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) 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 Ins13) 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)
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.

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 (Nippon-gene, 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 (Amr, Arx, Cyp11a1, Cyp17a1, Ddx4, Dbh, Dlx5, Dlx6, Gata4, Hsd17b3, Hsd3b1, Ins1, Nr5a1, Ptc1, 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-MAML1-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-MAML1-Ab was generated against mouse MAML1 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 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% H2O2 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-Müllerian 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.).

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 χ2 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.
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

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

<table>
<thead>
<tr>
<th></th>
<th>KO</th>
<th>WT</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g) (at birth)</td>
<td>1.48 ± 0.03 (n = 10)</td>
<td>1.44 ± 0.03 (n = 10)</td>
<td>0.40</td>
</tr>
<tr>
<td>AGD (mm) (at birth)</td>
<td>1.33 ± 0.02 (n = 10)</td>
<td>1.32 ± 0.02 (n = 10)</td>
<td>0.62</td>
</tr>
<tr>
<td>AGI (mm/g) (at birth)</td>
<td>0.90 ± 0.02 (n = 10)</td>
<td>0.92 ± 0.02 (n = 10)</td>
<td>0.55</td>
</tr>
<tr>
<td>Leydig cells (HSD3B-stained cells) (number/HPF) (at 14.5 dpc)</td>
<td>69.3 ± 8.2 (n = 3)</td>
<td>75.1 ± 7.6 (n = 3)</td>
<td>0.63</td>
</tr>
<tr>
<td>Testis weight (mg) (at birth)</td>
<td>1.46 ± 0.08 (n = 10)</td>
<td>1.35 ± 0.08 (n = 10)</td>
<td>0.34</td>
</tr>
</tbody>
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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 pregrenolone (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).
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 polypeptide 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.

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**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, preputial gland; u, urethra. Scale bars: 500 μm (A–F, I, and J), 1 mm (G and H), and 100 μm (K and L).
Discussion

The Mamld1 mRNA expression was gradually and steadily increased from 12.5 to 18.5 dpc in the fetal testis of WT male mice. In this regard, intratesticular T has also been reported to increase in a similar manner in the mouse (10, 11). In addition, human study has also revealed clear MAMLD1 expression in the fetal testis. These findings would argue for a positive role of MAMLD1/Mamld1 in the T production in the fetal testis (1, 21).

We generated and studied Mamld1 KO male mice. The results are summarized as follows: 1) mRNA levels of genes exclusively expressed in Leydig cells (Star, Cyp11a1, Cyp17a1, Hsd3b1, and Insl3) were mildly but significantly reduced, whereas those of genes expressed in other cell types or in Leydig and other cell types grossly remained normal (Hsd17b3 is expressed in Sertoli cells of the fetal testis, although it is expressed in Leydig cells of the adult testis) (22, 23); 2) despite such mild reduction of mRNA levels, CYP17A1 and HSD3B proteins were sufficiently produced; 3) no demonstrable abnormality was identified by detailed studies for the external and internal genital regions; and 4) the Mamld1 KO male mice retained normal fertility. Collectively, these findings imply that Mamld1 deficiency reduces mRNA expression levels of multiple, if not all, genes expressed in mouse fetal Leydig cells but permits normal genital development and reproductive function. In support of this notion, such discrepancy between mRNA levels and protein levels as well as phenotypic consequences has been reported previously (24–26). Indeed, Greenbaum et al. (27) have proposed three possible explanations for the poor correlations between mRNA and protein expression levels: 1) there are many complicated and varied posttranscriptional mechanisms involved in turning mRNA into protein that are not yet sufficiently well defined; 2) proteins may differ substantially in their in vivo half lives; and 3) there may be a significant amount of error and noise in both protein and mRNA experiments that limit our ability to get a clear picture. These explanations would also apply to our results indicating normal expression of CYP17A1 and HSD3B proteins, in the presence of mildly but significantly reduced expression of Cyp17a1 and Hsd3b1 mRNAs. Furthermore, because CYP17A1 and HSD3B protein levels increased in a manner grossly similar to that reported for intratesticular T (10, 11) in both the Mamld1 KO male mice and their WT littermates, this would be consistent with the apparently normal testicular function of the Mamld1 KO male mice.

The normal phenotype in the Mamld1 KO male mice is contrastive to the DSD phenotype in the MAMLD1 mutant mice.
tation positive patients (1, 3). In this regard, it is notable that male genital development is primarily induced by testicular T that is produced via Δ5-pathway during the stimulation of chorionic gonadotropin during the first trimester in the human (28–31), whereas it is primarily carried out by testicular T that is produced via Δ4-pathway independently of the chorionic gonadotropin stimulation during the late gestational period in the mouse (10, 31, 32). Thus, although the detailed mechanism(s) remains to be clarified, such species difference in the fetal male sex development may underlie the phenotypic difference between the Maml1 KO male mice and the MAML1 mutation positive patients. In addition, the bias that individuals with abnormal phenotypes only are usually examined in the human study may also be relevant to this matter.

The results of mRNA expression levels and intratesticular hormone concentrations in the Maml1 KO male mice are different from those identified by transient Maml1 knockdown experiments using siRNAs and MLTCs (6, 8), although the normal Leydig cell number of the Maml1 KO male mice appears to be consistent with the sustained proliferation of siRNA-transfected MLTCs (8). Indeed, Maml1 knockdown has predominantly affected Cyp17a1 expression (8) and significantly decreased T and other steroid metabolite after 17α-hydroxylation (6, 8). However, MLTCs are derived from adult Leydig tumor cells and are characterized by a markedly low 17α-hydroxylase activity and a well-preserved 17/20 lyase activity for both Δ4- and Δ5-pathways (33). Such unique properties of MLTCs may be relevant to the preferential impairment of Cyp17a1 expression and 17α-hydroxylation in siRNA-transfected MLTCs.

Two findings also appear to be worth pointing out in this study. First, Insl3 mRNA expression was significantly reduced and Amh mRNA expression was grossly normal, in the Maml1 KO mice. Such mRNA expression patterns, if they also take place in the human, would be relevant to the frequent occurrence of cryptorchidism and the lack of müllerian derivatives in patients with MAML1 mutations (1). Second, Maml1 KO male mice, WT male mice, homozygous (−/−) female mice, heterozygous (+/−) female mice, and WT female mice were born with frequencies consistent with the Mendelian mode of inheritance. Thus, although Maml1 is ubiquitously expressed with strong expressions in the central nervous system (1), Maml1 deficiency is unlikely to affect viability.

In summary, the present study implies that Maml1 enhances mRNA expression levels of multiple genes exclusively expressed in fetal Leydig cells, although the effects of Maml1 deficiency are insufficient to compromise the genital and reproductive development. Further studies will permit a better clarification of the biological function of MAML1/Maml1.

**Acknowledgments**

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