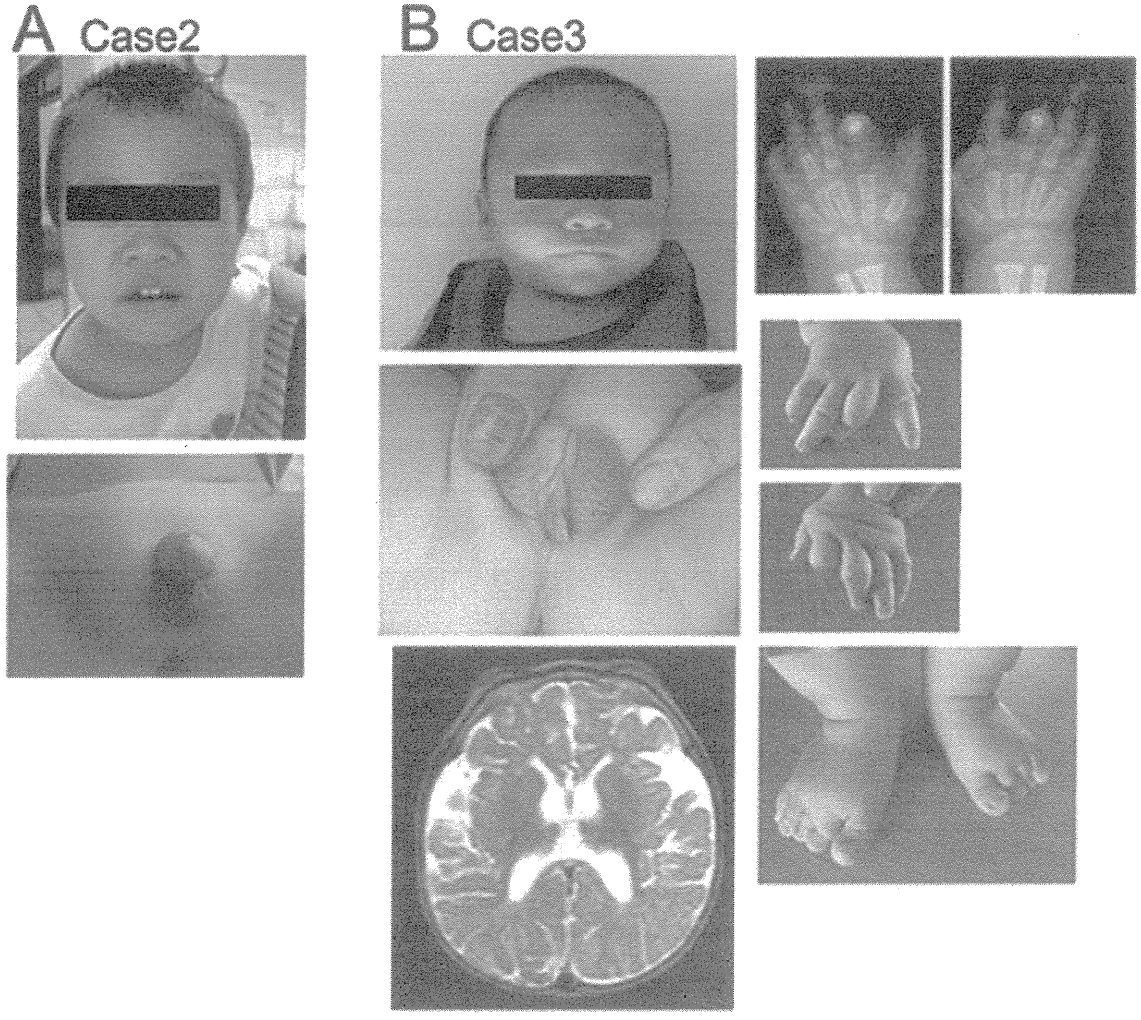

Figure 2. Clinical features of cases 2 and 3. A. Clinical findings of case 2 at 4.5 years of age. Images of the craniofacial region and external genitalia (after surgical intervention) are shown. B. Clinical findings of case 3 at 11 months of age. Multiple facial dysmorphisms and limb anomalies including syndactyly and camptodactyly are shown. Brain magnetic resonance imaging indicates delayed myelination, hypogenesis of the corpus callosum and prominent ventricular and CSF spaces. The parents of cases 2 and 3 have given written informed consent, as outlined in the PLOS consent form, to publication of the photographs of the patients.
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a role in social behavior [21]. Lack of social dysfunction in case 2 indicates that haploinsufficiency of *OXT* and *AVP* permits normal psychosocial development at least in childhood. However, this notion awaits further investigation.

Case 3 had a ~18.0 Mb interstitial deletion at 2q31.1–32.1. Clinical manifestations of case 3 including finger/toe anomalies, mental retardation and facial dysmorphism are compatible with the 2q31 microdeletion syndrome, a well-established contiguous gene deletion syndrome [22]. Notably, abnormal formation of the external genitalia has been reported in both male and female patients carrying 2q31 deletions [23], [24]. Previous studies have attributed the skeletal anomalies of 2q31 microdeletion syndrome to haploinsufficiency of the *HOXD* cluster [22], [25], and mental retardation and craniofacial abnormalities to deletions of certain genes located within the genomic interval spanning 174–175 Mb from the 2q telomere [22] (Fig. 3C). In this regard, while skeletal abnormalities are obviously milder in case 3 than the previously reported patients with deletions involving *HOXD* genes [25], this would be consistent with the assumption that haploinsufficiency of developmental genes is frequently associated with a broad phenotypic spectrum [26]. Since mouse *Hoxd* genes have been shown to play a role in the formation of external genitalia by regulating multiple target genes, genital abnormalities of 2q31 microdeletion syndrome could be associated with haploinsufficiency of *HOXD* genes [25], [27]. Indeed, the phenotype of case 3, such as hypomasculinized external genitalia without cryptorchidism and a normal blood testosterone value at birth, is indicative of perturbed organogenesis of the external genitalia rather than impaired hormone production in the gonads. However, since DSD has been described for only a small subset of males with 2q31 deletions [22], [23], [24], [25], impaired sex development in case 3 may be caused by other unknown genetic or environmental factors.

In summary, we identified cryptic genomic rearrangements in three of 24 individuals with 46,XY DSD. It appears that the genital abnormalities of case 1 result from gonadal dysgenesis due

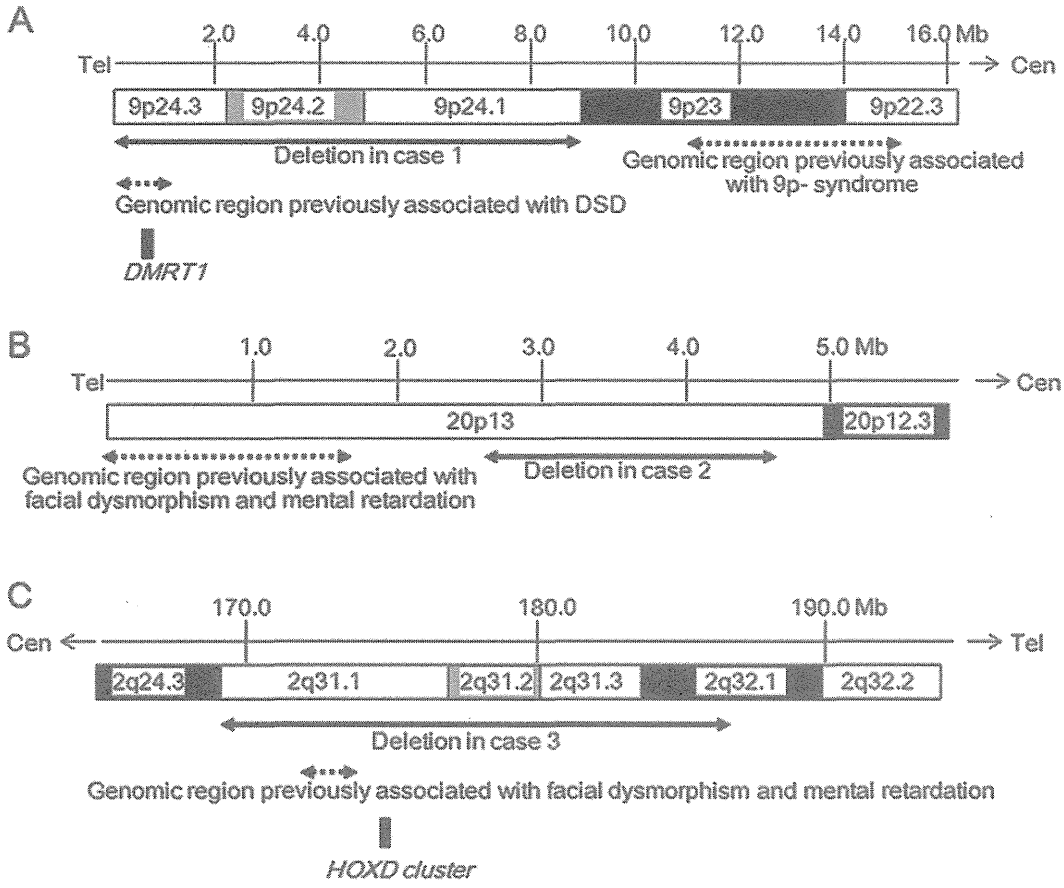

Figure 3. Schematic representation of the genomic regions around the deletions. A. Terminal part of the short arm of chromosome 9. The black arrow denotes the deletion identified in case 1. The dotted arrows indicate the genomic intervals associated with DSD and for 9p- syndrome [13]. The black box indicates the position of *DMRT1* that is likely to be associated with DSD in case 1. B. Terminal part of the short arm of chromosome 20. The black arrow denotes the deletion in case 2. The dotted arrow indicates the genomic region associated with facial dysmorphism and mental retardation [20]. C. The 2q24.3–2q32.2 region. The black arrow denotes the deletion in case 3. The dotted arrow indicates the genomic region associated with facial dysmorphism and mental retardation [22]. The black box indicates the position of the *HOXD* cluster possibly associated with DSD in case 3.
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to haploinsufficiency of *DMRT1*, while those of case 3 can be ascribed to perturbed organogenesis due to the deletion of the *HOXD* cluster. These data suggest that submicroscopic deletions can lead to various types of 46,XY DSD that occur as components of contiguous gene deletion syndromes. Moreover, the results obtained from case 2 provide a novel candidate locus for 46,XY DSD at 20p13. Further copy-number analyses on patients with 46,XY DSD and functional assays for genes involved in the genomic rearrangements will help to clarify novel causative mechanisms for 46,XY DSD.

Author Contributions

Conceived and designed the experiments: VCD KK YK TO MF. Performed the experiments: MI ES KN. Analyzed the data: MI ES TO MF. Contributed reagents/materials/analysis tools: VCD SI MN KM YH KK YK. Wrote the paper: TO MF.

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MAMLD1 and 46,XY Disorders of Sex Development

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Abstract

MAMLD1 (mastermind-like domain containing 1) is a recently discovered causative gene for 46,XY disorders of sex development (DSD), with hypospadias as the salient clinical phenotype. To date, microdeletions involving *MAMLD1* have been identified in six patients, and definitive mutations (nonsense and frameshift mutations that are predicted to undergo nonsense mediated mRNA decay [NMD]) have been found in six patients. In addition, specific *MAMLD1* cSNP(s) and haplotype may constitute a susceptibility factor for hypospadias. Furthermore, in vitro studies have revealed that (1) the mouse homolog is expressed in fetal Sertoli and Leydig cells around the critical period for sex development; (2) transient *Mamld1* knockdown results in significantly reduced testosterone production primarily because of compromised 17α-hydroxylation and *Cyp17a1* expression in Murine Leydig tumor cells; (3) MAMLD1 localizes to the nuclear bodies and transactivates the promoter activity of a non-canonical Notch target gene hairy/enhancer of split 3, without demonstrable DNA-binding capacity; and (5) *MAMLD1* is regulated by steroidogenic factor 1 (SF1). These findings suggest that the *MAMLD1* mutations cause 46,XY DSD primarily because of compromised testosterone production around the critical period for sex development. Further studies will provide useful information for the molecular network involved in fetal testosterone production.

Keywords
- *MAMLD1*
- 46
- XY DSD
- hypospadias
- testosterone

MAMLD1 (mastermind-like domain containing, 1), previously known as *CXORF6* (chromosome X open reading frame 6), is a recently discovered gene for 46,XY disorders of sex development (DSD) with abnormal external genitalia, especially hypospadias.[1] After the first report describing *MAMLD1* mutations in human 46,XY DSD, a remarkable progress has been made for *MAMLD1*. Here, we summarize the current knowledge about *MAMLD1*, including some hitherto unreported data.

Cloning of *CXORF6* as a Candidate Gene for 46, XY DSD

A gene for 46,XY DSD has been postulated around *MTM1* for myotubular myopathy on Xq28. Indeed, since genital development is normal in patients with intragenic *MTM1* mutations and invariably abnormal in six patients with microdeletions involving *MTM1* (patients 1–6 in ►**Table 1**),[2–5] this suggests that a gene for sex development resided in the vicinity of *MAM1*, and that loss or disruption of the putative sex development gene results in 46,XY DSD as a consequence of contiguous gene deletion syndrome.

In 1997, Laporte et al[6] identified a protein coding gene *CXORF6* from a 430-kb region deleted in two sporadic cases with myotubular myopathy and 46,XY DSD[2] (►**Fig. 1**). *CXORF6* consists of seven exons, and harbors a protein coding sequence on exons 3–6 that is predicted to produce two proteins of 701 and 660 amino acids because of in-frame alternative splicing with and without exon 4. Furthermore, subsequent studies have shown that *MAMLD1* is located

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Table 1 Genital findings in Patients with MAMLD1 Deletions or Mutations

Patient	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12
Deletion/Mutation	Deletion	Deletion	Deletion	Deletion	Deletion	Deletion	p.E124X	p.E124X	p.Q197X	p.R653X	p.E109fs121X	p.E109fs121X
Inheritance	Sporadic	Sporadic	Familial	Familial	Familial	Sporadic	Familial	Familial	Sporadic	Sporadic	Sporadic	Sporadic
Age at examination	Neonate	Neonate	Neonate	Neonate	Fetus	...	4 months	1 month	2 years	1 month	1 year	1 2/12 year
Ambiguity	Yes	No	No	No	No	No	No	No	No	No	No	No
Hypospadias	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
(Type)	Severe	Penoscrotal	Glandular	Penile	Penile	N.D.	Penoscrotal	Penoscrotal	Penoscrotal	Penoscrotal	Penile	Penoscrotal
Micropenis	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	No	No	Yes	Yes	No	No
Cryptorchidism	Yes (B)	N.D.	Yes (R)	Yes (B)	N.D.	N.D.	Yes (B)	No	No	Yes (B)	Yes (B)	No
Scrotal abnormalities	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	Yes	Yes	Yes	Yes	No	No
Other findings	Vaginal pouch											

Patients 1 & 2: cases 474 and 441 in Hu et al[2]; patient 3–5: cases III-1, III-2, and III-3 in Barsch et al[4]; patient 6: case CNM96 in Biancalana et al[5]; patients 7–10: cases 1–4 in Fukami et al[1]; and patients 11 and 12: cases 2 and 3 in Kalfa et al[11] N.D.: not described; B: bilateral; and R: right.

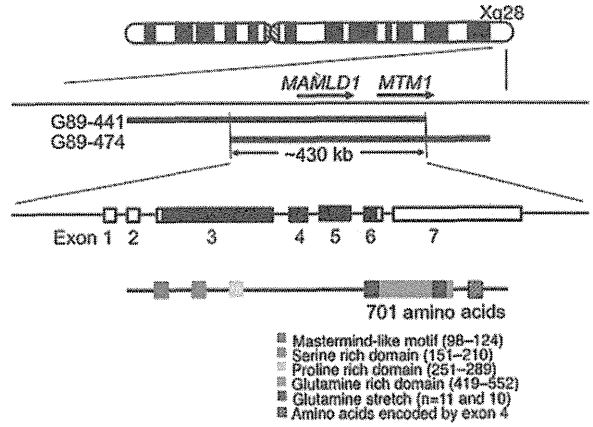

Figure 1 Positional cloning and the structure of *MAMLD1*. *MAMLD1* (*CXORF6*) has been isolated from a ~430 kb region commonly deleted in two patients with 46,XY DSD and myotubular myopathy (G89–441 and G89–474).[2] The horizontal bars indicate the deleted segments that involve *MAMLD1* and *MTM1* for myotubular myopathy. *MAMLD1* comprised 7 exons; the black and the white boxes represent the coding regions and the untranslated regions, respectively. MAMLD1 protein harbors mastermind-like domain and other characteristic domains.

within the smallest region of overlap in all patients with myotubular myopathy and 46,XY DSD,[7] and no other candidate gene for 46,XY DSD has been identified within the commonly deleted region. These findings imply that *MAMLD1* is an excellent candidate gene for 46,XY DSD.

MAMLD1 Mutations in 46,XY DSD Patients

The first evidence for *MAMLD1* being the causative gene for 46,XY DSD came from our group.[1] We performed direct sequencing for the coding exons 3–6 and their flanking splice sites of *MAMLD1* in 117 Japanese patients with various types of 46,XY DSD including 56 patients with hypospadias (16 with glandular type, 16 with penile type, 20 with pen scrotal type, and 4 with perineal type) associated with other external genital abnormalities, as well as in 49 European and Chinese patients with various types of abnormal genitalia ranging from hypospadias to feminized genitalia. Consequently, three nonsense mutations were identified in Japanese patients with hypospadias and other external abnormalities: p.E124X [Q9]on exon 3 in two maternally related half brothers, p.Q197X on exon 3 in a sporadic patient, and p.R653X on exon 5 in a sporadic patient (patients 7–10 in ←**Table 1**).[1] The mothers of families A and C were heterozygous for the mutations, although the mother of family B was not studied. Q9

The three nonsense mutations satisfy the conditions for the occurrence of nonsense mediated mRNA decay (NMD).[8] Consistent with this, RT-PCR [Q10]from leukocytes indicated drastically reduced transcripts for the three nonsense mutations.[1] Furthermore, the NMD was prevented by the NMD inhibitor cycloheximide, providing further support for the occurrence of NMD in the three nonsense mutations. The occurrence of NMD was also demonstrated in the carrier mothers.[9] Thus, although the NMD has not been confirmed in the testicular tissue, the results indicate that the three Q10

nonsense mutations are actually pathologic disease-causing mutations.

The occurrence of NMD would explain the apparently discordant genital phenotype between the patient with p.R653X and the Japanese patient with a microdeletion involving *MTM1* reported by Tsai et al.[10] In contrast to the p.R653X, the microdeletion resulted in the generation of a fusion gene between exons 1 to 4 of *MAMLD1* and exons 3 to[Q11] 16 of *MTMR1* (locus order: *MAMLD1–MTM1–MTMR1*) that escaped NMD and was expressed at least in the muscle. Thus, although both the cases retained *MAMLD1* exons 1 to 4 and were missing *MAMLD1* exons 5 to 7, the patient with p.R653X had 46,XY DSD because of NMD, and the patient with the microdeletion had apparently normal genital development because of its positive expression.

Subsequently, Kalfa et al[11] have identified p.E109fs121X (c.325delG) that is predicted to undergo NMD in two of 41 [Q12] 41[Q12] patients with hypospadias of variable degrees (patients 11 and 12 in ►**Table 1**). Furthermore, several mutations confirmed by functional studies have been identified to date (our unpublished observation).

Additional substitutions have also been identified in patients with 46,XY DSD. First, Kalfa et al[11] identified p.V432A and p.531ins3Q (expansion of the second polyglutamine domain from 10 to 13) in single sporadic patients. However, both variants were detected in normal individuals by subsequent examination.[12] In addition, we performed functional studies for p.V432A using Hes3 (see below), and found apparently normal transcriptional activity (►**Fig. 2**). Second, Chen et al[12] identified p.Q529K, which could affect splicing, in a patient with severe hypospadias. However, no functional studies have been performed for p.Q529K. Third, Brandao et al[13] detected p.H432Q, which interestingly appears to have an increased rather than a decreased function, in 4 of 50 patients with 46,XY DSD. However, this substitution is registered as a polymorphism at present, indicating the presence of this substitution in apparently normal individuals. Thus, there is no direct evidence for these substitutions being pathologic mutations. Rather, these substitutions appear to be variations rather than mutations. Nevertheless, p.531ins3Q, which may affect the three-dimensional protein structure, could function as a susceptibility factor, as has been shown for the polyglutamine expansion in exon 1 of *AR* for androgen receptor.[14]

Taken together, it is obvious that *MAMLD1* is a causative gene for 46,XY DSD with hypospadias as a salient phenotype, because of the identification of nonsense mutations and a frameshift mutation that should be subject to NMD. Furthermore, it might be possible that the identified substitutions may function as susceptibility factors.

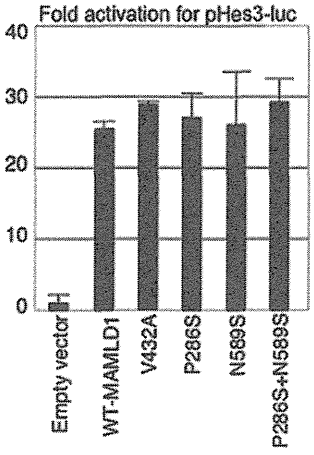

Figure 2 Functional studies for *MAMLD1* substitutions. The p.V432A, p.P286S, p.N589S, and p.P286S–p.N589S (S–S haplotype) have normal transactivating activities for the promoter of a non-canonical Notch target Hes3.

Phenotypes in Affected Patients

Genital findings in patients with microdeletions involving *MAMLD1* and in those with definitive intragenic *MAMLD1* mutations are shown in ►**Table 1**. Although detailed phenotypes are not examined in patients with microdeletions encompassing *MAMLD1*, affected patients almost invariably have hypospadias of variable degrees and often exhibit other genital features such as micropenis, cryptorchidism, and abnormal scrotum. Furthermore, patient 1 manifests rather ambiguous genitalia with virginal pouch, and patient 12 exhibits apparently isolated hypospadias phenotype. Thus, the phenotypic spectrum of *MAMLD1* mutations appears to be somewhat variable, with hypospadias as the core genital abnormality.

Detailed endocrine data are available in patients 7 to 10 in ►**Table 1**.[1] Serum testosterone was sufficiently high during the mini-puberty period, and response was well to human chorionic gonadotropin (hCG) stimulation during infancy to early childhood. This implies that *MAMLD1* mutations exert their deleterious effects primarily in the fetal period, as supported by the *Mamld1* [Q13]expression pattern in the fetal [Q13] and postnatal testes (see below). However, our long-term follow-up examinations have revealed that patients with *MAMLD1* mutations exhibit primary gonadal dysfunction in late childhood (our unpublished observation). This is consistent with weak but detectable *Mamald1* [Q14]expression in the [Q14] postnatal testis (our unpublished observation), and suggests deterioration in testicular function with age.

Expression Patterns of *MAMLD1*|*Mamld1*

In the human, PCR-based screening for cDNA samples has revealed ubiquitous expression of *MAMLD1* including fetal testis, with two in-frame splice variants, a major form with exon 4 and a minor form without exon 4.[1] Furthermore, RT-PCR analysis using human fetal testis has shown clear and gradually increasing expression of *MAMLD1* during the second trimester.[15]

More detailed expression studies have been performed in the mouse.[1] In situ hybridization (ISH) analysis has shown that, in the fetal testis, *Mamld1* is weakly expressed in the internal region at E11.5, and clearly expressed in Sertoli cells and in a small number of Leydig cells at E12.5. At E14.5, *Mamld1* is still clearly expressed in Sertoli cells and in the

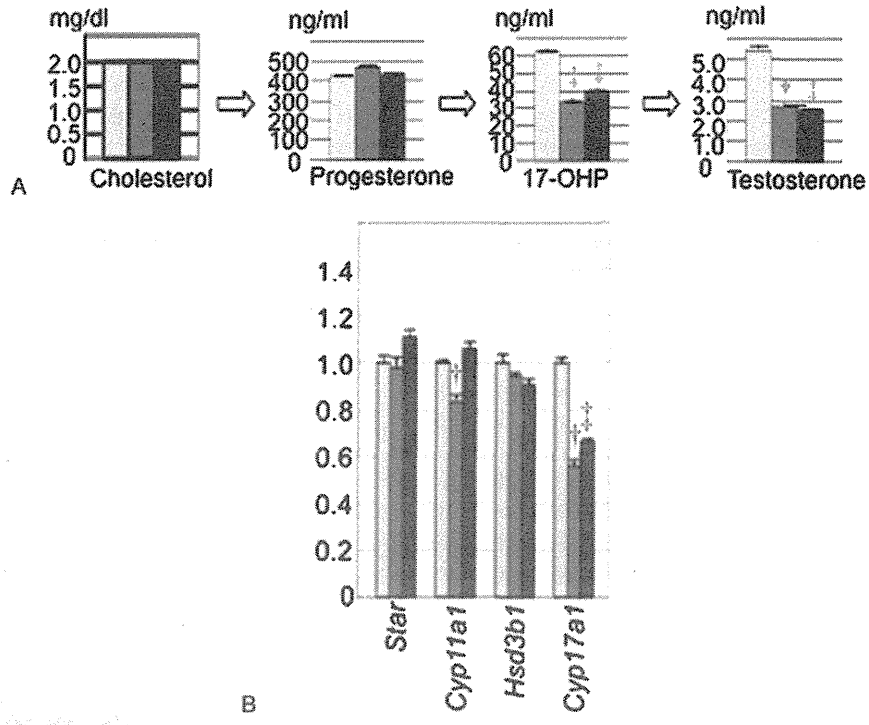

Figure 3 Representative data of the *Mamld1* knockdown experiments using MLTCs. Endogenous *Mamld1* expression has been markedly reduced to 10–15% by knockdown with si-RNAs. Shown are steroid metabolite concentrations in culture media and endogenous *Mamld1* expression levels in MLTCs. The white bars indicate the data obtained from MLTCs transfected with non-targeting RNA, and the light gray and the dark gray bars indicate the data obtained from MLTCs transfected with two different siRNAs.†: $P < 0.01$; and ‡: $P < 0.001$. (1) Representative steroid metabolite concentrations in culture media. (2) Real-time RT-PCR analysis for steroidogenic enzymes.

majority of Leydig cells. Such cell-type specific expression patterns were confirmed by co-localization of *Mamald1* mRNA and Nr5a1 (alias, steroidogenic factor 1 [SF1] or Ad4bp) protein as the marker for Sertoli and Leydig cells.[16,17] In the fetal ovary, *Mamld1* is expressed in a small number of somatic cells primarily at the boundary to the mesonephros at E11.5 and E12.5, and weakly expressed in a small number of somatic cells in the internal region at E14.5. In extragonadal tissues at E12.5, *Mamld1* is clearly expressed in the Müllerian ducts, forebrain, somite, neural tube, and pancreas, and weakly expressed in the external genital region. However, *Mamld1* expression is absent in the adrenals.

ISH analysis has revealed that, in the postnatal testis, *Mamld1* expression is weakly identified within the cords until one week of age and becomes faint thereafter; however, RT-PCT[Q15] analysis still detects clear expression of *Mamld1* in the postnatal testis (our unpublished observation). In the ovary, *Mamld1* expression is barely detected until 2 weeks of age and clearly identified in granulosa cells at the perifollicular regions of most of Graafian follicles at 3 and 8 weeks of age.

Relevance of *Mamld1* to Testosterone Production

The above data imply that MAMLD1 is involved in the testosterone production in the critical period for sex development during fetal life, and that *MAMLD1* deletions/mutations cause hypospadias primarily because of compromised testosterone production around the critical period for sex development. In this context, there are two major possibilities how *MAMLD1* mutations lead to compromised testosterone production: (1) compromised steroidogenic activity in Leydig cells; and (2) reduced proliferation of Leydig cells. To test which of the two possibilities is more relevant, we performed knockdown analysis with two different siRNAs for *Mamld1*, using mouse Leydig tumor cells (MLTCs[Q16]).[18] MLTCs are known to have the capacity to produce testosterone primarily via Δ^4-pathway, although the amount of testosterone production remains small primarily because of low 17α-hydroxylase and Hsd17b3 activities.[19] MLTCs are also known to retain responsiveness to hCG.[19–21]

Representative data of the steroidogenic activity are shown in ←**Fig. 3**; the data were obtained at 48 hours after the incubation of siRNA-transfected and non-transfected MLTCs followed by stimulation with hCG (for details, see reference[18]). The concentrations of pregnenolone and progesterone remained comparable between the culture media with siRNA-transfected MLTCs and those with nontargeted MLTCs, whereas the concentrations of 17-OH pregnenolone, 17-OH progesterone, dehydroepiandrosterone, androstenedione, and testosterone were significantly lower (~50–60%) 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 the Δ^4-pathway dominant testosterone 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. Consistent with these findings, real-time RT-PCR and microarray analyses showed significantly decreased *Cyp17a1* expression (~70%) in siRNA-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. By contrast, the proliferation capacity, which was examined for 120 hours, was comparable between siRNA-transfected MLTCs and non-transfected MLTCs.

These results imply that *MAMLD1* is involved in testosterone production via augmenting CYP17A1 (17α-hydroxylase) activity. In this regard, it is noteworthy that *Mamld1* is clearly expressed in fetal Leydig and Sertoli cells and is barely expressed in adrenal cells,[1,7] and that 17α-hydroxylase activity is indispensable for testosterone production in Leydig cells.[22] Thus, it appears likely that *Mamld1* enhances *Cyp17a1* expression primarily in Leydig cells, permitting the production of a sufficient amount of testosterone for male sex development. In addition, since the expressions of other genes involved in testosterone production and insulin-like 3 biosynthesis were not clearly affected in siRNA-transfected MLTCs,[18] this would argue against the possibility that *Mamld1* knockdown causes a global dysfunction of MLTCs, resulting in testosterone hyposecretion.

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.[22] 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.[22] 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.[19] Such a unique property of MLTCs may be relevant to the preferential impairment of 17α-hydroxylase activity in siRNA-transfected MLTCs. Thus, although the in vitro data strongly argue for a positive role of *MAMLD1* in testosterone production, further studies are necessary to examine the precise in vivo function of *MAMLD1*.

Transcriptional Regulation of *MAMLD1*/*Mamld1* by *NR5A1*/*Nr5a1*

Mouse *Mamld1* is coexpressed with *Nr5a1* (Sf1), and *NR5A1*/*Nr5a1* is known to regulate the transcription of a vast array of genes involved in sex development, by binding to specific DNA sequences.[16,17] This implies that *MAMLD1*/*Mamld1* is also controlled by *NR5A1*/*Nr5a1*. Consistent with this notion, human *MAMLD1* harbors a putative NR5A1 binding sequence "CCAAGGTCA" at intron 2 upstream of the coding region, and mouse *Mamld1* also carries a putative Nr5a1 binding site at intron 1 upstream of the coding region.[9] Furthermore, we performed DNA binding and luciferase assays, showing that NR5A1 protein binds to the putative target sequence and exerts a transactivation function.[9] These findings argue for the possibility that *MAMLD1*/*Mamld1* expression is regulated by *NR5A1*/*Nr5a1*.

In Vitro Function of MAMLD1 Protein

MAMLD1 protein has a unique structure with homology to that of mastermind like 2 (MAML2) protein (←**Fig. 1**).[9] In particular, both MAMLD1 and MAML2 contain a unique amino acid sequence to which we designate mastermind-like (MAML) motif. The MAML motif was well conserved among MAMLD1 orthologs identified in frog, bird, and mammals. In addition, glutamine-, proline-, and serine-rich domains reside on MAMLD1.

MAML2 is a non-DNA binding transcriptional co-activator in Notch signaling that plays an important role in cell differentiation in multiple tissues by exerting either inductive or inhibiting effects according to the context of the cells.[23–25] Upon ligand-receptor interaction, Notch intracellular domain (N-ICD) is translocated from the cell surface to the nucleus and interacts with a DNA-binding transcription factor, recombination signal binding protein-J (RBP-J), to activate target genes like hairy/enhancer of split 1 (*Hes1*) and *Hes5*.[26] In this canonical Notch signaling process, MAML2 forms a ternary complex with N-ICD and RBP-J at nuclear bodies, enhancing the transcription of the Notch target genes.[23,24,27–29] In addition to such canonical Notch target genes, recent studies have shown that *Hes3* can be induced by stimulation with a Notch ligand, via a STAT3 mediated pathway.[30] This finding, together with lack of *Hes3* induction by N-ICD,[25] implies that *Hes3* represents a target gene of a non-canonical Notch signaling.

Thus, we have performed functional studies of wildtype MAMLD1 in terms of Notch signaling, thereby revealing several findings.[9] First, MAMLD1 is distributed in a speckled pattern and co-localized with the MAML2 protein in the nuclear bodies. Second MAMLD1, as well as MAML2, is unlikely to have a DNA-binding capacity. Third, although MAMLD1 is incapable of enhancing the promoter activities of the canonical Notch target genes *Hes1* and *Hes5* with the RBP-J binding site, MAMLD1 transactivates the promoter activity of the non-canonical Notch target gene *Hes3* without the RBP-J binding site. These findings imply that MAML2 and MAMLD1 may have derived from a common ancestor and evolved as a co-activator for the canonical and the non-canonical Notch signaling.

We have also performed similar functional studies for the three nonsense mutants identified in patients 7 to 10 (p.E124X, p.Q197X, and p.R653X) and missense variants (p.P286S, p.Q507R, and p.N589S).[9] The p.E124X and p.Q197X proteins, though they localize to the nucleus, are incapable of

localizing to nuclear bodies and have no transactivation function for Hes3, whereas the p.R653X protein as well as the three variant proteins localize to the nuclear bodies and retained nearly normal transactivating activities. This suggests that the p.E124X and p.Q197X proteins have no transactivation function primarily because of the failure in localizing to the nuclear bodies, and that the p.R653X protein, when it is artificially produced, has a normal transactivating activity. Thus, if not all the mRNAs with nonsense mutations are subject to NMD,[8] this would permit the production of functional protein for p.R653X, but not for p.E124X and p.Q197X. In addition, the transactivation function has been shown to be significantly reduced in a p.L103P protein (an artificially constructed variant affecting the MAML motif) and normal in the ΔExon 4.[9] This implies the importance of the MAML motif, and the biological equivalence between exon 4 positive and negative *MAMLD1*.

MAMLD1 Variations as a Susceptibility Factor for Hypospadias

It is possible that single nucleotide polymorphisms (SNPs), especially those in the promoter region and the cDNA sequence (cSNPs), and haplotypes (a combination of SNPs) of disease-causing genes constitute susceptibility factors of the corresponding diseases. In this regard, *MAMLD1* harbors several cSNPs, and the p.P286S and p.N589S cSNPs are fairly common in Caucasian populations, although they remain rare in the Japanese population.[1,12] In this regard, Chen et al[12] have revealed that the p.P286S allele, the p.N589S allele, and the p.P286S–p.N 589S haplotype (S–S haplotype) are more frequent in patients with hypospadias than in control males. While functional studies using the non-canonical Notch target Hes3 showed normal transactivation function for p.P286S allele, the p.N589S allele, and the 286S–589S haplotype (►**Fig. 2**), it is known that a substitution exerts differential effects on different promoters.[31] Thus, it remains possible that a specific SNP(s) or haplotype(s) may form a susceptibility factor for the development of hypospadias and other forms of 46,XY DSD.

Implications for Primary Ovarian Insufficiency

MAMLD1 may also have a certain role in the ovarian development. This notion is primarily based on two findings: (1) murine *Mamld1* is clearly identified in granulosa cells at the perifollicular regions of most of Graafian follicles at 3 and 8 weeks of age; and (2) a female with a heterozygous microdeletion involving *MAMLD1*, who gave birth to a boy with the same microdeletion and 46,XY DSD, has exhibited ovarian dysfunction from her late teens,[10] although ovarian dysfunction has not been identified in other obligatory carrier females. These findings may suggest that *MAMLD1* is involved in the normal ovarian development, and that rather exceptional females with a heterozygous *MAMLD1* mutation/deletion and skewered inactivation of the X chromosome carrying the normal allele manifest primary ovarian dysfunction. In this context, we examined a total of 78 females with primary ovarian insufficiency and 46,XX karyotype, and identified p.P494S and p.428delQ (shortening of the first polyglutamine domain from 11 to 10) (our unpublished observation). However, functional studies using Hes3 system showed apparently normal transcription activities for the two variants. Thus, further studies are necessary to reveal the relevance of *MAMLD1* to ovarian development.

Conclusions

MAMLD1 is a causative gene for 46,XY DSD with hypospadias as the salient clinical phenotype. It appears to play a supportive role in the testosterone production around the critical period for sex development. Interestingly, *Mamald1* knockout mice exhibit normal genital findings and reproductive functions, although they manifest metabolic syndrome (our unpublished observation). Further studies will permit to reveal the frequency of *MAMLD1* mutations in 46,XY DSD and the in vivo function of *MAMLD1*.

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