specific susceptibility factors, and false positive results might be obtained in association studies with multiple comparisons. This matter awaits further studies.

One may argue that although the present study indicates an association of the specific *ESR2* haplotype with SF, there is no direct evidence for estrogenic EEDs being involved in the development of SF. Indeed, it may be possible that an interaction between the specific *ESR2* haplotype and endogenous estrogens rather than estrogenic EEDs actually underlie the development of SF. However, estrogenic effects of EEDs are known to be primarily mediated by ER.[1,3] In addition, as all the SF patients and the control males examined in this study were apparently free from high exposure to EEDs, the amount of exposed EEDs would be similar between the two groups of subjects. Thus, although further studies such as the investigation of subjects with a high risk of EEDs exposure (for example, workers at chemical factories) are necessary, our results would suggest that the specific *ESR2* haplotype constitutes a susceptibility factor for the development of SF in response to estrogenic EEDs in males who live in an ordinary condition with no high risk of EEDs exposure.

Several points should be made with respect to the present study. First, the number of subjects analyzed remains rather small. Second, the true susceptibility factor(s) on the specific haplotype remains to be identified, although the specific 'TGTAGA' haplotype would facilitate the development of SF by enhancing the ERβ signaling. Third, it remains possible that another susceptibility factor(s) is present on *ESR2*. In particular, as only a few of SNPs were examined in non-LD block regions, a different susceptibility factor(s) may be present on the non-LD block regions of *ESR2*. Fourth, several patients may have some unidentified pathologic cause(s) for SF such as single gene disorders. Fifth, there may be some unknown minor genetic and environmental differences between the patients and the control males. In this context, as SF becomes discernible in adulthood, such minor differences, if they exist, may exert unfavorable influences on spermatogenic function for a long time, leading to SF. This may explain why the OR obtained in this study remained low, in contrast to the high ORs identified in cryptorchidism (7.55) and hypospadias (13.75)[6,7] (and our unpublished updated observation) which develop during the fetal life. Sixth, although it is known that EEDs also exert anti-androgenic effects and influence aromatization,[25,26] these have not been examined in this study. Lastly, it remains to be determined whether similar results can be reproduced in other case–control studies.

Despite the above caveats, this study provides a useful clue to clarify the genetic susceptibility to estrogenic EEDs. In summary, we propose that the specific *ESR2* haplotype raises the susceptibility to the development of SF in response to estrogenic EEDs. Further studies including similar haplotype analyses in different ethnic groups from both developed and developing countries will serve to clarify the relative importance of the dosage of exposed EEDs and the genetic heterogeneity obtained in the process of natural human selection, in the presumably EEDs-related phenomenon such as SF.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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human reproduction ORIGINAL ARTICLE *Reproductive genetics*

Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies

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BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors.

METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well-known imprinting diseases to determine any association with ART. Using bisulfite sequencing, we examine the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann syndrome (BWS, $n = 1$) and also Silver-Russell syndrome (SRS, $n = 5$) born after ART, and compared these with patients conceived naturally.

RESULTS: We found a 10-fold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by a small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest due to the differences in the disease phenotypes in these imprinting disorders.

CONCLUSION: A possible association between ART and BWS/SRS was found, and we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Key words: assisted reproduction technologies / genomic imprinting / DNA methylation / gametic differentially methylated regions / genomic imprinting disorders

Introduction

Human assisted reproduction technologies (ARTs) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith-Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270; DeBaun *et al.*, 2003; Gosden *et al.*, 2003; Svensson *et al.*, 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Bowdin *et al.*, 2007; Kagami *et al.*, 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al.*, 2011) and are reset with every reproductive cycle (Lucifero *et al.*, 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without the use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al.*, 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al.*, 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with the loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R*) gDMR (Young *et al.*, 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes. Previously, Chang *et al.* (2005) reported no phenotypic differences between BWS patients who were conceived after ART and naturally. However, Lim *et al.* (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al.*, 2006; Bliek *et al.*, 2009; Lim *et al.*, 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases: BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico-epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. Ltd (Nagoya, Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded to the earlier deadline (at the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Estimation of prevalence of imprinting disorders

The number of patients, who were diagnosed by genetic and cytogenetic testing and by clinical phenotypes, was obtained from data from the departments who responded to the first survey. The 95% confidence interval (CI) was calculated as previously described (Wakai *et al.*, 1997). The prevalence was determined, based on the population of Japan in 2009 (127 510 000) with data from the Statistics Bureau of the Ministry of Internal Affairs and Communications.

DNA preparation

Genomic DNA was obtained from blood or buccal mucosal cell samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi *et al.*, 2007). For control DNAs, DNA was prepared from the sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patients or their parents' consent.

Bisulfite-treatment PCR including the SNPs

We first searched for single nucleotide polymorphisms (SNPs) within 22 previously reported human gDMRs (Kikyo *et al.*, 1997; Smith *et al.*, 2003; Kobayashi *et al.*, 2006, 2009; Wood *et al.*, 2007) using 20 control Japanese blood DNA samples. PCR primer sets were designed to span these SNPs (Supplementary data, Table SI) and human sperm DNA and blood DNA was used to confirm that these PCR assays detected the methylation status of the 22 DMRs. Paternal DMRs were shown to be fully methylated in sperm DNA, maternal DMRs were fully unmethylated and in blood DNA, both paternal and maternal DMRs showed ~50% methylation (Supplementary data, Fig. S1). The human gDMRs and the non-imprinted repetitive long interspersed nucleotide element (*LINE1*) and *Alu* repetitive sequences were examined by bisulfite sequencing using established protocols (Kobayashi *et al.*, 2007). Briefly, PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On average, 20 clones were sequenced for each sample.

Statistics

The frequency of the manifestation in patients who were conceived after ART was compared with that observed in patients conceived naturally using Fisher's exact test.

Results

Frequency of four imprinting disorders and their association with ART

We first investigated the nationwide frequency of four imprinting disorders (BWS, AS, PWS and SRS) in Japan in the year 2009. Of a total of 3158 departments contacted, 1602 responded to the first-stage survey questionnaire (50.7%). The total number of cases was calculated using a second-stage survey ensuring the exclusion of duplicates (Table I). Using this information, and taking into account the number of patients with suspect clinical signs but without a formal diagnosis, we identified 444 BWS patients (95% CI: 351–538), 949 AS patients (95% CI: 682–1217), 2070 PWS patients (95% CI: 1504–2636) and 326 SRS patients (95% CI: 235–416). From these figures (and using the 2009 population of Japan: 127 510 000) we estimated the prevalence of these syndromes to be 1 in 287 000, 1 in 134 000, 1 in 62 000 and 1 in 392 000, respectively, for BWS, AS, PWS and SRS. Further details are given in Supplementary data, Table SII and Supplementary data, Fig. S2.

Table I The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART).

Imprinting disorders	Total estimated patient number (95% CI)	The total prevalence of the syndrome	The number of patients after ART/ total (%)
BWS	444 (351–538)	1 in 287 000	6/70 (8.6)
AS	949 (682–1217)	1 in 134 000	2/123 (1.6)
PWS	2070 (1504–2636)	1 in 62 000	4/261 (1.5)
SRS	326 (235–416)	1 in 392 000	4/42 (9.5)

Results of a nationwide epidemiological investigation of four imprinting disorders in Japan, under the governance of the Ministry of Health, Labor and Welfare of the Japanese government. Precise diagnosis was performed using fluorescence *in situ* hybridization and DNA methylation analyses. The type of ART, obtained from the questionnaires, was compared with the frequencies of these diseases and the epimutation rates. BWS, Beckwith-Wiedemann syndrome, AS, Angelman syndrome, PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome.

Between 1997 and 2008, the period during which the ART babies in this study were born, 0.64–0.98% of the total number of babies born in Japan were born as a result of IVF and ICSI. We ascertained the frequency of ART procedures in the cases of BWS, AS, PWS and SRS via the questionnaire sent to doctors (Table I, Supplementary data, Table SIII). The numbers of patients with PWS and AS we identified was low; however, the frequency of ART in these cases was not dissimilar to that expected, based on the population rate of ART use, with 2/123 (1.6%) cases of AS and 4/261 (1.5%) cases of PWS born after ART. In contrast, for BWS and SRS the frequency of ART was nearly 10-fold higher than anticipated with 6/70 (8.6%) BWS and 4/42 (9.5%) SRS patients born after ART.

After analyzing the second questionnaire, the blood or buccal mucosal cell samples were obtained from 15 individuals with BWS, 23 with SRS, 73 with AS and 29 with PWS. Using polymorphic bisulfite-PCR sequencing, we examined the methylation status of gDMRs within these samples at the imprinted regions implicated in these syndromes. For BWS we assayed *H19* and *KCNQ1OT1* (*LIT1*) gDMRs, for SRS we assayed the *H19* gDMR and for PWS and AS we assayed the *SNRPN* gDMR. For all patients (conceived naturally and with ART), the frequencies of DNA methylation errors (epimutations) corrected were 7/15 (46.7%) for BWS, 9/23 (39.1%) for SRS, 6/73 (8.2%) for AS and 2/29 (6.9%) for PWS. When looking at the ART cases exclusively, epimutation rates were 3/5 (BWS), 3/7 (SRS), 0/2 (AS) and 0/2 (PWS).

Abnormal methylation patterns in the ART and naturally conceived SRS patients with epimutations.

While hypomethylation of *H19* at chromosome 11 is known to be a frequent occurrence in SRS (Bliek *et al.*, 2006), various additional loci at chromosomes 7, 8, 15, 17 and 18 have been implicated as having a

role in this syndrome (OMIM 180860). We first identified SNPs in the previously reported 22 human DMRs using genomic DNA isolated from human sperm and blood from unaffected individuals, which could then be used in bisulfite-PCR methylation assays to assign methylation to the parental allele. We next collected a total of 15 SRS samples, including previously collected samples (ART: 2, naturally conceived: 4), which had DNA methylation errors at the paternal gDMR at *H19*. Five of these were born from ART and 10 were from natural conceptions. We analyzed and compared the DNA methylation status of the 3 other paternal gDMRs and the 19 maternal gDMRs (Supplementary data, Fig. S3, Table, Supplementary data, Table SIV). In four out of the five ART cases, DNA methylation errors were not restricted to the *H19* gDMR, and were present at both maternally and paternally methylated gDMRs. These four cases showed a mixture of hyper- and hypomethylation with mosaic (partial) patterns. In contrast, only 3 of the 10 naturally conceived patients showed DNA methylation errors at loci other than *H19* gDMR.

To determine whether DNA methylation errors occurred in patients at a broader level in the genomes, we assessed the methylation profiles of the non-imprinted *LINE1* and *Alu* elements. We examined a total of 28 CpG sites in a 413-bp fragment of *LINE1* and 12 CpG sites in a 152-bp fragment of *Alu* (Supplementary data, Table SIV), and no significant differences were found in the methylation ratios between patients conceived by ART and naturally.

The abnormal methylation pattern in BWS patients with epimutations

In BWS, hypermethylation of *H19* or hypomethylation of *KCNQ1OT1(LIT1)* at human chromosome 11 are both frequently reported (Choufani *et al.*, 2010). We collected seven BWS samples with DNA methylation errors of the *LIT1* gDMR, one of which was derived from ART patient and six from naturally conceived patients (Supplementary data, Fig. S3, Table II, Supplementary data, Table SIV). In the one ART (ICSI) case, we identified four additionally gDMR methylation errors, again present at both maternally and paternally methylated gDMRs and with mixed hyper- and hypomethylation patterns. Furthermore, the methylation error at the *NESPAS* DMR was mosaic in this patient. One of the six naturally BWS cases had similar changes. Although we had only one BWS case conceived by ART, widespread methylation errors were similar to those for the DNA methylation error pattern in SRS.

Phenotypic differences between ART patients and those conceived naturally

The increased frequency of DNA methylation errors at other loci in the ART cases suggested that the BWS and SRS cases born after ART might exhibit additional phenotypic characteristics. However, when we compared in detail the clinical features from both categories of conception (Supplementary data, Table SV), we found no major differences between ART and naturally conceived patients with BWS and SRS.

Discussion

Our key finding from this study was a possible association between ART and the imprinting disorders, BWS and SRS. We did not find a similar association with PWS and AS but our numbers were quite low in this study and a larger due to the questionnaire return rate and relative rarity of the diseases, international study will be required to reach definitive conclusions. Furthermore, factors such as PCR and/or cloning bias in the bisulfite method and correction for changing rate of ART over time must be considered when analyzing any results.

In addition to the possible association between ART and BWS/SRS, we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART shown by us and others is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable. The process of ART exposes the developing epigenome to many external influences, which have been shown to influence the proper establishment and maintenance of genomic imprints, including hormone stimulation (Sato *et al.*, 2007), *in vitro* culturing (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003), cryopreservation (Emiliani *et al.*, 2000; Honda *et al.*, 2001) and the timing of embryo transfer (Shimizu *et al.*, 2004; Miura and Niikawa, 2005). Furthermore, we and others have also shown that some infertile males, particularly those with oligozoospermia, carry pre-existing imprinting errors in their sperm (Marques *et al.*, 2004; Kobayashi *et al.*, 2007; Marques *et al.*, 2008) which might account for the association between ART and imprinting disorders.

Imprinting syndromes and their association with ART

We report the first Japanese nationwide epidemiological study to examine four well-known imprinting diseases and their possible association with ART. We found that the frequency of ART use in both BWS and SRS was higher than anticipated based on the nationwide frequency of ART use at the time when these patients were born. Several other reports have raised concerns that children conceived by ART have an increased risk of disorders (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Lim and Maher, 2009). However, the association is not clear in every study (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007). The studies reporting an association were mainly from case reports or case series whereas the studies where no association was reported were cohort studies. Therefore, the differences in the epidemiological analytical methods might accounts for the disparity in findings.

Owing to the rare nature of the imprinting syndromes, statistical analysis is challenging. In addition, the diagnosis of imprinting diseases is not always clear cut. Many of the syndromes have a broad clinical spectrum, different molecular pathogenesis, and the infant has to have reached a certain age before these diseases become clinically detectable. It is therefore likely that some children with these diseases are not recorded with the specific diagnosis code for these syndromes. Nonetheless, in this study we were examining the relationship between ART and the imprinting syndromes and these confounding factors are likely to apply equally to both groups.

Both BWS and SRS occurred after ART but our numbers for PWS and AS were low, precluding any definitive conclusion for these two disorders. However, while most cases of BWS and SRS are caused by an epimutation, epimutations are very rare in PWS and AS (only 1–4%) and ART would not be expected to increase chromosome 15

Table II Abnormal methylation in patients with SRS and BWS.

Case	ART	Abnormal methylation			
SRS					
SRS-1	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated	PEG10 hypermethylated (mosaic)	GRB10 hypermethylated; ZNF597 hypomethylated
SRS-2	IVF-ET	H19 hypomethylated (mosaic)			
SRS-3	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated (mosaic)		
SRS-4	IVF-ET	H19 hypomethylated	GRB10 hypermethylated		
SRS-5	IVF-ET	H19 hypomethylated (mosaic)	INPP5F hypermethylated		
SRS-6		H19 hypomethylated			
SRS-7		H19 hypomethylated (mosaic)	ZNF597 hypermethylated (mosaic)	ZNF331 hypomethylated (mosaic)	
SRS-8		H19 hypomethylated			
SRS-9		H19 hypomethylated (mosaic)			
SRS-10		H19 hypomethylated			
SRS-11		H19 hypomethylated (mosaic)	PEG1 hypermethylated		
SRS-12		H19 hypomethylated			
SRS-13		H19 hypomethylated (mosaic)	FAM50B hypomethylated		
SRS-14		H19 hypomethylated			
SRS-15		H19 hypomethylated			
BWS					
BWS-1	ICSI	LIT1 hypomethylated	ZDBF2 hypermethylated	PEG1 hypermethylated	NESPAS hypomethylated (mosaic)
BWS-2		LIT1 hypomethylated			
BWS-3		LIT1 hypomethylated			
BWS-4		LIT1 hypomethylated			
BWS-5		LIT1 hypomethylated			
BWS-6		LIT1 hypomethylated	ZDBF2 hypomethylated	ZNF331 hypomethylated (mosaic)	
BWS-7		LIT1 hypomethylated			

ET, embryo transfer. Summary of the abnormal methylation patterns in the ART conceived and naturally conceived patients with Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) with epimutations. Numbers in parentheses show the results of the methylation rates obtained using bisulfite-PCR sequencing. The % of DNA methylation of 22 gDMRs in all patients with SRS and BWS examined are presented in Supplementary data, Table SIV. Depictions in red represent DMRs normally exclusively methylated on the maternal allele, while blue represent paternally methylated sites.

deletions or uniparental disomy, consistent with our findings. Prior to this investigation, there was some evidence for an increased prevalence of BWS after ART but less evidence for an increased prevalence of SRS, with five SRS patients reported linked to ART (Svensson *et al.*, 2005; Bliek *et al.*, 2006; Kagami *et al.*, 2007; Galli-Tsinopoulou *et al.*, 2008). Our population-wide study provides evidence to suggest that both BWS and SRS occur more frequently after ART in the Japanese population.

Mechanisms of epimutation in the patients conceived by ART

By performing a comprehensive survey of all the known gDMRs in a number of patients with BWS and SRS, we found that multiple loci were more likely to be affected in ART cases than those conceived naturally. Lim *et al.* (2009) have reported a similarly increased frequency of multiple errors after ART, with 37.5% of patients conceived with ART and 6.4% of naturally conceived patients displaying abnormal methylation at additional imprinted loci. However, while Bliek *et al.* (2009) reported alterations in multiple imprinted loci in 17 patients out of 81 BWS cases with hypomethylation of *KCNQ1OT1*(*LIT1*) ICR, only 1 of the cases with multiple alterations was born after ART. Similarly, Rossignol *et al.* (2006) reported that 3 of 11 (27%) ART-conceived patients and 7 of 29 (24%) naturally conceived patients displayed abnormal methylation at additional loci. In these four earlier studies, not all gDMRs were assayed and it may be that by doing so, these incongruities will be resolved.

The pattern of cellular mosaicism we observed in some patients suggested that the imprinting defects occurred after fertilization rather than in the gamete as DNA methylation alterations arising in the gamete would be anticipated to be present in every somatic cell. This suggested the possibility that the DNA methylation errors occurred as a consequence of impaired maintenance of the germline imprints rather than a failure to establish these imprints in the germline or a loss of these imprints in the sperm or oocytes *in vitro*. Furthermore, some patients conceived by ART with SRS and BWS showed

alterations at both maternally and paternally methylated gDMRs suggesting that the defects were not limited to one parental germline. The mechanisms controlling the protection of imprinted loci against demethylation early in the development remain unclear. Our data suggested that this protection may fail in ART resulting in the tissue-specific loss of imprints, though it remains unclear if this ever occurs naturally. Potential factors involved could include the culture conditions for the newly fertilized oocyte and the length of exposure to specific media or growth factors, as part of the ART procedure. Some of the naturally conceived patients also had abnormal methylation at both maternally and paternally methylated gDMRs, which were in some cases mosaic. This could indicate that fertility issues arise as a consequence of pre-existing mutations in factors required to protect and maintain imprints early in life and it may therefore be possible to identify genetic mutations in these factors in this group of patients.

Clinical features

In our large-scale epidemiological study, we found differences in the frequency of some classic features of SRS and BWS between patients conceived by ART and those conceived naturally. We found that 7/7 (100%) ART conceived SRS patients showed body asymmetry, whereas only 30/54 (55.5%) who were conceived naturally possessed this feature. Similarly in BWS, earlobe creases were present in 4/7 (57.1%) ART conceived cases and 44/89 (49.4%) naturally conceived, bulging eyes in 3/7 (42.8%) versus 21/89 (23.6%), exomphalos in 6/7 (85.7%) versus 61/89 (68.5%) and nephromegaly in 2/7 (28.6%) versus 18/89 (20.2%), respectively. It is therefore possible that the dysregulation of the additional genes does modify the typical SRS and BWS phenotypes (Azzi *et al.*, 2010). BWS patients with multiple hypermethylation sites have been reported with complex clinical phenotypes (Bliek *et al.*, 2009) and a recently recognized BWS-like syndrome involving overgrowth with severe developmental delay was reported after IVF/ICSI (Shah *et al.*, 2006).

In our study patients with diagnosed imprinting disorders that presented with defects at additional loci (i.e. other than the domain responsible for that disorder) did not display additional phenotypes not normally reported in BWS or SRS. Since we were effectively selecting for classic cases of BWS and SRS in the first instance, it is possible that there are individuals born through ART showing entirely novel or confounding phenotypes that were not identified in our survey. Alternatively, as many of the alterations we observed showed a mosaic pattern, it is possible that mosaic individuals have more subtle phenotypes. In light of this new information on mosaicism, we may be able to use our knowledge of the individual's epigenotype to uncover these subtle changes.

This study, and the work of our colleagues, highlights the pressing need to conduct long-term international studies on ART treatment and the prevalence of imprinting disorders, particularly as the use of ART is increasing worldwide. It remains to be seen if other very rare epigenetic disorders will also have a possible association with the use of ART. Furthermore, it is not yet known what other pathologies might be influenced by ART. For example, in addition to general growth abnormalities, many imprint methylation errors also lead to the occurrence of various cancers (Okamoto *et al.*, 1997; Cui *et al.*, 1998). Further molecular studies will be required to understand the pathogenesis of these associations, and also to identify preventative methods to reduce the risk of occurrence of these syndromes following ART.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.H., H.O., N.M., F.S. and A.S. performed the DNA methylation analyses. M.K., K.N. and H.S. collected the samples of the patients. K.N. did the statistical analyses. H.H., M.V.D.P., R.M.J. and T.A. wrote this manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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PRKAR1A Mutation Affecting cAMP-Mediated G Protein-Coupled Receptor Signaling in a Patient with Acrodysostosis and Hormone Resistance

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Context: Acrodysostosis is a rare autosomal dominant disorder characterized by short stature, peculiar facial appearance with nasal hypoplasia, and short metacarpotarsals and phalanges with cone-shaped epiphyses. Recently, mutations of *PRKAR1A* and *PDE4D* downstream of *GNAS* on the cAMP-mediated G protein-coupled receptor (GPCR) signaling cascade have been identified in acrodysostosis with and without hormone resistance, although functional studies have been performed only for p.R368X of *PRKAR1A*.

Objective: Our objective was to report a novel *PRKAR1A* mutation and its functional consequence in a Japanese female patient with acrodysostosis and hormone resistance.

Patient: This patient had acrodysostosis-compatible clinical features such as short stature and brachydactyly and mildly elevated serum PTH and TSH values.

Results: Although no abnormality was detected in *GNAS* and *PDE4D*, a novel *de novo* heterozygous missense mutation (p.T239A) was identified at the cAMP-binding domain A of *PRKAR1A*. Western blot analysis using primary antibodies for the phosphorylated cAMP-responsive element (CRE)-binding protein showed markedly reduced CRE-binding protein phosphorylation in the forskolin-stimulated lymphoblastoid cell lines of this patient. CRE-luciferase reporter assays indicated significantly impaired response of protein kinase A to cAMP in the HEK293 cells expressing the mutant p.T239A protein.

Conclusions: The results indicate that acrodysostosis with hormone resistance is caused by a heterozygous mutation at the cAMP-binding domain A of *PRKAR1A* because of impaired cAMP-mediated GPCR signaling. Because *GNAS*, *PRKAR1A*, and *PDE4D* are involved in the GPCR signal transduction cascade and have some different characters, this would explain the phenotypic similarity and difference in patients with *GNAS*, *PRKAR1A*, and *PDE4D* mutations. ***(J Clin Endocrinol Metab* 97: E1808–E1813, 2012)**

Acrodysostosis is a rare autosomal dominant disorder characterized by short stature, peculiar facial appearance with nasal hypoplasia, short metacarpotarsals and phalanges with cone-shaped epiphyses, and variable degrees of mental retardation (1, 2). Recent studies have shown that acrodysostosis is caused by mutations of *PRKAR1A* (protein

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Abbreviations: AHO, Albright's hereditary osteodystrophy; CRE, cAMP-responsive element; CREB, CRE-binding; DMR, differentially methylated regions; GNAS, stimulatory G protein α-subunit; GPCR, G protein-coupled receptor; PDE4D, phosphodiesterase 4D, cAMP-specific; PHP-Ia, pseudohypoparathyroidism type Ia; PKA, protein kinase A; PRKAR1A, protein kinase, cAMP-dependent, regulatory type 1, α; RIα, type 1α regulatory subunit.

kinase, cAMP-dependent, regulatory type 1,α) and *PDE4D* (phosphodiesterase 4D, cAMP-specific) involved in the cAMP-mediated G protein-coupled receptor (GPCR) signaling cascade (3–5). *PRKAR1A* consists of 11 exons and encodes type 1α regulatory subunit (RIα) of protein kinase A (PKA) with a dimerization domain, an inhibitory site, and two cAMP-binding domains A and B (6). The PKA holoenzyme is a tetramer consisting of two regulatory subunits and two catalytic subunits, and cooperative binding of two cAMP molecules to each regulatory subunit leads to the dissociation of the catalytic subunits from the regulatory subunits (7). The regulatory subunit-associated catalytic subunits remain inactive, whereas the free catalytic subunits released from the regulatory subunits can phosphorylate a variety of substrate proteins including the cAMP-responsive element (CRE)-binding (CREB) protein (7, 8). It is likely, therefore, that the *PRKAR1A* mutations hinder the cAMP-mediated dissociation of the catalytic subunits from the regulatory subunits, thereby leading to reduced PKA signaling (3). *PDE4D* comprises 15 exons and encodes cAMP-dependent phosphodiesterase 4D (PDE4D) that regulates intracellular cAMP concentrations by converting cAMP to AMP (9). Thus, the *PDE4D* mutations appear to result in desensitization to cAMP because of persistently elevated intracellular cAMP concentrations, thereby affecting the cAMP-mediated GPCR signaling cascade (4). However, functional studies have been performed only for the *PRKAR1A* p.R368X mutation that resides on the last exon and is predicted to escape nonsense-mediated mRNA decay (3), whereas protein modeling analysis argues for the pathological consequences of the remaining *PRKAR1A* and *PDE4D* mutations (4, 5).

Notably, nine of 10 *PRKAR1A* mutation-positive patients and two of seven *PDE4D* mutation-positive patients identified to date exhibit resistance to PTH and/or TSH. Such clinical findings, *i.e.* acrodysostosis plus hormone resistance, overlap with those of pseudohypoparathyroidism type Ia (PHP-Ia), because PHP-Ia is associated with Albright's hereditary osteodystrophy (AHO) reminiscent of acrodysostosis and resistance to several hormones such as PTH and TSH. Indeed, although acrodysostosis and AHO have been classified as different skeletal disorders (2), it is often difficult to distinguish between acrodysostosis and AHO on the basis of clinical and radiological findings (10). Consistent with such phenotypic similarities, PHP-Ia is primarily caused by heterozygous loss-of-function mutations of *GNAS* (the stimulatory G protein α-subunit) (11) that resides in the upstream of *PRKAR1A* and *PDE4D* on the cAMP-mediated GPCR signaling cascade.

Here, we report on a novel *de novo PRKAR1A* mutation and its functional consequence in a patient with acrodysostosis and hormone resistance and discuss phenotypic findings in patients with *PRKAR1A*, *PDE4D*, and *GNAS* mutations.

Patients and Methods

Case report

This Japanese female patient was born to nonconsanguineous parents at 38 wk of gestation. At birth, her length was 46.5 cm (−0.75 SD) and her weight 1.81 kg (−2.8 SD). Neonatal screening tests were normal. Her gross motor milestones were somewhat delayed, with sitting alone without support at 10 months and walking alone at 21 months of age. Her stature remained below −2.0 SD of the mean.

At 3 yr and 10 months of age, she was referred to us because of short stature. Her height was 86.9 cm (−3.1 SD) and her weight 10.6 kg (−2.3 SD). She exhibited round face, nasal hypoplasia, anteverted nostrils, severe brachydactyly of the hands, and mild developmental retardation, and hand roentgenograms showed generalized shortening of the tubular bones with cone shaped epiphyses (Fig. 1A). Brain computerized tomography showed neither sc nor intracranial calcifications. Biochemical and endocrine studies revealed 1) increased serum PTH and plasma cAMP values and normal serum calcium, phosphate, and vitamin D values; 2) decreased urine calcium/creatinine ratio and normal percent tubular reabsorption of phosphate; 3) slightly elevated serum TSH value and normal free T_4 value; 4) age-appropriate serum LH and FSH values; and 5) normal GH response to GHRH stimulation (Table 1). Thus, she was suspected as having acrodysostosis with mild resistance to PTH and TSH.

The parents showed neither brachydactyly nor abnormal endocrine findings (Table 1), although the mother had short stature (144 cm, −2.6 SD).

Molecular and functional studies

We performed 1) direct sequencing for coding exons and their splice sites of *PRKAR1A*, *PDE4D*, and *GNAS*; 2) methylation analysis for four differentially methylated regions (DMR) around *GNAS*; 3) parental testing by microsatellite genotyping; 4) conservation of a substituted amino acid; 5) the forskolin-induced PKA activity of lymphoblastoid cell lines in terms of the phosphorylation status of the CREB protein using Western blot analysis; and 6) forskolin-induced PKA activity using HEK293 cells expressing the wild-type and the mutant proteins. The primers used in this study are shown in Supplemental Table 1, and the detailed methods are described in Supplemental Methods (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Results

Analysis of *PRKAR1A*, *PDE4D*, and *GNAS*

No pathological mutation was found for *PDE4D* and *GNAS*, nor was an aberrant methylation pattern detected for the DMR around *GNAS*. By contrast, a novel heterozygous missense mutation (c.715A→G; p.T239A)

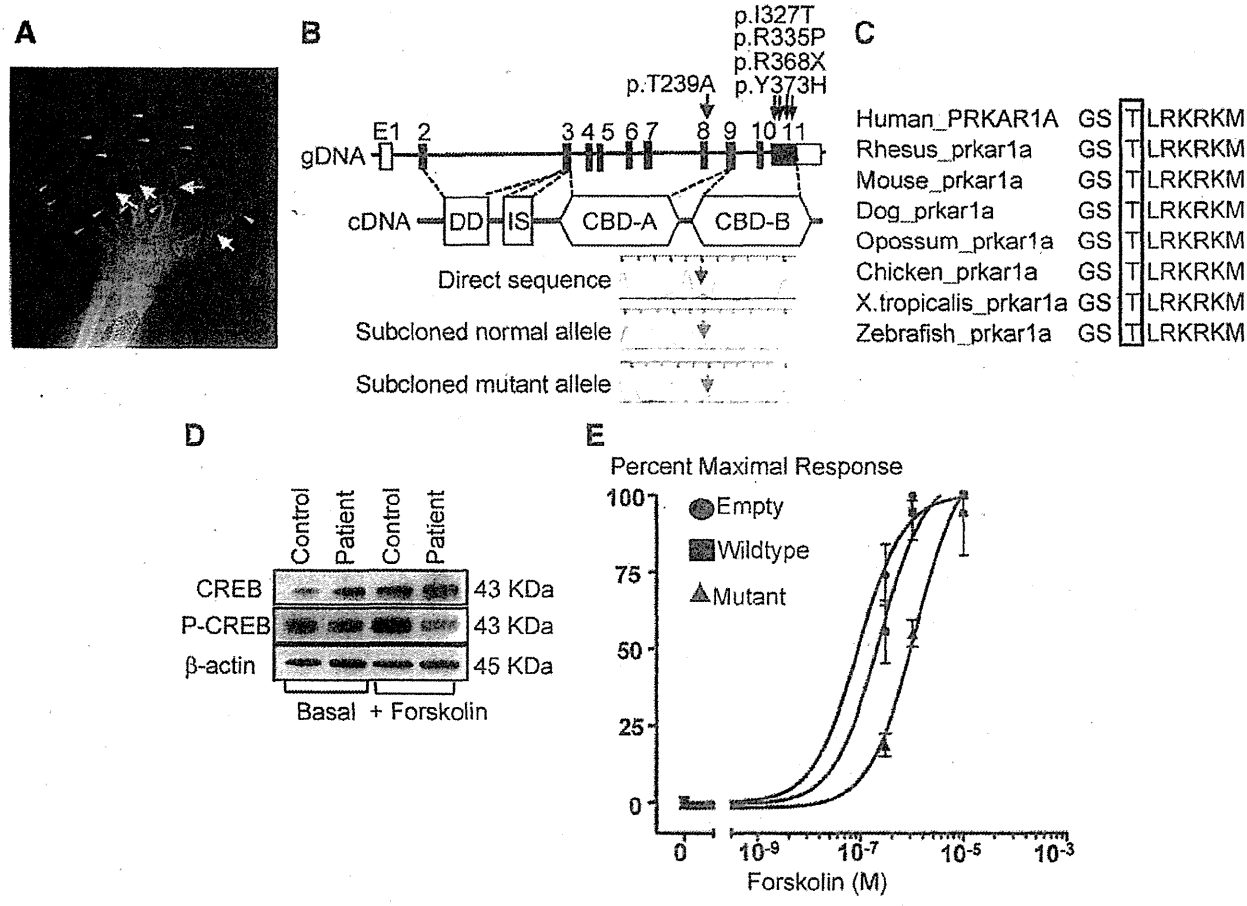

FIG. 1. Representative clinical and experimental findings of this patient. A, Radiograph of the left hand at 3 yr and 10 months of age. Note the shortening of all tubular bones with cone-shaped epiphyses (*arrows*) and early infusions (*arrowheads*). B, The structure of *PRKAR1A* and the position of the mutations identified. The *black and white boxes* on genomic DNA (gDNA) denote the coding regions on exons 2–11 and the untranslated regions, respectively. *PRKAR1A* encodes a dimerization domain (DD), an inhibitory site (IS), and two cAMP-binding domains A and B (CBD-A and -B). A missense mutation (p.T239A) was identified on exon 8 for the cAMP-binding domain A of this patient, whereas the previously described one nonsense and three missense mutations have been found on exon 11 for cAMP-binding domain B (3–5). C, Amino acid sequence of PRKAR1A. Note that the T239 residue is well conserved among species. D, Representative results of Western blot analysis for lymphoblastoid cell lines of the patient and a control subject. The cells were collected before (basal) and after stimulation with 10 μM forskolin. The samples were probed with antibodies for phospho-CREB protein (Ser 133) (P-CREB) and CREB protein, together with those for β-actin used as an internal control. E, Transactivating activities of the wild-type and the mutant PRKAR1A for the CRE-luc reporter. HEK293 cells were transfected with an empty expression vector or with vectors containing either the wild-type PRKAR1A or the p.T239A mutant. Samples were treated with various concentrations of forskolin. The values (percentages to the maximal CRE-luciferase activity) are expressed as the mean ± SE. Curves are fitted with sigmoidal dose-response models. In cells expressing the mutant protein, forskolin induced a concentration-dependent increase in CRE-luciferase activity, yet with a shift to the right in the dose-response curve. The EC_{50} values were significantly higher in the cells expressing the mutant protein than those expressing the wild-type protein ($P < 0.001$). The results are obtained from three independent experiments.

was identified on exon 8 at the cAMP-binding site A of *PRKAR1A* (Fig. 1B). This mutation was absent from her parents and 100 Japanese control subjects.

Parental testing

Microsatellite genotyping data were consistent with paternity as well as maternity of the parents (Supplemental Table 2).

Functional characterization of the mutant *PRKAR1A*

The T239 residue was well conserved among species (Fig. 1C). Protein modeling analysis indicated that the p.T239A resulted in loss of the hydrogen bond between the M236 and the T239 residues and in aberration of the random coils in the mutant PRKAR1A protein, although there was no gross conformational alteration affecting α-helices and β-strands (Supplemental Fig. 1). Western blot analysis indicated obviously reduced forskolin-induced CREB protein phosphorylation in the presence of the apparently normal amount of CREB protein in the lymphoblastoid cell line of this patient (Fig. 1D). Similarly, forskolin-induced PKA activity was significantly lower in the HEK293 cells expressing the mutant PRKAR1A protein than in those expressing the wild-type protein (Fig. 1E).

TABLE 1. Clinical and laboratory data of the patient and her parents

	Patient	Father	Mother[a]
Age (yr)	3 10/12	31	30
Height (cm) (SDS)	**86.9** (−3.1)	177 (+1.1)	**144** (−2.6)
Weight (kg) (SDS)	**10.9** (−2.3)	**89 (+2.5)**	45[b]
Blood			
Intact PTH (pg/ml)	**128** (10–65)	42 (10–65)	23 (10–65)
Calcium (mg/dl)	9.4 (8.5–10.2)	9.4 (8.5–10.2)	9.0 (8.5–10.2)
Phosphate (mg/dl)	5.6 (3.5–5.9)	3.2 (2.4–4.3)	3.6 (2.4–4.3)
25-Hydroxyvitamin D (ng/ml)	26 (7–41)	NA	NA
TSH (mU/liter)	**7.2** (0.5–5.0)	1.7 (0.5–5.0)	1.2 (0.5–5.0)
Free T_4 (ng/ml)	1.2 (0.9–1.6)	1.3 (0.9–1.6)	1.1 (0.9–1.6)
LH (IU/liter)	<0.1 (<0.7)	4.1 (0.8–5.7)	0.17 (1.8–10.2)[c]
FSH (IU/liter)	0.9 (0.6–5.3)	6.6 (2.0–8.3)	0.07 (3.0–14.7)[c]
GH (ng/ml) stimulated[d]	19.5 (>9)	NA	NA
cAMP (pmol/ml)	**39.6** (6.4–20.8)	NA	NA
Urine			
Calcium/creatinine ratio	**0.04** (0.13–0.25)	NA	NA
% TRP	91 (81.3–93.3)	NA	NA

The values in *parentheses* indicate the SD score (SDS) for heights and weights and the age-and sex-matched reference blood and urine hormone and laboratory data. The conversion factors to the SI unit are as follows: intact PTH, 1.0 (nanograms per liter); serum calcium, 0.25 (millimoles per liter); serum phosphate, 0.3229 (millimoles per liter); 25-hydroxyvitamin D, 2.496 (nanomoles per liter); free T_4, 12.9 (picomoles per liter); GH, 1.0 (micrograms per liter); and cAMP, 1.0 (nanomoles per liter). Hormone values have been evaluated by the age- and sex-matched Japanese reference data; abnormal data are in *bold*. NA, Not available; TRP, tubular reabsorption of phosphate.

[a] During the second trimester of pregnancy.

[b] Not assessed because of pregnancy.

[c] Low LH/FSH values are consistent with pregnant status of the mother (18).

[d] Blood sampling during the provocation tests were done at 0, 30, 60, 90, and 120 min after GHRH stimulation (1 μg/kg).

Discussion

We identified a novel *de novo* heterozygous *PRKAR1A* mutation in a patient with acrodysostosis and mild resistance to PTH and TSH. In this regard, several findings are noteworthy. First, although the phenotypic findings of this patient are similar to those of PHP-Ia, the severe skeletal lesion would be regarded as acrodysostosis rather than AHO. Consistent with this, a mutation was identified in *PRKAR1A* rather than *GNAS*. Second, the p.T239A was present at the cAMP-binding domain A, in contrast to the previously reported *PRKAR1A* mutations that were invariably located at the cAMP-binding domain B (3–5). In this regard, because cAMP binds first to the binding domain B and then to the binding domain A, it has been suggested that the binding domain B acts as the gatekeeper of the PKA activation, and that the binding domain A is relatively inaccessible to cAMP (8). Despite such a hierarchical phenomenon, this study indicates that mutations at the cAMP-binding domains A and B lead to a similar clinical phenotype. Third, functional analyses showed obviously reduced PKA signaling of the mutant PRKAR1A protein. Thus, the p.T239A mutation appears to impair the dissociation of the catalytic subunits from the RIα regulatory subunits, thereby leading to the reduced GPCR signaling, as has been stated by the functional studies for the p.R368X mutation (3). Although the underlying factors remains to be elucidated, loss of the hydrogen bond and aberration of the random coils in the mutant PRKAR1A protein may be relevant to this functional alteration.

To date, *GNAS*, *PRKAR1A*, and *PDE4D* mutations have been identified in patients with overlapping skeletal and endocrine phenotypes (3–5). It appears, however, that *GNAS* abnormalities usually lead to relatively mild skeletal phenotype and clinically discernible hormone resistance, whereas *PRKAR1A* and *PDE4D* mutations usually result in relatively severe skeletal lesion and mild or absent hormone resistance. In this regard, it is predicted in patients with maternally derived *GNAS* mutations that normally functioning *GNAS* is absent from several tissues including renal proximal tubules where *GNAS* is paternally imprinted and is present in a single copy in other tissues including skeletal tissues where *GNAS* is biparentally expressed (11, 12). By contrast, it is likely in patients with *PRKAR1A* and *PDE4D* mutations that normally functioning *PRKAR1A* and *PDE4D* are present in a single copy in all the tissues because of the absence of DMR around these genes (13). Such a difference in the functional gene dosage in several *GNAS*-imprinted tissues may more or less be relevant to the prevalent hormone resistance in *GNAS* mutations. Furthermore, because there are four genes encoding the regulatory subunits of PKA (RIa, RIb,

RIIa, and RIIb) and three genes encoding the catalytic subunits (14), such redundancy would also be related to the apparently mild hormone resistance in *PRKAR1A* mutations. However, these factors are unlikely to account for the apparently severe skeletal lesion in *PRKAR1A* and *PDE4D* mutations. Furthermore, although aberrant signaling via PTHrP receptor belonging to the GPCR families may play an important role in the development of skeletal lesions in *PRKAR1A* mutations, this perturbation is also relevant to the occurrence of skeletal lesions in *GNAS* abnormalities (15). Thus, there may be a hitherto unknown factor involved in the development of severe skeletal phenotype in *PRKAR1A* and *PDE4D* mutations. Notably, hormone resistance is apparently infrequent in acrodysostosis (2). It remains to be clarified whether acrodysostosis with and without hormone resistance may represent genetically heterogeneous conditions, and whether hormone resistance may have been overlooked or remained at a subclinical level in a certain fraction of patients with *PRKAR1A* and *PDE4D* mutations.

For *PRKAR1A*, more than 100 different mutations have been identified in Carney complex with multiple neoplasias and lentiginosis (16). Because most mutations reported in Carney complex are frameshift, nonsense, and splice mutations that are predicted to undergo nonsense-mediated mRNA decay and cause *PRKAR1A* haploinsufficiency, they would result in the increased amount of the free-lying intracellular catalytic subunits, leading to excessive PKA signaling in target tissues (16, 17). Furthermore, other types of mutations have also been identified in Carney complex, such as missense mutations at the cAMP-binding domain A (*e.g.* p.D183Y and p.A213D) and an in-frame deletion of 53 amino acids from the binding domain A (c.708 + 1 g→t) (16). Thus, in conjunction with the results of this study, we presume that *PRKAR1A* mutations can cause a mirror image of disorders in terms of the PKA activity, *i.e.* Carney complex resulting from defective association between the regulatory and the catalytic subunits and acrodysostosis with hormone resistance ascribed to impaired dissociation between the two subunits.

In summary, we identified a heterozygous *PRKAR1A* mutation affecting cAMP-mediated GPCR signaling in a patient with acrodysostosis with hormone resistance. Additional studies will permit a better clarification of the underlying causes in acrodysostosis with and without hormone resistance.

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Mamld1 Deficiency Significantly Reduces mRNA Expression Levels of Multiple Genes Expressed in Mouse Fetal Leydig Cells but Permits Normal Genital and Reproductive Development

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Although mastermind-like domain containing 1 (*MAMLD1*) (*CXORF6*) on human chromosome Xq28 has been shown to be a causative gene for 46,XY disorders of sex development with hypospadias, the biological function of *MAMLD1*/*Mamld1* remains to be elucidated. In this study, we first showed gradual and steady increase of testicular *Mamld1* mRNA expression levels in wild-type male mice from 12.5 to 18.5 d postcoitum. We then generated *Mamld1* knockout (KO) male mice and revealed mildly but significantly reduced testicular mRNA levels (65–80%) of genes exclusively expressed in Leydig cells (*Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, and *Insl3*) as well as grossly normal testicular mRNA levels of genes expressed in other cell types or in Leydig and other cell types. However, no demonstrable abnormality was identified for cytochrome P450 17A1 and 3β-hydroxysteroid dehydrogenase (HSD3B) protein expression levels, appearance of external and internal genitalia, anogenital distance, testis weight, Leydig cell number, intratesticular testosterone and other steroid metabolite concentrations, histological findings, *in situ* hybridization findings for *sonic hedgehog* (the key molecule for genital tubercle development), and immunohistochemical findings for anti-Müllerian hormone (Sertoli cell marker), HSD3B (Leydig cell marker), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (germ cell marker) in the KO male mice. Fertility was also normal. These findings imply that *Mamld1* deficiency significantly reduces mRNA expression levels of multiple genes expressed in mouse fetal Leydig cells but permits normal genital and reproductive development. The contrastive phenotypic findings between *Mamld1* KO male mice and *MAMLD1* mutation positive patients would primarily be ascribed to species difference in the fetal sex development. (***Endocrinology* 153: 6033–6040, 2012**)

Mastermind-like domain containing 1 (*MAMLD1*) (alias *CXORF6*) on human chromosome Xq28 is a causative gene for 46,XY disorders of sex development (DSDs) with hypospadias as a salient clinical phenotype. Indeed, several pathologic nonsense and frameshift mutations (p.E124X, p.Q197X, p.R653X, and p.E109fsX121) have been identified in patients with various types of hypospadias with and without other associated genital abnormalities, such as micropenis and cryptorchidism (1–3). In addition, a specific polymorphism(s) and a haplotype of *MAMLD1* appear to constitute a genetic risk factor for hypospadias (2, 4, 5).

To date, several important findings have been revealed for *MAMLD1* and its mouse homolog *Mamld1*. First, the

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Abbreviations: Ab, Antibody; AGD, anogenital distance; AGI, AGD index; CYP17A1, cytochrome P450 17A1; dpc, days postcoitum; DSD, disorder of sex development; HSD3B, 3β-hydroxysteroid dehydrogenase; KO, knockout; *MAMLD1*, mastermind-like domain containing 1; MLTC, mouse Leydig tumor cell; *Shh*, *sonic hedgehog*; siRNA, small interfering RNA; T, testosterone; WT, wild type.

upstream region of *MAMLD1/Mamld1* harbors a putative binding site for *NR5A1* (alias *SF-1* and *AD4BP*) (6) that regulates the transcription of a vast array of genes involved in sex development (7). Second, nuclear receptor subfamily 5, group A, member 1 protein can bind to the putative target site and exert a transactivation function for *Mamld1* (6). Third, *Mamld1* is clearly coexpressed with mouse *Nr5a1* in fetal Leydig and Sertoli cells in the fetal testis (1). Fourth, transient *Mamld1* knockdown using small interfering RNAs (siRNAs) significantly reduces *Cyp17a1* expression (8) and testosterone (T) production in cultured mouse Leydig tumor cells (MLTCs) (6, 8). These findings imply that *MAMLD1/Mamld1* is involved in the molecular network for T production probably via the transactivation of *CYP17A1/Cyp17a1* under the regulation of *NR5A1* and that *MAMLD1* mutations result in 46,XY DSD phenotype with hypospadias primarily because of compromised, but not abolished, T production around the critical period for sex development.

However, the biological function of *MAMLD1/Mamld1* during testis development remains to be elucidated. Thus, we examined testicular *Mamld1* mRNA expression pattern in wild-type (WT) male mice and performed molecular and phenotypic analyses in *Mamld1* knockout (KO) male mice.

Materials and Methods

WT and *Mamld1* KO male mice

We examined WT male mice of the C57BL/6 strain purchased from Sankyo Labo Service Corp., Inc. (Tokyo, Japan) and *Mamld1* KO male mice generated by Macrogen, Inc. (Seoul, Korea). This study was approved by the Animal Ethics Committee of National Research Institute for Child Health and Development.

Mamld1 KO male mice were produced by a standard gene-targeting procedure (9). In brief, a targeting vector was designed to replace *Mamld1* exon 3, which harbors a translation start codon and approximately two thirds of the coding sequence, with a *PGK-neo* cassette (Fig. 1A). After transfection of the targeting vector into 129/Sv embryonic stem cells by electroporation, two clones of recombination-positive embryonic stem cells were selected by Southern blot analysis using probes at the 5′ and 3′ flanking regions of *Mamld1* and injected into blastocysts. The blastocysts were then transferred into pseudopregnant ICR female mice, to generate chimeric male mice. The chimeric male mice were mated with C57BL/6 female mice, and germline transmission of the mutant gene was confirmed by Southern blot analysis. Subsequently, *Mamld1* KO male mice were produced by mating heterozygous (+/−) female mice with WT male mice. The *Mamld1* KO mouse strain was backcrossed with the C57BL/6 strain and maintained for multiple generations by cross-mating between heterozygous (+/−) female mice and WT male mice.

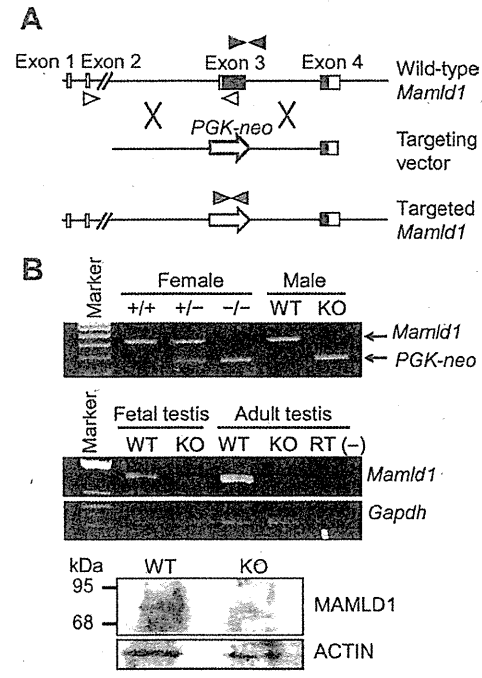

FIG. 1. Generation of *Mamld1* KO mice. A, Schematic representation of the gene targeting procedure. Exon 3 of WT *Mamld1* was replaced by the *PGK-neo* cassette (*PGK-neo*) through homologous recombination indicated by *cross symbols*. The *black and white boxes* denote the coding regions and the untranslated regions, respectively. *Paired black, white, and gray arrowheads* indicate the primer set for amplification of WT *Mamld1* genomic sequence, that for amplification of *Mamld1* transcripts, and that for amplification of Neomycin-resistant gene. B, Confirmation of *Mamld1* KO. Genotyping analysis (*upper panel*), RT-PCR analysis (*middle panel*), and Western blot analysis (*lower panel*) are consistent with successful *Mamld1* KO. +/+, WT female mice; +/−, heterozygous female mice; −/−, homozygous female mice; RT (−), negative control without reverse transcriptase.

In this study, KO male mice of the ninth generation were examined. The noon of the day when a vaginal plug was observed was designated 0.5 d postcoitum (dpc). PCR-based genotyping analysis with tail tissue genomic DNA was performed for *Mamld1*, *PGK-neo*, and *Sry*, using primers shown in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. Body weight and testis weight were measured at birth.

Genital and testicular sample preparation

In the male mice, androgen synthesis starts after approximately 13.5 dpc (10, 11), and morphological characteristics of the male external genitalia are established around 16.5 dpc (12, 13). Thus, genital and testicular samples were prepared from genotype- and embryonic day-matched KO male mice and their WT littermates in the latter half of the fetal life and at birth.

Real-time RT-PCR analyses

Testes from three mice were pooled in a single tube, and five tubes were prepared for each embryonic day. Total RNA was extracted from homogenized samples using ISOGEN (Nippongene, Tokyo, Japan), and cDNA was synthesized from 200 ng of total RNA using High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA). Real-time RT-PCR was per-

formed for *Mamld1* and 17 genes involved in sex development and expressed in the fetal testis (*Amh*, *Ar*, *Arx*, *Cyp11a1*, *Cyp17a1*, *Ddx4*, *Dhh*, *Dlx5*, *Dlx6*, *Gata4*, *Hsd17b3*, *Hsd3b1*, *Insl3*, *Nr5a1*, *Ptch1*, *Sox9*, and *Star*) as well as *Gapdh* used as an internal control, using the ABI 7500 Fast real-time PCR system (Life Technologies) and TaqMan gene expression assay kit. Primers and probes used are shown in Supplemental Table 2.

Western blot analysis

Testes collected as described above were homogenized, diluted in Laemmli buffer, and heated at 95 C. Protein extracts were subjected to a standard SDS-PAGE (12% gel) and were hybridized with anti-MAMLD1-antibody (Ab), anti-cytochrome P450 17A1(CYP17A1)-Ab, and anti-3β-hydroxysteroid dehydrogenase (HSD3B)-Ab, as well as anti-ACTIN-Ab (A2066; Sigma, St. Louis, MO) used as an internal control. Anti-MAMLD1-Ab was generated against mouse MAMLD1 peptide (CGSESFLPGSSFAHE) using rabbits, anti-CYP17A1-Ab was purchased from Santa Cruz Biotechnology, Inc. (sc-46081; Santa Cruz, CA), and anti-HSD3B-Ab was as reported previously (14). Chemiluminescence signals were detected using ECL Plus Western Blot Detection kit (GE Healthcare UK Ltd., Buckinghamshire, UK), and signal densities were assessed using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Stereoscopic observation

Morphological findings of external and internal genital regions were examined, as were anogenital distance (AGD) (the distance between the anus and the penoscrotal junction) and AGD index (AGI) (AGD divided by body weight) as indicators for the androgen action during the embryonic period (15–17). Furthermore, whole mount *in situ* hybridization was performed for *sonic hedgehog* (*Shh*), one of the key molecules for the development of genital tubercle (18, 19), using an antisense cRNA fragment as a probe (GenBank accession no. BC063087; nucleotide position, 138-1499). Sense cRNA was used as a negative control. Hybridization was performed using the Wilkinson procedure (20), and signals were visualized with the BM Purple AP Substrate (Roche, Mannheim, Germany).

Histological and immunohistochemical examinations

Histological examination was performed for tissue samples that were fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. Serial 6-μm sections were mounted on Superfrost slides, and every tenth section was stained with hematoxylin-eosin.

Immunohistochemical examination was carried out for the remaining section slides that were deparaffinized and incubated with 3% H_2O_2 in PBS to inactivate endogenous peroxidases. The slides were then incubated in blocking solution (Roche) and transferred into a new solution containing polyclonal primary Abs against anti-Mülerian hormone (sc-46081; Santa Cruz Biotechnology, Inc.) as a marker for Sertoli cells, HSD3B as a marker for Leydig cells, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (ab13840; Abcam, Cambridge, UK) as a marker for germ cells, and proliferating cell nuclear antigen (PC10; Dako, Glostrup, Denmark) as a marker for proliferating cells. The samples were washed and incubated with secondary Abs conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.). The Simple Stain DAB Solution (Nichirei, Tokyo, Japan) was used for color development. Apoptotic cells were detected by terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling staining using an *In Situ* Apoptosis Detection kit (TaKaRa Bio, Shiga, Japan). Furthermore, HSD3B-positive cells in four randomly selected fields of each testis were counted, to estimate the number of Leydig cells.

Measurement of intratesticular T and steroid metabolites

Intratesticular T and steroid metabolites were measured at 18.5 dpc by liquid chromatography tandem mass spectrometry (ASKA Pharma Medical, Kanagawa, Japan) using samples stored at −80 C, because intratesticular T usually peaks at 18.5 dpc in normal mice (10, 11).

Cross-mating experiments

Cross-mating was performed between *Mamld1* KO male mice and WT or heterozygous (+/−) female mice and between WT male mice and WT or heterozygous (+/−) female mice.

Statistical analysis

The data are expressed as the mean ± SEM. Statistical significance of the mean between two groups was examined by Student's *t* test, and that of the frequency between two groups was examined by χ^2 test. $P < 0.05$ was considered significant.

Results

Mamld1 expression in the fetal testis of WT male mice

Real-time RT-PCR analyses indicated a gradual and steady increase in the *Mamld1* mRNA levels from 12.5 to 18.5 dpc (Fig. 2).

Generation of *Mamld1* KO male mice

Mamld1 KO male mouse was successfully produced. *Mamld1* exon 3 was deleted from the genome of the KO

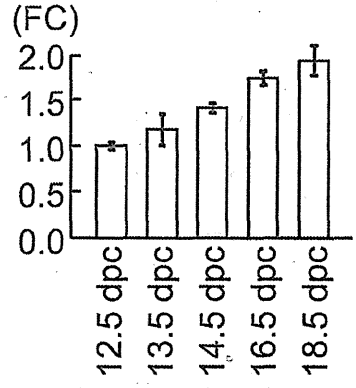

FIG. 2. Testicular *Mamld1* expression levels during the latter half of the fetal life in WT male mice. Figure indicates the data obtained by real-time RT-PCR analyses. Fold change (FC) represents relative mRNA levels of *Mamld1* against *Gapdh*. The relative expression level of *Mamld1* mRNA at 12.5 dpc was designated as 1.0.

TABLE 1. Comparison between *Mamld1* KO mice and their WT littermates

	KO	WT	*P* value
Body weight (g) (at birth)	1.48 ± 0.03 (n = 10)	1.44 ± 0.03 (n = 10)	0.40
AGD (mm) (at birth)	1.33 ± 0.02 (n = 10)	1.32 ± 0.02 (n = 10)	0.62
AGI (mm/g) (at birth)	0.90 ± 0.02 (n = 10)	0.92 ± 0.02 (n = 10)	0.55
Leydig cells (HSD3B-stained cells) (number/HPF) (at 14.5 dpc)	69.3 ± 8.2 (n = 3)	75.1 ± 7.6 (n = 3)	0.63
Testis weight (mg) (at birth)	1.46 ± 0.08 (n = 10)	1.35 ± 0.08 (n = 10)	0.34
Intratesticular steroid metabolites (at 18.5 dpc)			
Pregnenolone (pg/two testes)	17.9 ± 4.0 (n = 4)	15.4 ± 1.4 (n = 4)	0.57
Progesterone (pg/two testes)	16.5 ± 4.6 (n = 4)	15.0 ± 1.7 (n = 4)	0.56
17-OH pregnenolone (pg/two testes)	15.2 ± 2.9 (n = 4)	15.4 ± 1.3 (n = 4)	0.77
17-OH progesterone (pg/two testes)	10.4 ± 1.7 (n = 4)	13.5 ± 2.5 (n = 4)	0.15
Androstenedione (ng/two testes)	0.44 ± 0.15 (n = 4)	0.51 ± 0.07 (n = 4)	0.25
T (ng/two testes)	2.31 ± 0.30 (n = 4)	2.38 ± 0.31 (n = 4)	0.89

Expressed as mean ± SEM. HPF, High power field (234.1 × 175.5 μm).

mice, and neither *Mamld1* mRNA nor MAMLD1 protein was identified in the testis of the KO mice (Fig. 1B). Body weight was comparable between the KO male mice and their WT littermates (Table 1).

Gene and protein expression pattern in the fetal testes of *Mamld1* KO mice

The results are shown in Fig. 3. Relative mRNA levels of *Cyp17a1*, *Hsd3b1*, and *Insl3* mRNAs were mildly but

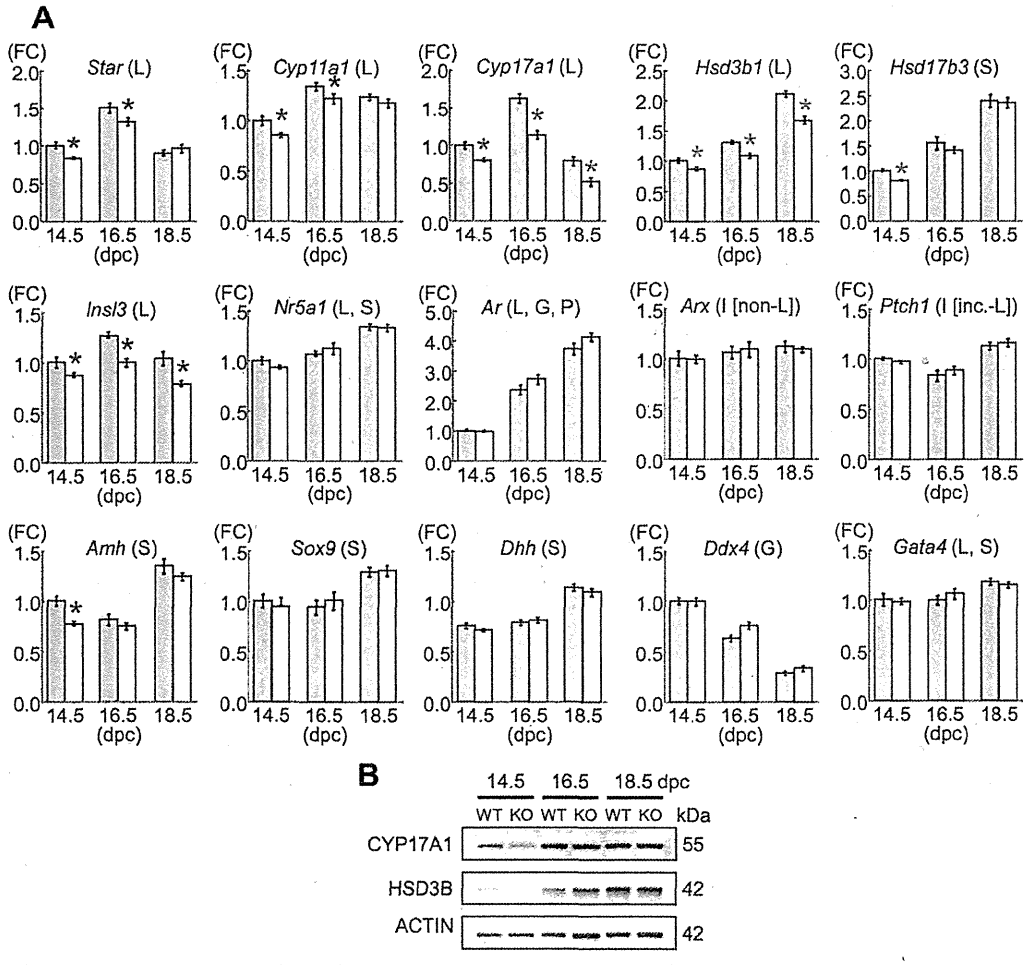

FIG. 3. Gene and protein expression patterns in the fetal testes. A, Relative mRNA levels of examined genes against *Gapdh*. FC, Fold change; L, Leydig cells; S, Sertoli cells; G, germ cells; P, peritubular cells; I [non-L], interstitial cells excluding Leydig cells; I [inc.-L], interstitial cells including Leydig cells. The *green and the yellow bars* indicate the data obtained from WT male mice and *Mamld1* KO male, respectively. For each gene, the relative expression level of mRNA in WT male mice at 14.5 dpc was designated as 1.0. *Red asterisks* indicate significant results ($P < 0.05$). B, Western blot analysis for CYP17A1 and HSD3B, as well as for ACTIN.

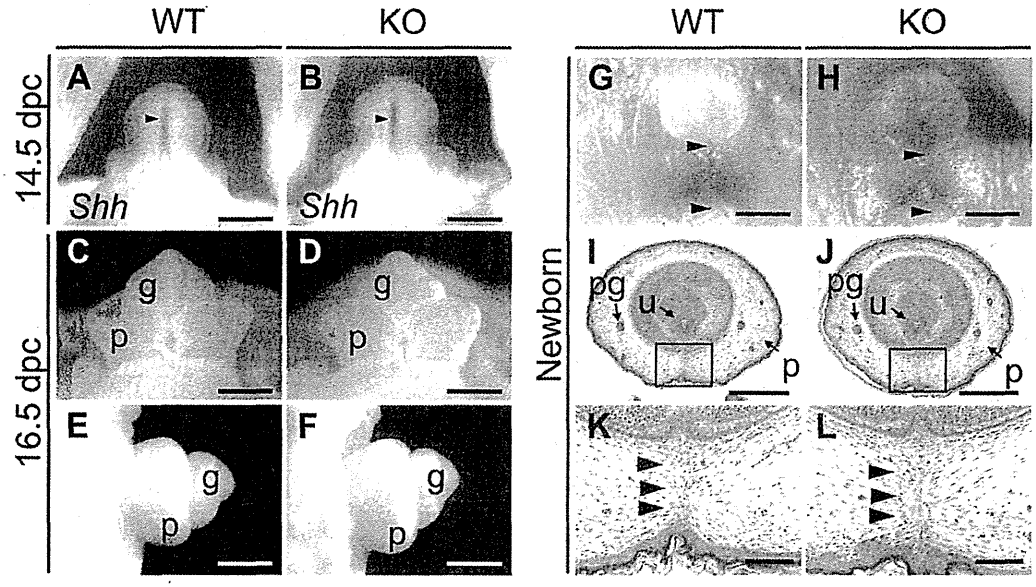

FIG. 4. External genitalia of WT and *Mamld1* KO male mice. A and B, Whole mount *in situ* hybridization for *Shh* (*arrowheads*) in the developing genital region at 14.5 dpc. C–F, Appearance of the genital tubercle at 16.5 dpc. G and H, Appearance of the external genitalia at birth. The distance between the anus and the penoscrotal junction (*arrowheads*) represents the AGD. I–L, Histological findings of the external genitalia at birth. *Arrowheads* in K and L indicate the fused prepuce. g, Glans; p, prepuce; pg, preputal gland; u, urethra. *Scale bars*: 500 μm (A–F, I, and J), 1 mm (G and H), and 100 μm (K and L).

significantly lower in the KO male mice than in their WT littermates at 14.5, 16.5, and 18.5 dpc, as were those for *Star* and *Cyp11a1* at 14.5 and 16.5 dpc (65–80%) (*Dlx5* and *Dlx6* expression levels were extremely low). By contrast, relative mRNA levels of the remaining genes were comparable between the KO male mice and their WT littermates, except for relative mRNA levels of *Hsd17b3* and *Amh* at 14.5 dpc. However, expression levels of CYP17A1 and HSD3B proteins were similar between the KO male mice and their WT littermates and were obviously higher at 16.5 and 18.5 dpc than at 14.5 dpc.

External genital findings of *Mamld1* KO male mice

External genitalia were obviously normal in the *Mamld1* KO male mice (Fig. 4 and Table 1). *Shh* was normally expressed in the urethral epithelium of the KO male mice at 14.5 dpc, and subsequent outgrowth of genital tubercle and fusion of the urethral folds at the ventral midline occurred in the KO male mice at the same embryonic stages as in their WT littermates. Furthermore, external genitalia were normally developed at birth, with the comparable AGD and AGI between the KO mice and their WT littermates.

Internal genital findings of *Mamld1* KO mice

Internal genitalia of the *Mamld1* KO male mice were also free from demonstrable abnormality (Fig. 5 and Table 1). Intraabdominal testicular descent, wolffian development, and müllerian regression were normally observed in the KO male mice at 16.5 dpc. Testicular histological findings were comparable between the KO mice and their WT littermates at 14.5 dpc and at birth. Immunohistochemical findings indicated the presence of similar numbers of Sertoli cells (anti-Müllerian hormone-stained cells), Leydig cells (HSD3B-stained cells), and germ cells [DEAD (Asp-Glu-Ala-Asp) box polyoeotide 4-stained cells] at 14.5 dpc as well as the presence of a similar number of Leydig cells (HSD3B-stained cells) at birth between the KO mice and their WT littermates. A relatively large number of mitotic cells (proliferating cell nuclear antigen-stained cells) was also identified in both the KO mice and their WT littermates, as were a small number of apoptotic cells (terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling-stained cells) (data not shown). In addition, testis weights at birth and intratesticular concentrations of T and other steroid metabolites at 18.5 dpc were also similar between the KO mice and their WT littermates.

Cross-mating experiments

The results are shown in Table 2. *Mamld1* KO male mice produced offspring with WT and heterozygous (+/−) female mice, as did WT male mice. Furthermore, the frequency of littermate offspring [*Mamld1* KO male mice, WT male mice, homozygous (−/−) female mice, heterozygous (+/−) female mice, and WT female mice] was in agreement with the expected Mendelian mode of inheritance.