawa F, Miyado M, Nonomura K, et al. (2011) *Mamld1* Knockdown Reduces Testosterone Production and *Cyp17a1* Expression in Mouse Leydig Tumor Cells. PLoS ONE 6(4): e19123. doi:10.1371/journal.pone.0019123

Editor: Michael Polymenis, Texas A&M University, United States of America

Received January 31, 2011; **Accepted** March 17, 2011; **Published** April 29, 2011

Funding: Grant for Research on Intractable Diseases (H22-98) from the Ministry of Health, Labor and Welfare (http://www.mhlw.go.jp/english/), and Grants-in-Aid for Scientific Research (B) (20390265) and for Young Scientists (B-22791715) from the Japan Society for the Promotion of Science (JSPS) (http://www.jsps.go.jp/english/), and by Grant-in-Aid for Scientific Research on Innovative Areas (22132004 A01) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (http://www.mext.go.jp/english/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

MAMLD1 (mastermind-like domain containing 1, alias *CXorf6*) on human chromosome Xq28 is a causative gene for hypospadias, a mild form of 46,XY disorders of sex development (DSD) [1]. To date, multiple mutations have been identified in patients with various types of hypospadias [1–3]. In this regard, the mouse homologous gene *Mamld1* is transiently expressed in fetal Sertoli and Leydig cells around the critical period for sex development [1], and transient *Mamld1* knockdown using small interfering RNAs (siRNAs) reduces testosterone (T) production in cultured mouse Leydig tumor cells (MLTCs) [4]. Furthermore, the upstream region of *MAMLD1/Mamld1* harbors a putative binding site "CCAAGGTCA" for *NR5A1* (alias, *SF-1* and *AD4BP*) [4] that regulates the transcription of a vast array of genes involved in sex development [5], and NR5A1 protein has been shown to bind to the putative target site and exert a transactivation function for *Mamld1* [4]. These findings imply that *MAMLD1/Mamld1* is involved in fetal T production under the regulation of NR5A1, and that *MAMLD1* mutations result in hypospadias primarily because of compromised T production around the critical period for sex development.

However, the underlying mechanism(s) by which impaired *MAMLD1/Mamld1* leads to compromised T production remains to be clarified, although there are several possibilities such as defective activities of enzyme(s) involved in T production and compromised proliferation of Leydig cells. Furthermore, although previous functional studies have indicated that *MAMLD1* has a transactivation function for the non-canonical Notch target *Hes3* [4], its relevance to biological function including T production remains unknown. To examine these matters, we performed detailed analyses in *Mamld1* knockdown experiments using MLTCs.

Methods

Knockdown experiments

MLTCs (ATCC, CRL-2065™) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, and were

transiently transfected with two siRNAs, i.e., siRNA1 (sense: GCUUCCAGUUCAGAUGCCATT; and anti-sense: UGGCAUCUGAACUGGAAGCTT) and siRNA2 (sense: GGAACUAACCAAAAUUCAATT; and anti-sense: UUGAAUUUUGGUUAGUUCCTC) or with non-targeting control RNA (4611G) (final concentration 20 nM), using Lipofectamine RNAiMAX (Life Technologies). Relative amount of endogenous *Mamld1* mRNA against *B2m* (β2-microglobulin) was determined by the TaqMan real-time PCR method using the probe-primer mix on ABI PRISM 7000 (Life Technologies) (Assay No.: Mm01293665_m1 for *Mamld1*; and Mm00437762_m1 for *B2m*).

Steroid metabolite measurements

MLTCs are known to have the capacity to produce T primarily via Δ^4-pathway, although the amount of T production remains small primarily because of low 17α-hydroxylase and Hsd17b3 activities [6]. MLTCs are also known to retain responsiveness to human chorionic gonadotropin (hCG) [6–8]. Thus, after 48 hours of incubation of transfected MLTCs in 12-well plates with 1 ml of culture medium (an initial cell count: 1×10^5 cells/well), hCG (Mochida Pharmaceutical) was added to the media at a final concentration of 50 IU/L, and the culture media were obtained at one hour after the addition of hCG. Subsequently, steroid metabolites in the T production pathway were measured by the liquid chromatography-tandem mass spectrometry (ASKA Pharma Medical). This experiment was performed three times.

Gene expression analyses

Real-time reverse transcriptase (RT)-PCR and microarray analyses were performed using total RNA extracted from MLTCs that were harvested at the time of steroid metabolite measurements. For real-time RT-PCR analysis, 1 μg of total RNA was examined for relative mRNA dosage against *B2m* by the TaqMan Gene Expression Assay on ABI PRISM 7000 (Assay No.: Mm00446826_m 1 for *Nr5a1* (*Sf1*); Mm00441558_m1 for *Star*; Mm00490735_m1 for *Cyp11a1*; Mm01261921_mH for *Hsd3b1*; and Mm00484040_m1 for *Cyp17a1*). In addition to the genes for steroidogenic enzymes involved in T biosynthesis, we also studied *Insl3* (Mm01340353_m1) for gubernacular development that is expressed in Leydig cells [9,10]. This experiment was repeated three times. For microarray analysis, 300 ng of total RNA was converted into cRNA associated with Cyanine-3 labeled CTP using RNA Spike-In Kit and Quick Amp Labeling Kit, and was subjected to hybridization on Whole Mouse Genome Oligo Microarray in triplicate (4×44 K G4122F) (Agilent Technologies). Subsequently, fluorescent signals were detected by Agilent Scanner, and were analyzed by GeneSpring GX10 (Tomy Digital Biology). The microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE26913 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26913). All data is MIAME compliant and the raw data has been deposited in a MIAME compliant database (GEO), as detailed on the MGED Society website (http://www.mged.org/Workgroups/MIAME/miame.html).

Cell proliferation assays

The number of viable MLTCs transfected with two siRNAs or with non-targeting RNA was calculated by the colorimetric method [11,12], using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). The detailed procedure has been described in the manufacturer's protocol. In brief, MLTCs were cultured in 96-well plates (an initial cell count: 1×10^4 cells/well), and the cell number was determined every 24 hours by measuring the absorbance on a plate reader (Molecular Device) at 490 nm. This method is based on a positive correlation between the number of viable cells and the absorbance until the cells become confluent, and our preliminary studies showed a good correlation until the absorbance of ~2.0 (~6×10^5 cells/well) (Figure S1). This experiment was performed three times.

Statistical analysis

Statistical significance was examined by Student's *t*-test or by Mann-Whitney's *U*-test. $P<0.05$ was considered significant.

Results

Steroid metabolite measurements

The mean steroid metabolite concentrations are shown in Figure 1, together with the mean endogenous *Mamld1* mRNA levels that were markedly reduced in both siRNA1- and siRNA2-transfected MLTCs at the time of steroid metabolite measurements. The concentrations of pregnenolone and progesterone remained comparable between the culture media with siRNA-transfected MLTCs and those with non-targeted MLTCs, whereas the concentrations of 17-OH pregnenolone, 17-OH progesterone, dehydroepiandrosterone, androstenedione, and T were significantly lower in the culture media with siRNA-transfected MLTCs than in those with non-targeted MLTCs. Furthermore, comparison of the steroid metabolite concentrations in the media with non-targeted MLTCs confirmed revealed the Δ^4-pathway dominant T production, markedly low 17α-hydroxylase activity and well preserved 17/20 lyase activity for both Δ^4- and Δ^5-pathways, and extremely low Hsd17b3 activity in MLTCs. These results indicated that *Mamld1* knockdown further reduced 17α-hydroxylase activity that was originally low in MLTCs.

Gene expression analyses

Real-time RT-PCR and microarray analyses showed significantly decreased *Cyp17a1* expression (~70%) in both siRNA1- and siRNA2-transfected MLTCs (Figure 2). Although *Cyp11a1* and *Hsd3b1* expressions were found to be reduced in siRNA1-transfected MLTCs by real-time RT-PCR and microarray analyses respectively, such reduced activities were not reproduced in siRNA2-transfected MLTCs. The siRNAs knockdown did not affect the expressions of *Nr5a1* (*Sf1*), *Star*, *Por*, and *Insl3*. The assessment of *Hsb17b3* was impossible, because of its extremely low expression.

In addition, 47 genes including a Notch-related gene *Hey1* were significantly up-regulated (fold change >2.0×) and 38 genes were significantly down-regulated (fold change <0.5×) in both siRNA1- and siRNA2-transfected MLTCs (Table S1 and Table S2). However, *Mamld1* knockdown had no discernible effect on the *Hes3* expression level (siRNA1: fold change 0.92, $P=0.80$; siRNA2: fold change 1.43, $P=0.35$). The microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE26913 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26913).

Cell proliferation assays

The results are shown in Figure 3. The mean endogenous *Mamld1* mRNA levels were sufficiently suppressed for 120 hours in both siRNA1- and siRNA2-transfected MLTCs. Under this condition, the absorbance values for the siRNA-targeted and non-targeted MLTCs showed a roughly linear increase until 72 hours (absorbance ~2.0). In this linear proliferative phase, although the absorbance values were significantly decreased in siRNA2-treated MLTCs at 24 and 48 hours after the transfection,

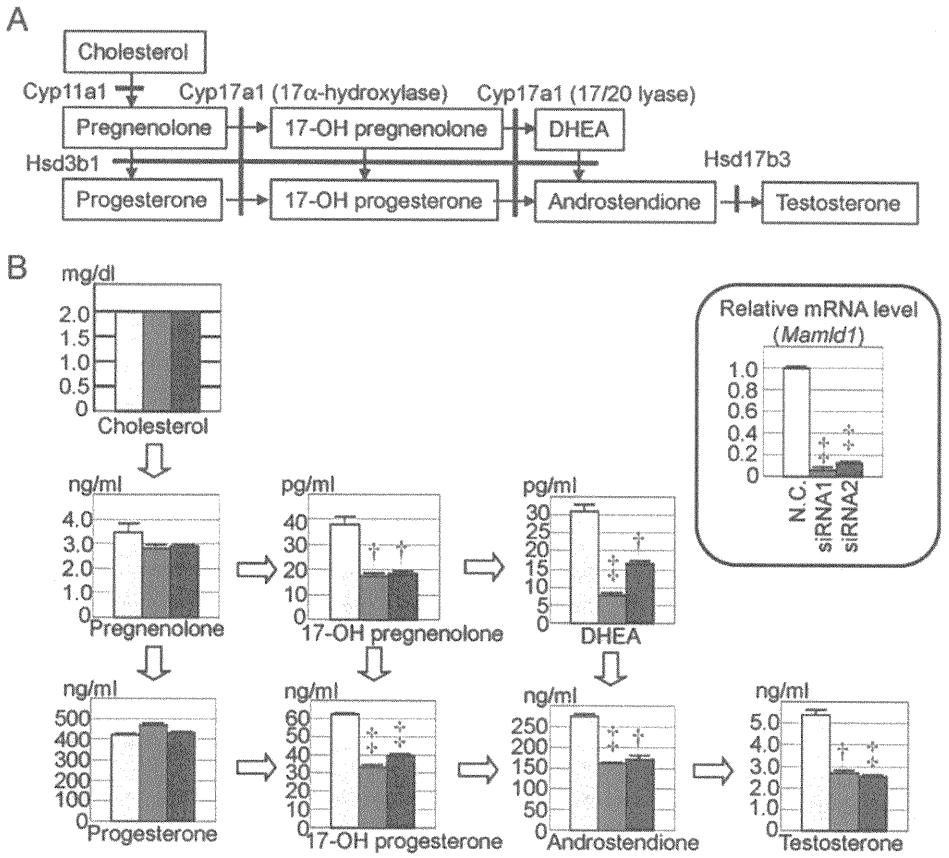

Figure 1. Steroid metabolite concentrations. A. Steroid metabolic pathway from cholesterol to testosterone and enzymes involved in each conversion. Pregnenolone, 17-OH pregnenolone, and DHEA (dehydroepiandrosterone) are Δ^4-steroid metabolites (Δ^4-pathway), and progesterone, 17-OH progesterone, and androstenedione are Δ^5-steroid metabolites (Δ^5-pathway). Hsd3b1 also functions as $\Delta^{5\,4}$ isomerase. B. Steroid metabolite concentrations in culture media and endogenous *Mamld1* expression levels in MLTCs. The yellow, the green, and the blue bars indicate the data obtained from MLTCs transfected with non-targeting RNA, siRNA1, and siRNA2, respectively. †: $P<0.01$; and ‡: $P<0.001$. The conversion factor to the SI unit: cholesterol 0.026 (mmol/L), pregnenolone 3.16 (nmol/L), progesterone 3.18 (nmol/L), 17-OH pregnenolone 3.00 (pmol/L), 17-OH progesterone 3.03 (nmol/L), DHEA 3.46 (pmol/L), androstenedione 3.49 (nmol/L), and testosterone 3.46 (nmol/L).
doi:10.1371/journal.pone.0019123.g001

this was not reproduced in siRNA1-transfected MLTCs. After 72 hours of incubation, the MLTCs became confluent, and the absorbance values became a plateau phase around ~2.0. In this plateau phase, although the absorbance values at 96 hours after the transfection were significantly low in both siRNA1- and siRNA2-treated MLTCs, this was not reproduced at 120 hours after the transfection.

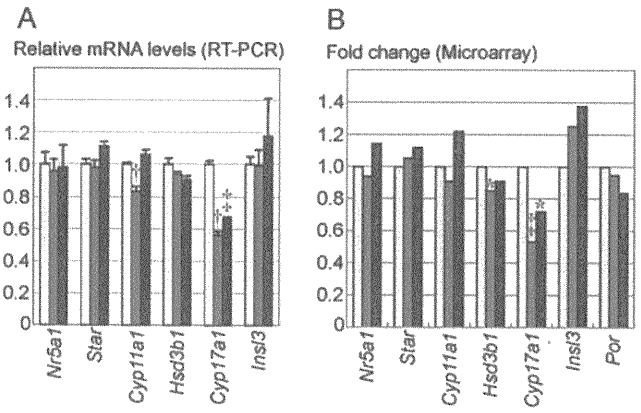

Figure 2. Gene expression analysis. The yellow, the green, and the blue bars indicate the data obtained from MLTCs transfected with non-targeting RNA, siRNA1, and siRNA2, respectively. *: $P<0.05$; †: $P<0.01$; and ‡: $P<0.001$. A. Real-time RT-PCR analysis. B. Microarray analysis.
doi:10.1371/journal.pone.0019123.g002

Discussion

Mamld1 knockdown with two siRNAs resulted in compromised T production, together with reduced 17α-hydroxylase activity and *Cyp17a1* expression in MLTCs. This provides further support for a positive role of *Mamld1* in T production [4], and implies for the first time a possible interaction between *Mamld1* and *Cyp17a1*, at least in MLTCs. In this regard, it is noteworthy that *Mamld1* is clearly expressed in fetal Leydig and Sertoli cells and is barely expressed in adrenal cells [1,13], and that *Cyp17a1* expression is indispensable for T production in Leydig cells [14]. Thus, it appears likely that *Mamld1* enhances *Cyp17a1* expression primarily in Leydig cells, permitting the production of a sufficient amount of T for male sex development. In addition, since the expressions of other genes involved in T production and insulin-like 3 biosynthesis were not clearly affected in siRNA-transfected MLTCs, this would argue against the possibility that *Mamld1* knockdown causes a global dysfunction of MLTCs, resulting in T hyposecretion.

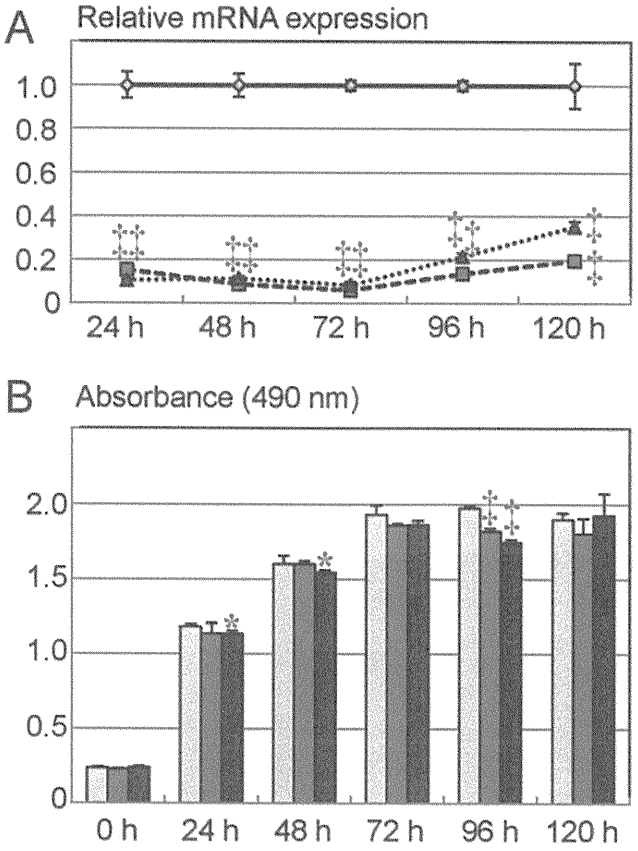

Figure 3. Cell proliferation assay. The yellow, the green, and the blue line graphs and bars indicate the data obtained from MLTCs transfected with non-targeting RNA, siRNA1, and siRNA2, respectively. *: $P<0.05$; and ‡: $P<0.001$. A. Endogenous *Mamld1* expression levels. B. Absorbance values.
doi:10.1371/journal.pone.0019123.g003

However, a straightforward explanation appears to be difficult between impaired 17α-hydroxylase activity and reduced *Cyp17a1* expression. Indeed, 17/20 lyase activity was well preserved in siRNA-transfected MLTCs, although the same Cyp17a1 enzyme is utilized for both17α-hydroxylase and 17/20 lyase reactions [14]. In addition, defective 17α-hydroxylase activity occurred in the presence of ~70% of *Cyp17a1* expression, despite 17α-hydroxylase deficiency being an autosomal recessive disease in which 50% of enzyme reduction has no major effect on the steroid metabolism [14]. In this context, it is notable that MLTCs originally have a markedly low 17α-hydroxylase activity and a well preserved 17/20 lyase activity for both Δ^4- and Δ^5-pathways (Figure 1) [6]. Such a unique property of MLTCs may be relevant to the preferential impairment of 17α-hydroxylase activity in siRNA-transfected MLTCs.

Mamld1 knockdown had no discernible effect on the *Hes3* expression. In addition, while a Notch-related gene *Hey1* [15–17] was up-regulated in siRNA-transfected MLTCs, there are no data suggesting a possible interaction between *Hes3* and *Hey1* in the T production process. Furthermore, while *Hes3* is weakly expressed in the MLTCs [4], *Hes3* expression is apparently absent from mouse fetal gonads around the critical period for sex development [18]. Thus, it is unlikely that *Hes3*-related non-canonical Notch signaling underlies a link between *Mamld1* and *Cyp17a1*. In addition, while microarray analysis revealed multiple up-regulated and down-regulated genes in siRNA-transfected MLTCs, none of them is known to be involved in the T production at present. It remains to be clarified, therefore, how *Mamld1* enhances *Cyp17a1* expression and T production.

The cell proliferation analysis revealed no clear evidence for the reduced number of viable MLTCs transfected with siRNAs. This implies that the reduced T and several other steroid metabolite concentrations observed at 48 hours after the transfection (Figure 1) are inexplicable by impaired proliferation of MLTCs. However, since the cell doubling time of MLTCs is 35–40 hours [8], a slight difference in cell proliferation would not be detected by the present analysis. Thus, it might remain tenable at this time that impaired cell proliferation becomes discernible after multiple cell divisions, and that such a possibly reduced cell proliferation underlies the development of hypospadias phenotype in patients with *MAMLD1* mutations, in addition to compromised T production in Leydig cells.

In summary, the present study implies that *Mamld1* enhances *Cyp17a1* expression primarily in Leydig cells and permit to produce a sufficient amount of T for male sex development, independently of the *Hes3*-related non-canonical Notch signaling. Although the data were obtained from *in vitro* studies using MLTCs, they provides a useful clue to clarify the underlying factors for the development of hypospadias and other forms of 46,XY DSD.

Supporting Information

Figure S1 Cell proliferation assay by the colorimetric method, using non-transfected MLTCs. The absorbance value is well correlated with cell number until the absorbance value of ~2.0, but does not reflect the cell number after the absorbance value of ~2.0.
(TIF)

Table S1 List of up-regulated genes in MLTCs trasnfected with siRNAs for *Mamld1*.
(DOC)

Table S2 List of down-regulated genes in MLTCs trasnfected with siRNAs for *Mamld1*.
(DOC)

Author Contributions

Conceived and designed the experiments: MF KN TO. Performed the experiments: MN MF FS MM. Analyzed the data: MN MF. Contributed reagents/materials/analysis tools: MF TO. Wrote the paper: TO.

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Proximal Promoter of the Cytochrome P450 Oxidoreductase Gene: Identification of Microdeletions Involving the Untranslated Exon 1 and Critical Function of the SP1 Binding Sites

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Context: *POR* (cytochrome P450 oxidoreductase) is a ubiquitously expressed gene encoding an electron donor to all microsomal P450 enzymes and several non-P450 enzymes. *POR* mutations cause an autosomal recessive disorder characterized by skeletal dysplasia, adrenal dysfunction, and disorders of sex development. Although recent studies have indicated the presence of a CpG-rich region characteristic of housekeeping genes around the untranslated exon 1 (exon 1U) and a tropic effect of thyroid hormone on *POR* expression via thyroid hormone receptor-β, detailed regulatory mechanisms for the *POR* expression remain to be clarified.

Objective: Our objective was to report a pivotal element of the proximal promoter of *POR*.

Results: We first studied three patients (cases 1–3) with POR deficiency due to compound heterozygosity with an p.R457H mutation and transcription failure of an apparently normal allele, by oligoarray comparative genomic hybridization and serial direct sequencing of the deletion fusion points. Consequently, a 2,487-bp microdeletion involving exon 1U was identified in case 1 and an identical 49,604-bp deletion involving exon 1U and exon 1 was found in cases 2 and 3. We next analyzed the 2,487-bp region commonly deleted in cases 1–3 by *in silico* analysis, DNA binding analysis, luciferase assays, and methylation analysis. The results showed a critical function of the evolutionally conserved SP1 binding sites just upstream of exon 1U, especially the binding site at the position −26/−17, in the transcription of *POR*.

Conclusions: The results suggest that the SP1 binding sites constitute an essential element of the *POR* proximal promoter. (***J Clin Endocrinol Metab* 96: E1881–E1887, 2011**)

Cytochrome P450 (CYP) oxidoreductase (POR) deficiency (PORD) is a rare autosomal recessive disorder caused by mutations in the gene encoding a flavoprotein that functions as an electron donor to all microsomal P450 enzymes and several non-P450 enzymes (1–3). Salient clinical features of PORD include skeletal dysplasia referred to as Antley-Bixler syndrome, adrenal dysfunction, 46,XY and 46,XX disorders of sex development (DSD), and maternal virilization during pregnancy (1–4). Such features are primarily explained by impaired activities of POR-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2011-1337 Received April 25, 2011. Accepted August 18, 2011.
First Published Online September 7, 2011

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Abbreviations: CGH, Comparative genomic hybridization; CYP, cytochrome P450; DSD, disorders of sex development; exon 1U, untranslated exon 1; HEK, human embryonic kidney; POR, CYP oxidoreductase; PORD, POR deficiency; SL2, Schneider line 2.

dependent CYP51A1 and squalene epoxidase involved in cholesterologenesis and CYP17A1, CYP21A2, and CYP19A1 involved in steroidogenesis (1–4). Anorectal and urinary anomalies are also occasionally observed in PORD, probably due to decreased activity of CYP26 relevant to retinoic acid metabolism (5). The complete absence of POR activity is assumed to be lethal (4), and consistent with this, all the patients identified to date have at least one missense mutation that is likely to preserve some residual activity (1, 2, 6, 7). In addition, heterozygosity with one apparently normal allele has been reported in approximately 12% of PORD patients (4).

The *POR/Por* gene is transcribed ubiquitously with more or less variable expression levels among different tissues (8, 9). Consistent with the ubiquitous expression pattern, rat *Por* is known to be associated with a CpG-rich region (CpG islands) (9) characteristic of housekeeping genes (10). Similarly, human *POR* consists of a single untranslated exon 1 (exon 1U) and coding exons 1–15, and the region around exon 1U harbors a CpG-rich region (11). In addition, the SP1 binding sites as a potential proximal promoter element reside in the CpG-rich region of rat *Por* (9), whereas they have not yet been reported in the CpG-rich region of human *POR*. Furthermore, Tee *et al.* (12) have recently studied the approximately 300-bp proximal promoter region just upstream of exon 1U of human *POR*, showing that thyroid hormone exerts a major trophic effect on *POR* expression primarily via thyroid hormone receptor-β, with thyroid hormone receptor-α, estrogen receptor-α, Smad3, and Smad4 exerting lesser modulatory effects. However, the detailed regulatory mechanisms for the transcription of human *POR* remain to be clarified.

Here, we report two types of microdeletions, one involving exon 1U alone and the other involving exon 1U and exon 1, in patients with PORD and suggest a pivotal role of the SP1 binding sites in the transcriptional regulation of *POR*. The results, in conjunction with the previous data (12), provide significant progress in the clarification of the regulatory machinery for the expression of *POR*.

Patients and Methods

Patients

We examined three nonconsanguineous patients (case 1 with 46,XY and cases 2 and 3 with 46,XX) reported in our previous paper describing 35 patients with PORD (7); cases 1, 2, and 3 in this report correspond to cases 18, 26, and 27 in the previous paper, respectively. Cases 1–3 manifested Antley-Bixler syndrome-compatible skeletal features, adrenal dysfunction with

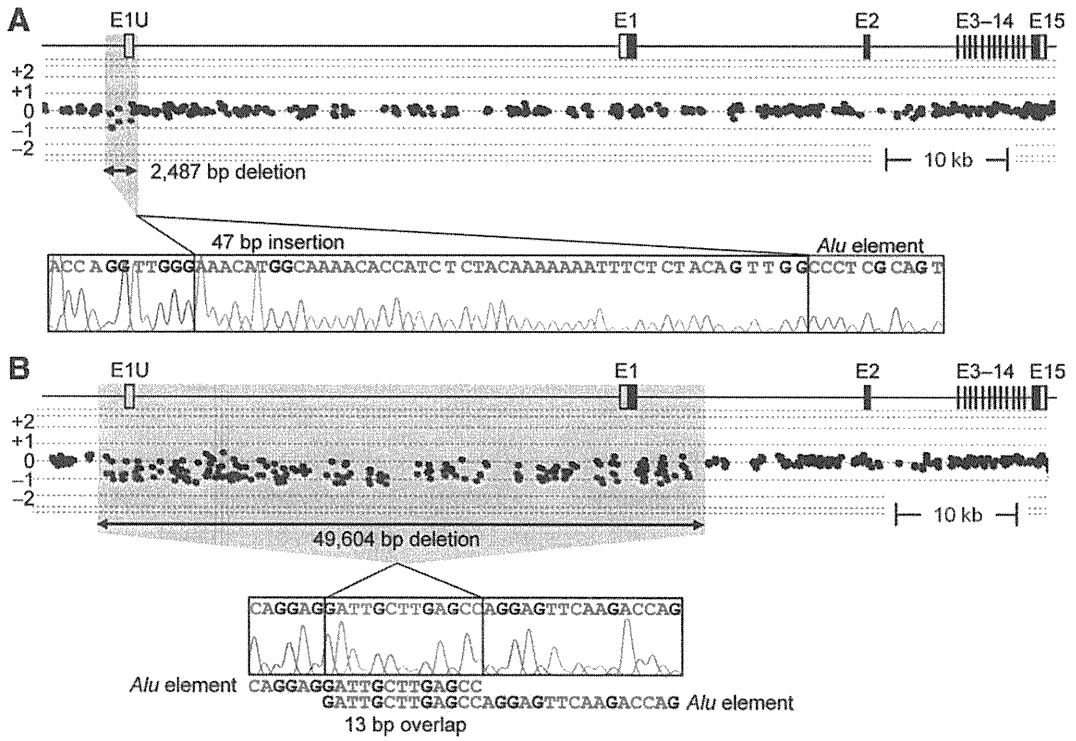

FIG. 1. Identification and characterization of the microdeletions in case 1 (panel A) and cases 2 and 3 (panel B) by CGH analysis and direct sequencing of the deletion junctions. The position of *POR* exons (E1U–E15) is shown on the CGH findings; the *black and white boxes* denote the coding regions and the untranslated regions, respectively. In the CGH results, the *black and green dots* denote signals indicative of the normal and the decreased (<−0.5) copy numbers, respectively. In the direct sequencing findings, the 47-bp segment inserted into the fusion point in case 1 is *highlighted* with *light yellow*, and the 13-bp overlapping sequence at the fusion point in cases 2 and 3 is *highlighted* with *light blue*. The *Alu* elements are indicated with *light blue bars*.

drastically compromised cortisol response to ACTH stimulation, and DSD (bilateral cryptorchidism in case 1, partial labial fusion in case 2, and mild clitoromegaly in case 3). Cases 2 and 3 also experienced adrenal crisis, whereas maternal virilization during pregnancy was not identified in cases 1–3. In addition, case 2 had right vesicoureteral reflux, and case 3 manifested imperforated anus. In cases 1–3, direct sequencing for leukocyte genomic DNA indicated apparent heterozygosity for the Japanese founder mutation p.R457H, and that for leukocyte cDNA demonstrated transcription failure of an apparently normal allele (7). Thus, although cases 1–3 were found to have compound heterozygosity for p.R457H and transcription failure, the cause of transcription failure remained to be clarified.

Primer and probe

The primers and probes used in the present study are shown in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Genome structure analysis

Oligoarray comparative genomic hybridization (CGH) was performed for leukocyte genomic DNA, using a custom-build oligo-microarray containing 39,169 probes for an approximately 8-Mb region around *POR* and 26,662 reference probes for a different genomic interval (2x105K format, design ID 022431) (Agilent Technologies, Palo Alto, CA). The procedure was as described in the manufacturer's instructions. To determine the deletion size and the junction structure, serial direct sequencing was performed for long PCR products obtained with primer pairs flanking the deleted region, and the obtained junction sequence was compared with the reference sequence at the NCBI Database (NT_007933.15). The presence or absence of repeat sequences around the breakpoints was examined with Repeatmasker (http://www.repeatmasker.org).

In silico analysis

In silico analysis was performed for CpG islands, evolutionally conserved sequences, and promoter-associated histone marks, using UCSC genome browser (http://genome.ucsc.edu/). Putative transcription factor binding sites were searched by TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). In addition, because animal *Por* has been well studied in rats (9), conservation status of identified sites was examined using rat data. The transcription start site of *POR* exon 1U (+1) was determined on the basis of the *POR* cDNA sequence (NM_000941) obtained from the NCBI database.

Luciferase assays

A series of promoter-reporter constructs were generated by inserting PCR-amplified DNA fragments into PGL3-enhancer vector or pGL3-basic vector (Promega, Madison, WI). Deletion mutants were created by site-directed mutagenesis. Transient transfection was carried out using human embryonic kidney (HEK) 293 cells with endogenous SP families, because of their stable transfection efficiency and usefulness in *in vitro* functional studies for SP1 binding sites (13). HEK 293 cells were cultured in DMEM at 37 C, seeded in 12-well dishes, and transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) with 0.6 μg of the reporter plasmids. As an internal control for the transfection, 20 ng pRL-CMV vector (Promega) was used. In addition, transient transfection was also performed using *Drosophila* Schneider line 2 (SL2) cells (CRL-1963; American Type Culture Collection, Manassas, VA) that lack endogenous SP families. SL2 cells were grown in Schneider's medium at 25 C, seeded in six-well dishes, and transfected using calcium phosphate (14) with 1.0 μg of the reporter plasmid and a total of 50 ng of various combinations of the SP1 expression vector (pPAC-SP1) and an empty pPAC vector, as well as 50 ng of the SP3 expression vector (pPAC-SP3). As an internal control for the transfection, 50 ng pPAC-β-galactosidase vector was used. For both experiments using HEK 293 cells and SL2 cells, luciferase activities were determined at 48 h after the transfections.

Transfections were performed in triplicate within a single experiment, and the experiments were repeated three times. The results are expressed as mean ± SEM, and statistical significance was examined by the t test. $P < 0.05$ was considered significant.

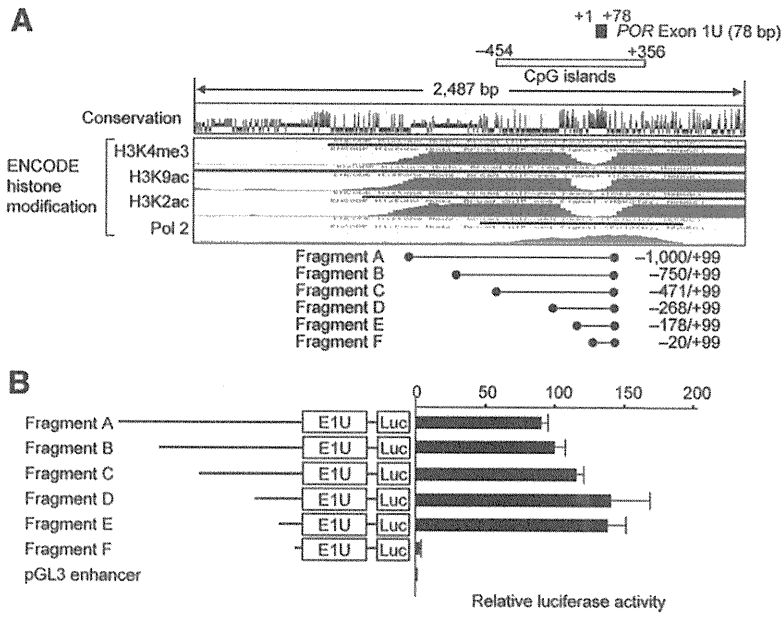

FIG. 2. Localization of the promoter region to a 178-bp segment just upstream of exon 1U. Panel A, *In silico* analysis in search of the promoter-compatible sequences. The transcription start site of *POR* exon 1U (+1) is based on the *POR* cDNA sequence at the NCBI database (NM_000941). The CpG-rich region spans from −454 to +356 bp. The ENCODE histone modification analysis indicates the presence of a highly conserved promoter-compatible sequence just upstream of exon 1U. The fragments A–F denote the DNA sequences used for the luciferase assays. Panel B, Luciferase reporter assays using the fragments A–F. The results are expressed as fold-change of the target vectors over the empty pGL3 enhancer vector (mean ± SEM). Transfections were performed in triplicate within a single experiment, and the experiments were repeated three times. Although the increase in the relative luciferase activity is significant for fragment A (92.6 ± 5.2, $P = 0.0006$), fragment B (101.6 ± 5.8, $P = 0.0006$), fragment C (106.0 ± 5.5, $P = 0.0004$), fragment D (137.7 ± 29.0, $P = 0.0009$), and fragment E (131.3 ± 13.4, $P = 0.0006$), it is not significant for fragment F (2.6 ± 1.1, $P = 0.25$).

DNA binding analysis

EMSA was performed as described previously (15). In brief, 10 μg of nuclear extracts of HEK 293 cells were incubated with ^{32}P-labeled oligonucleotides and unlabeled polydeoxyinosinic-deoxycytidylic acids and subjected to polyacrylamide gel electrophoresis (4%). For a competition experiment, a 200-fold molar excess of unlabeled competitor DNA was added. Supershift assay was performed by preincubating the nuclear extracts with anti-SP1 antisera (PEP2) and/or anti-SP3 antisera (D-20) (Santa Cruz Biotechnology, Santa Cruz, CA).

Methylation analysis

Bisulfite sequencing was performed for human leukocyte- and HEK 293-derived genomic DNA samples treated with the EZ DNA Methylation Kit (Zymo Research, Orange, CA) that converts all the cytosines except for methylated cytosines at the CpG dinucleotides into uracils and subsequently thymines. A 282-bp CpG-rich region containing SP1 binding sites just upstream of exon 1U was amplified with primer sets that hybridize to both methylated and unmethylated alleles because of absent CpG dinucleotides within the primer sequences. Subsequently, the PCR products were subcloned with the TOPO TA Cloning Kit (Life Technologies), and multiple clones were subjected to direct sequencing on the CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA).

Results

Identification and characterization of microdeletions in cases 1–3

Oligoarray CGH analysis indicated cryptic heterozygous deletions in cases 1–3 (Fig. 1). Furthermore, sequencing of the long PCR products harboring the fusion points revealed a 2,487-bp microdeletion (13,575,403–13,577,889 bp) encompassing exon 1U in case 1 and an identical 49,604-bp deletion (13,571,326–13,620,929 bp) involving exon 1U and exon 1 in cases 2 and 3. Thus, the 2,487-bp microdeletion on the noncoding upstream region was common to cases 1–3. The microdeletion in case

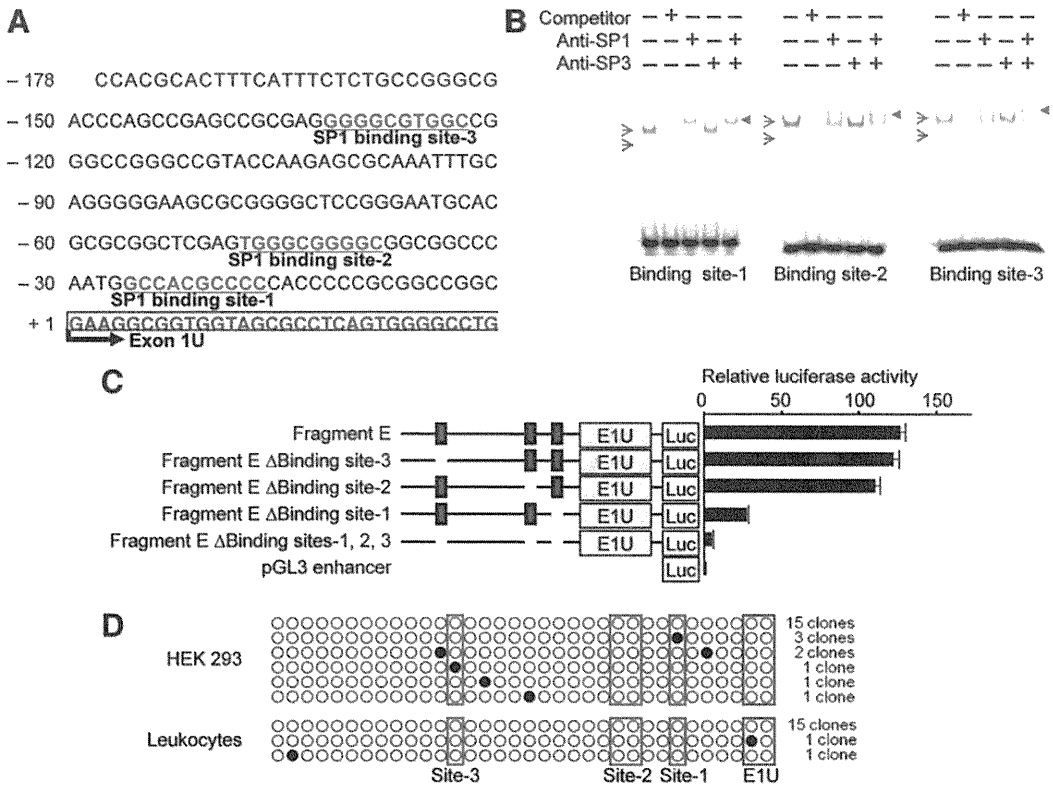

FIG. 3. Functional studies of the SP1 binding sites. Panel A, The three potential SP1 binding sites 1–3 at the position just upstream of exon 1U. The transcription start site of *POR* exon 1U (+1) is based on the *POR* cDNA sequence at the NCBI database (NM_000941). Panel B, EMSA showing positive bindings of SP1 and SP3 proteins to the SP1 binding sites 1–3. The *red arrows* indicate the strong bands derived from the SP1 protein binding to the probes containing the SP1 binding sites. These bands become weak, and supershifted bands (*red arrowheads*) are seen by adding anti-SP1. In addition, the *blue arrows* denote specific bands derived from the SP3 protein binding to the same probes. These bands become very weak by adding anti-SP3; the extremely faint supershifted bands are not visible in this figure. The band shift pattern is more obvious for SP1 protein than for SP3 protein. Panel C, Luciferase reporter assays using fragment E and its deletion mutants. The results are expressed as fold change of the target vectors over the empty pGL3 enhancer vector (mean ± SEM). Transfections were performed in triplicate within a single experiment, and the experiments were repeated three times. Although the relative luciferase activity is similar between Fragment E (121.8 ± 3.4) and ΔBinding site-3 (117.8 ± 3.1) ($P = 0.22$), it is significantly different between Fragment E and ΔBinding site-2 (105.7 ± 3.5) ($P = 0.015$), ΔBinding site-1 (25.8 ± 1.2) ($P = 0.0007$), and ΔBinding site-1, -2, and -3 (5.2 ± 0.5) ($P = 0.0004$). Panel D, Methylation analysis of the CpG-rich region. Each *circle* denotes a CpG island, and *filled* and *open circles* represent methylated and unmethylated cytosines, respectively. The CpG dinucleotides within the exon 1U are surrounded by *blue squares*, and those within the SP1 binding sites 1, 2, and 3 by *red squares*.

1 occurred between an *Alu* element and a nonrepeat sequence and was associated with an addition of a 47-bp segment of unknown origin, whereas that in cases 2 and 3 occurred between two *Alu* elements with an overlap of a 13-bp segment.

Critical function of the SP1 binding sites

In silico analysis for the noncoding 2,487-bp region showed an 810-bp long CpG-rich region involving exon 1U, an approximately 350-bp long evolutionally conserved sequence-rich region encompassing exon 1U, and an approximately 1.3-kb region with promoter-associated histone marks (Fig. 2A). The TATA box was not identified. Thus, relative luciferase activity was examined for fragments A–F with various lengths of the candidate promoter region, localizing a critical sequence for the *POR* promoter to a 178-bp segment defined by fragment E and fragment F (Fig. 2B).

The 178-bp segment was found to harbor three SP1 binding sites, *i.e.* site 1 at the position −26/−17, site 2 at the position −48/−39, and site 3 at the position −132/−123 (Fig. 3A). The three binding sites were well conserved in rats. EMSA indicated specific binding of SP1 and SP3 proteins to the three binding sites, with the band shift pattern being more obvious for SP1 protein than for SP3 protein (Fig. 3B). Deletion of the binding site 1 and the binding site 2 significantly reduced the relative luciferase activity (by ~80 and ~15%, respectively), although deletion of the binding site 3 had no significant effect on the relative luciferase activity; furthermore, loss of the binding sites 1–3 virtually abolished the relative luciferase activity (Fig. 3C). The 282-bp segment containing the three SP1 binding sites was almost completely unmethylated (Fig. 3D).

Furthermore, relative luciferase activity was examined for a 170-bp fragment (−120/+50) harboring the SP1 binding site 1 and the SP1 binding site 2, using SL2 cells devoid of endogenous SP families. Relative luciferase activity was clearly increased in a dose-dependent manner by adding the *SP1* expression vector but was barely elevated by adding the *SP3* expression vector (Fig. 4).

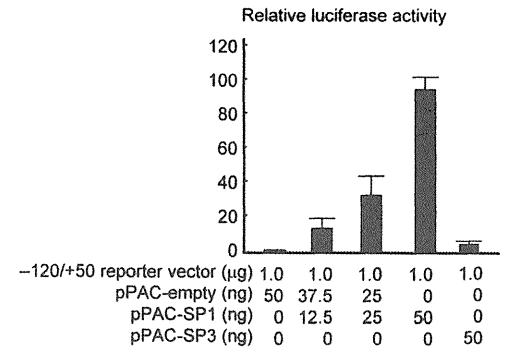

FIG. 4. Luciferase assays of a fragment containing the SP1 binding sites 1 and 2, using SL2 cells lacking endogenous SP families. The results are expressed as fold change of the target vectors over the empty pPAC vector (mean ± SEM). Transfections were performed in triplicate within a single experiment, and the experiments were repeated three times. The relative luciferase activity is significantly increased by adding the *SP1* expression vector of 12.5 ng (14.7 ± 4.4) ($P = 0.037$), 25.0 ng (31.8 ± 7.6) ($P = 0.035$), and 50 ng (95.8 ± 7.1) ($P = 0.0002$), although it is barely elevated by adding the *SP3* expression vector of 50 ng (5.2 ± 1.5) ($P = 0.054$).

Discussion

We identified two types of cryptic deletions, one involving exon 1U alone and the other encompassing exon 1U and exon 1, in three cases with PORD. The microdeletion in case 1 is explained by nonhomologous end joining that occurs between nonhomologous sequences and is frequently accompanied by an insertion of a short segment at the fusion point (16). The microdeletion in cases 2 and 3 is compatible with a repeat sequence mediated nonallelic intrachromosomal or interchromosomal recombination (16). Although cases 2 and 3 were apparently nonconsanguineous, it would not be unexpected that the same repeat-mediated genomic rearrangement took place in unrelated individuals. Notably, because the apparently normal allele in cases 1–3 was not transcribed (7), this implies that the 2,487-bp microdeletion common to cases 1–3 has affected the promoter function for *POR*. In this context, because approximately 12% of patients with PORD are known to be heterozygotes with one apparently normal *POR* allele (4), it might be possible that some, if not all, of them have similar microdeletions or other genetic aberrations affecting the *POR* transcription.

The present study revealed a pivotal role of the SP1 binding sites, especially the binding site 1, in the transcription of *POR*. This implies that the SP1 binding sites constitute an essential element of the *POR* proximal promoter. Indeed, SP1 binding sites as well as other noncore promoter elements are usually located in multiple copies within the proximal promoter region (~250 bp upstream of the transcription initiation site) of a ubiquitously expressed gene like *POR* (10). In this regard, several findings are noteworthy. First, the TATA box was apparently absent from the *POR* promoter region. This is compatible with the ubiquitous expression of *POR*, because the TATA box is usually identified in genes with a tissue-specific expression pattern (10). Second, the SP1 binding sites were highly conserved between the human and the rat. This finding, in conjunction with the previous data indicating absence of polymorphism for the three SP1 binding sites in 842 individuals (17), implies that the wild-type sequences of the SP1 binding sites are indispensable for the regulation of *POR* transcription. Third, the functional

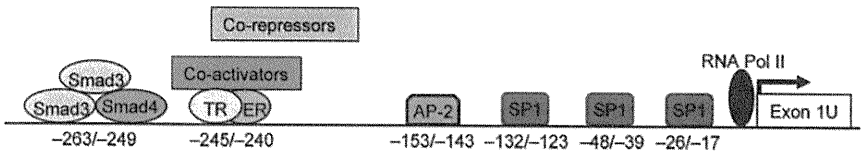

FIG. 5. Schematic representation indicating the binding sites for various factors in the proximal promoter region of *POR*. The diagram of the promoter upstream of −143 has been taken from Tee *et al.* (12). ER, Estrogen receptor; Pol II, polymerase II; TR, thyroid hormone receptor; AP-2, activator protein 2.

data using SL2 cells indicated a major role of SP1, rather than SP3, in the *POR* transcription. This is consistent with the notion that although both SP1 and SP3 can bind to the same cognate SP1 binding site, the DNA binding properties and regulatory functions are quite different between SP1 and SP3, depending on the promoter context and the cell type (18). Lastly, the SP1 binding sites were almost completely unmethylated. This argues for a transcriptionally active status of *POR*, because SP1 protein binding is known to be reduced when the CpG-rich region around the SP1 binding sites is methylated (19).

The proximal promoter region of *POR* has been studied previously (11, 12). Scott *et al.* (11) analyzed the 5′ region of *POR* coding exons by means of comparative genomics and characterized human *POR* exon 1U and its flanking sequences. Subsequently, Tee *et al.* (12) examined a 361-bp region around the transcription start site of exon 1U (−325/+36) using adrenal NCI-H295A and liver Hep-G2 cells and found a major trophic effect of thyroid hormone on *POR* expression primarily via thyroid hormone receptor-β as well as modulatory effects of thyroid hormone receptor-α, estrogen receptor-α, Smad3, and Smad4 on *POR* expression. The binding sites for these factors reside in a −263/−240 region upstream of the SP1 binding sites (Fig. 5). Furthermore, Tee *et al.* (12) screened functional alterations of polymorphisms within the 325-bp region, suggesting that the common −152C→A polymorphism may play a certain role in the genetic variation of steroid biosynthesis and drug metabolism. In this regard, whereas the −152C→A polymorphism resides on the AP-2 (activator protein 2) binding site, the functional difference of the polymorphism is obviously independent of the recruit of AP-2 (12). Thus, the underlying factors for the reduced activity of the −152A allele remain to be clarified.

Taken together, multiple regulatory elements have been identified in the proximal promoter region of *POR* (Fig. 5). Although the regulatory machinery has not yet been fully elucidated, we suggest that the presence of the SP1 binding sites has permitted the ubiquitous expression of *POR* and that the presence of other sites including thyroid hormone receptors is relevant to the variability in *POR* expression level among different tissues. In this regard, although the present study failed to identify the effects of the −263/−240 regulatory sequence identified by Tee *et al.* (12) (fragment D *vs.* fragment E in Fig. 2), this may be due to the difference in the cell type and/or in the promoter-luciferase construct used in the study by Tee *et al.* (+36) and in this study (+99). In addition, the hormonal effects on the *POR* transcription have not been examined in this study.

Finally, it would be useful to refer to clinical phenotypes of cases 1–3. In this context, we have previously compared clinical phenotype between Japanese PORD patients with homozygosity for the hypomorphic p.R457H mutation (group A) and those with compound heterozygosity for p.R457H and one apparently null mutation including nonsense and frameshift mutations (group B) and found that skeletal features are definitely more severe and adrenal dysfunction and 46,XY DSD are somewhat more severe in group B than in group A, whereas 46,XX DSD, maternal virilization during pregnancy, and anorectal and urinary anomalies are similarly identified in the two groups (5, 7). It is likely, therefore, that the residual POR activity reflected by the p.R457H dosage constitutes the underlying factor for clinical variability in some features but not in other features, probably due to the simplicity and complexity of POR-dependent metabolic pathways relevant to each phenotype. The clinical features of cases 1–3 are quite comparable to those of group B patients and, therefore, are consistent with transcription failure of one allele being a null mutation.

In summary, we identified microdeletions involving exon 1U and its upstream region in PORD patients, and revealed the critical function of the SP1 binding sites in the transcription of *POR*. Additional studies will permit to elucidate the regulatory machinery for *POR* expression.

Acknowledgments

We thank Dr. Timothy F. Osborne, University of California, Irvine, CA, and Dr. Guntram Suske, Philipps Universität, Marburg, Germany, for providing us with pPAC-β-galactosidase and pPAC-SP1/Sp3, respectively.

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This work was supported by the Grant-in-Aid for Scientific Research on Innovative Areas (22132004) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT); by the Grant-in-Aid for Scientific Research (S) (22227002) from

the Japan Society for the Promotion of Science (JSPS); by the Grants for Research on Intractable Diseases (H22-098) and for Health Research on Children, Youth, and Families (H21-005) from the Ministry of Health, Labor and Welfare; by the Grant from Japan Foundation for Pediatric Research (10-001); and by the Grant of National Center for Child Health and Development (23A-1).

Disclosure Summary: The authors have nothing to declare.

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Original Article

Sexual Development

Sex Dev 330122
DOI: 10.1159/000330122

Accepted: June 3, 2011
by M. Schmid
Published online: ■■■

Association of Primary Ovarian Insufficiency with a Specific Human Leukocyte Antigen Haplotype (A*24:02-C*03:03-B*35:01) in Japanese Women

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Key Words
Association study · Haplotype analysis · Human leukocyte antigen · Primary ovarian insufficiency · Susceptibility

Abstract
Primary ovarian insufficiency (POI) is a heterogeneous condition defined by the triad of oligo/amenorrhea, elevated gonadotropins and estrogen deficiency in women under the age of 40 years. Although autoimmune abnormalities appear to be involved in the development of POI, there are only a few studies with respect to human leukocyte antigen (HLA). The objective of this study was to identify an HLA allele(s) and/or haplotype(s) constituting a susceptibility factor(s) for POI. We examined 83 Japanese women with apparently idiopathic isolated POI. For controls, Japanese HLA reference data registered in the HLA Laboratory were utilized. No significant association was found for a total of 94 alleles for HLA-A, B, C, DRB1, and DQB1 loci, after both stringent Bonferroni correction and less stringent Benjamini-Hochberg (B-H) correction for multiple comparisons. By contrast, of 86 haplotypes identified for MHC class I (HLA-A, B, and C) and 31 haplotypes detected for MHC class II (HLA-DRB1 and DQB1), a single haplotype (A*24:02-C*03:03-B*35:01) remained significant after Bonferroni and B-H corrections (frequency: 4.82% in women with POI and 1.06% in the control data; p = 0.00049). The results imply that a specific HLA haplotype (A*24:02-C*03:03-B*35:01) constitutes a susceptibility factor for apparently isolated POI in Japanese women.

Primary ovarian insufficiency (POI), or premature ovarian failure, is a heterogeneous condition defined by the triad of oligo/amenorrhea, elevated gonadotropins and estrogen deficiency in women under the age of 40 years [Kalantaridou et al., 1998]. While POI is frequently observed in women with sex chromosome aberrations, it also occurs in women with normal karyotype [Laml et al., 2000]. In this regard, although underlying factors for POI remain to be elucidated in most women with normal karyotype, various genetic and environmental factors have been implicated in the development of POI in such women. Indeed, mutations of several genes including *BMP15* [Di Pasquale et al., 2004], *NOBOX* [Qin et al., 2009] and *NR5A1* (alias *SF-1* and *AD4BP*) [Lourenço et al., 2009] are known to cause POI, as well as premutations

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of *FMR1* [Cronister et al., 1991]. In addition, several candidate genes such as *LHX8* [Qin et al., 2008] and *GDF9* [Kovanci et al., 2007] have been identified to date. Furthermore, endocrine disruptors, chemotherapy, radiation, smoking, and viral infections also constitute risk factors for POI [Morrison et al., 1975; Howell and Shalet, 1998; Sharara et al., 1998; Di Prospero et al., 2004].

Autoimmune abnormalities also appear to be involved in the development of POI in women with normal karyotype. Indeed, POI can occur as a part of autoimmune polyendocrine syndromes [Kauffman and Castracane, 2003]. Moreover, women with POI occasionally have autoimmune hypothyroidism, type 1 diabetes mellitus (T1DM), hypoadrenalism, myasthenia gravis and systemic lupus erythematosus and, conversely, women with autoimmune disorders occasionally exhibit POI [Moncayo-Naveda et al., 1989; Belvisi et al., 1993; Bakalov et al., 2002; Ryan and Jones, 2004]. Non-specific autoantibodies such as antinuclear antibodies are often identified in women with POI [Ishizuka et al., 1999], as are organ-specific autoantibodies such as anti-thyroid autoantibodies [Belvisi et al., 1993] and steroid-producing cell autoantibodies [Betterle et al., 1993].

Human leukocyte antigen (HLA) of the major histocompatibility complex (MHC) plays an essential role in the human immune system. Consistent with this, specific HLA alleles or haplotypes are known to constitute susceptibility factors for several autoimmune disorders such as T1DM [Todd et al., 1988]. For POI, however, there are only a few studies with respect to HLA, and there is no haplotype association study. Thus, we performed HLA allele and haplotype analyses in Japanese women with POI. Here, we focused on apparently idiopathic isolated POI as a relatively homogeneous group, because POI with associated autoimmune abnormalities may represent genetically heterogeneous disorders.

Subjects and Methods

Subjects

This study consisted of 83 Japanese women with apparently idiopathic isolated POI who satisfied the following selection criteria: (1) lack of somatic abnormalities; (2) absence of clinically discernible autoimmune diseases; (3) no history of chemotherapy or radiation; (4) 46,XX karyotype in all the ≥30 lymphocytes examined; (5) no demonstrable mutations in *BMP15, NOBOX, NR5A1, LHX8*, and *GDF9*, and (6) no *FMR1* premutations. Two women were familial cases with a similarly affected sister or mother, and the remaining 81 women were sporadic cases. Organ-specific autoantibodies such as anti-thyroid autoantibodies as well as non-specific antinuclear antibodies were not detected in 60 patients examined, although they were not examined in the remaining 23 patients. The menarchial age ranged from 10 to 15 years (median, 13 years) (menarchial age in normal Japanese girls, 12.25 ± 1.25 years), and the age of POI onset (amenorrhea persisting ≥6 months) ranged from 13 to 39 years (median, 31 years). Serum FSH was above 40 IU/l and estradiol was undetectable or below the normal range on at least 2 occasions. Serum testosterone was undetectable, and polycystic ovary was excluded by abdominal ultrasound studies.

Thus, the following patients with overt autoimmune diseases were excluded from the present study: (1) ten patients with Hashimoto thyroiditis accompanied by anti-thyroid peroxidase autoantibodies; (2) one patient with Basedow disease; (3) one patient with Sjögren syndrome; (4) two patients with systemic lupus erythematosus, and (5) two patients with anti-phospholipid antibody syndrome.

Human Leukocyte Antigen Analysis

This study was approved by the Institutional Review Board Committees of the investigators' affiliations. After obtaining written informed consent, genotyping was performed for HLA-A, B, C, DRB1, and DQB1 loci by the polymerase chain reaction-based sequence-specific oligonucleotide probe method, using leukocyte genomic DNA samples. Haplotype inference was performed for MHC class I (HLA-A, B, and C) and class II (DRB1 and DQB1) by the maximum likelihood method using expectation maximization algorithm [Excoffier and Slatkin, 1995] implemented in the software LDSUPPORT [Kitamura et al., 2002]. For controls, we utilized Japanese HLA reference data registered in the HLA Laboratory (http://www.hla.or.jp/) that have been obtained from 14,631 control subjects for HLA-A, B, and DRB1 loci, 8,240 control subjects for HLA-C locus and MHC class I haplotype, and 2,934 control subjects for HLA-DQB1 locus and MHC class II haplotype. The sex ratio was nearly equal in the control subjects, and HLA allele and haplotype patterns have been shown to be comparable between men and women control subjects (http://www.hla.or.jp/).

Statistical Analysis

Allele and haplotype frequencies were compared between women with POI and control subjects by the Fisher's exact probability test, using R environment version 2.7.1 (http://www.r-project.org/). The odds ratio and the 95% confidence interval were also calculated using the same environment. To address the problem of multiple comparisons, the Bonferroni correction and the Benjamini-Hochberg (B-H) correction were employed in this study [Shaffer, 1995]. The Bonferroni correction is the most stringent method that sets the corrected significance level by dividing the empirical significance level (0.05) by the number of tests, and corrected p values by multiplying the observed p values by the number of tests. The B-H correction is a subsequently developed less stringent method that defines the significance level by multiplying the Bonferroni significance level by the frequency order of observed p values, and corrected p values by dividing the Bonferroni corrected p values by the frequency order of observed p values. In both corrections, observed p values are regarded as significant when corrected p values remain below the empirical significance level (0.05). Since individual HLA genotyping data are not available in the control reference data, it was impossible to perform the permutation test that is frequent-

Table 1. Representative results of the association studies between POI and each HLA allele ($p < 0.05$)

Allele	Frequency, %		Statistical analysis			Bonferroni correction		Benjamini-Hochberg correction		
	women with POI	control subjects	p value	odds ratio	95% CI	significance level	corrected p	significance level	corrected p	frequency order of p value
B*27:07	0.60	0.00	0.0056	530.4	21.51–13,080	0.0017	0.16	0.0017	0.16	1
B*35:03	0.60	0.00	0.0056	530.4	21.51–13,080	0.0017	0.16	0.0017	0.16	1
B*51:02	1.20	0.19	0.034	6.60	1.59–27.29	0.0017	1.00	0.0069	0.25	4
B*55:04	1.20	0.13	0.0071	9.38	2.24–39.21	0.0017	0.21	0.0052	0.07	3
C*15:05	0.60	0.02	0.043	24.96	2.77–224.70	0.0033	0.65	0.0033	0.64	1
DRB1*04:01	3.01	0.94	0.019	3.29	1.34–8.07	0.0023	0.42	0.0023	0.41	1
DRB1*12:02	4.22	1.82	0.045	2.38	1.11–5.09	0.0023	0.99	0.0068	0.33	3
DRB1*14:01	6.63	3.36	0.035	2.04	1.11–3.78	0.0023	0.77	0.0046	0.38	2

CI = Confidence interval.

ly utilized to estimate significant p values in multiple comparisons [Shaffer, 1995].

The power ($1 - \beta$ [type II error]: the probability of rejecting a false null hypothesis) was estimated using GDesignPlus (StaGen Co., Ltd.). The power of >50% is usually required to approve the results obtained in a pilot study like the present study, while the power of >80% is usually necessary to admit the results obtained in a replication study [Cohen, 1988]. Furthermore, false positive report probability (FPRP), the probability of no true association between a genetic variant and disease given a statistically significant finding, was also calculated using α (the significance level), power ($1 - \beta$), and π (assumed frequency of susceptibility allele or haplotype; in this study, π was assumed as 0.02) [Wacholder et al., 2004]. The FPRP of <5% is usually regarded as verifying the quality of the obtained data.

Results

HLA genotyping identified 16 alleles for the HLA-A locus, 29 alleles for HLA-B, 15 alleles for HLA-C, 22 alleles for HLA-DRB1, and 12 alleles for HLA-DQB1 in women with POI (online suppl. table 1; for all suppl. material, see www.karger.com/doi/10.1159/000330122). While low p values ($p < 0.05$) were identified for 8 of these alleles (table 1), none of them were significant after the Bonferroni and B-H corrections.

Haplotype estimation indicated 86 haplotypes for MHC class I and 31 haplotypes for MHC class II in women with POI (online suppl. table 2). Low p values ($p < 0.05$) were identified for 34 of these haplotypes (table 2), and one of them (A*24:02-C*03:03-B*35:01) remained significant after the Bonferroni and B-H corrections. This haplotype was invariably present in a heterozygous condition, and accounted for 4.82% of haplotypes in women with POI (the 2nd most frequent haplotype in this group) and 1.06% of haplotypes in the control subjects (the 15th most common haplotype in this group). The power was calculated as 72.2%, and the FPRP was 3.8%. Notably, each of the A*24:02, C*03:03, and B*35:01 alleles constituting the specific haplotype was fairly common in both women with POI and control subjects and was identified with similar frequencies (online suppl. table 1). There was no haplotype that was regarded as significant by the B-H correction but not by the Bonferroni correction.

Discussion

We studied Japanese patients with apparently idiopathic isolated POI who had no associated overt autoimmune diseases. This selection was performed to reduce genetic heterogeneity among patients. Indeed, POI in patients with Sjögren syndrome and systemic lupus erythematosus may represent specific groups of POI, as well as POI in patients with single gene diseases such as autoimmune polyendocrine syndromes [Kauffman and Castracane, 2003]. Similarly, thyroid disease-associated POI may also exhibit a specific group of POI. Thus, we excluded such patients from this study. It should be pointed out, however, that not all patients with POI received examination of autoantibodies, and that several patients may have subclinical autoimmune diseases or may develop autoimmune diseases at later ages.

The present study revealed significant association between apparently isolated POI and a specific HLA haplotype (A*24:02-C*03:03-B*35:01) in Japanese women. In this regard, since the frequency of each allele for the specific haplotype was similar between women with POI and

Table 2. Representative results of the association studies between POI and each HLA haplotype ($p < 0.05$)

Haplotype	Frequency, %		Statistical analysis			Bonferroni correction		Benjamini-Hochberg correction		
	women with POI	control subjects	p value	odds ratio	95% CI	significance level	corrected p	significance level	corrected p	frequency order of p value
A*02:01-C*01:02-B*54:01	2.41	0.73	0.036	3.37	1.23–9.23	0.00058	1.00	0.01	0.13	23
A*02:01-C*03:03-B*55:04	1.20	0.07	0.0083	16.74	3.72–75.40	0.00058	0.71	0.0017	0.24	3
A*02:01-C*03:04-B*40:02	2.41	0.66	0.026	3.74	1.36–10.28	0.00058	1.00	0.01	0.13	17
A*02:01-C*08:01-B*40:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*02:01-C*08:01-B*48:01	1.81	0.16	0.0029	11.65	3.49–38.88	0.00058	0.25	0.0012	0.12	2
A*02:06-C*01:02-B*15:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*02:06-C*01:02-B*40:06	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*02:06-C*07:02-B*67:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*02:07-C*01:02-B*54:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*02:10-C*01:02-B*15:01	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*11:01-C*03:04-B*15:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*11:01-C*03:04-B*51:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*11:01-C*07:02-B*40:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*11:01-C*08:03-B*15:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*24:02-C*01:02-B*46:01	2.41	0.80	0.047	3.06	1.12–8.37	0.00058	1.00	0.02	0.16	26
A*24:02-C*01:02-B*56:03	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*24:02-C*03:03-B*35:01	4.82	1.06	0.00049	4.75	2.30–9.81	0.00058	0.042	0.00058	0.042	1
A*24:02-C*04:01-B*27:07	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*24:02-C*08:01-B*07:05	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*24:02-C*08:01-B*40:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*26:01-C*01:02-B*15:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*26:01-C*03:04-B*40:01	1.20	0.18	0.049	6.69	1.58–28.22	0.00058	1.00	0.01	0.17	25
A*26:01-C*07:02-B*39:02	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*26:01-C*08:01-B*35:01	1.20	0.17	0.036	7.17	1.69–30.34	0.00058	1.00	0.01	0.13	24
A*26:01-C*12:02-B*52:01	1.20	0.21	0.0499	5.90	1.41–24.77	0.00058	1.00	0.02	0.14	31
A*26:01-C*14:02-B*51:01	1.81	0.26	0.01	7.20	2.21–23.48	0.00058	0.86	0.0093	5.5E-02	16
A*29:01-C*15:05-B*15:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*31:01-C*03:04-B*51:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*31:01-C*07:02-B*67:01	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*32:01-C*15:02-B*35:03	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*33:03-C*07:02-B*67:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
DRB1*04:01-DQB1*03:01	3.01	1.06	0.046	2.91	1.15–7.33	0.0016	1.00	0.0048	0.48	3
DRB1*12:02-DQB1*03:01	4.22	1.70	0.034	2.54	1.16–5.55	0.0016	1.00	0.0032	0.53	2
DRB1*14:06-DQB1*06:02	0.60	0.00	0.0039	106.40	4.31–2,623	0.0016	0.12	0.0016	0.12	1

CI = Confidence interval.

the control subjects, this would imply that an additive or a synergic effect of the alleles constituting the specific haplotype increases susceptibility to the development of POI. Thus, this study exemplifies the importance of haplotype analysis in the investigation of genetic susceptibility.

The mechanism(s) by which this specific haplotype raises the susceptibility to POI remains to be clarified. It may be possible, however, that this haplotype has some ovary-specific effect such as the production of hitherto unknown autoantibodies against an ovary-specific tissue(s), because this haplotype was identified as a susceptibility factor for the apparently isolated POI, and because there is no study suggesting a positive association between this haplotype and non-ovarian autoimmune disorders. In support of this notion, it is known in T1DM that pancreatic β-cell-specific insulinoma-associated antigen 2 autoantibodies are preferentially detected in patients with a specific HLA allele [Qu and Polychronakos, 2009].

HLA association studies have previously been performed for POI in non-Japanese populations. Walfish et al. [1983] found that the prevalence of DR3 antigen was significantly higher in 19 women with POI than in 80 control women. Anasti et al. [1994] reported that the frequency of DR4 antigen was significantly higher in 102