TABLE 2. Cross-mating experiments for *Mamld1*

Offspring produced by cross-mating between *Mamld1* KO male mice (n = 5) and WT female mice (n = 24)					
Sex and *Mamld1* genotype	Male (−)	Male (+)	Female (−/−)	Female (+/−)	Female (+/+)
Number and frequency	n/o	89 (45.6%)	n/o	106 (54.4%)	n/o
Offspring produced by cross-mating between *Mamld1* KO male mice (n = 14) and heterozygous female mice (n = 49)					
Sex and *Mamld1* genotype	Male (−)	Male (+)	Female (−/−)	Female (+/−)	Female (+/+)
Number and frequency	84 (23.6%)	96 (27.0%)	94 (26.4%)	82 (23.0%)	n/o
Offspring produced by cross-mating between WT male mice (n = 6) and WT female mice (n = 12)					
Sex and *Mamld1* genotype	Male (−)	Male (+)	Female (−/−)	Female (+/−)	Female (+/+)
Number and frequency	n/o	58 (59.8%)	n/o	n/o	39 (40.2%)
Offspring produced by cross-mating between WT male mice (n = 9) and heterozygous female mice (n = 46)					
Sex and *Mamld1* genotype	Male (−)	Male (+)	Female (−/−)	Female (+/−)	Female (+/+)
Number and frequency	86 (25.3%)	85 (25.0%)	n/o	84 (24.7%)	85 (25.0%)

WT or +, WT; KO or −, *Mamld1* KO; n/o, not obtained.

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 Δ^5-pathway under 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 Δ^4-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 *Mamld1* KO male mice and the *MAMLD1* 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 *Mamld1* KO male mice are different from those identified by transient *Mamld1* knockdown experiments using siRNAs and MLTCs (6, 8), although the normal Leydig cell number of the *Mamld1* KO male mice appears to be consistent with the sustained proliferation of siRNA-transfected MLTCs (8). Indeed, *Mamld1* 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 Δ^4- and Δ^5-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 *Mamld1* 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 *MAMLD1* mutations (1). Second, *Mamld1* 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 *Mamld1* is ubiquitously expressed with strong expressions in the central nervous system (1), *Mamld1* deficiency is unlikely to affect viability.

In summary, the present study implies that *Mamld1* enhances mRNA expression levels of multiple genes exclusively expressed in fetal Leydig cells, although the effects of *Mamld1* deficiency are insufficient to compromise the genital and reproductive development. Further studies will permit a better clarification of the biological function of *MAMLD1/Mamld1*.

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Neuromuscular symptoms in a patient with familial pseudohypoparathyroidism type Ib diagnosed by methylation-specific multiplex ligation-dependent probe amplification

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Abstract. Pseudohypoparathyroidism type Ib (PHP-Ib) is a rare genetic disorder characterized by hypocalcemia and hyperphosphatemia due to imprinting defects in the maternally derived *GNAS* allele. Patients with PHP-Ib are usually identified by tetany, convulsions, and/or muscle cramps, whereas a substantial fraction of patients remain asymptomatic and are identified by familial studies. Although previous studies on patients with primary hypoparathyroidism have indicated that hypocalcemia can be associated with various neuromuscular abnormalities, such clinical features have been rarely described in patients with PHP-Ib. Here, we report a 12-year-old male patient with familial PHP-Ib and unique neuromuscular symptoms. The patient presented with general fatigue, steppage gait, and myalgia. Physical examinations revealed muscular weakness and atrophies in the lower legs, a shortening of the bilateral Achilles' tendons and absence of deep tendon reflexes. Laboratory tests showed hypocalcemia, hyperphosphatemia, elevated serum intact PTH level, and impaired responses of urinary phosphate and cyclic AMP in an Ellsworth-Howard test, in addition to an elevated serum creatine kinase level. Clinical features of the patient were significantly improved after 1 month of treatment with alfacalcidol and calcium. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) and subsequent PCR analyses identified a methylation defect at exon A/B of *GNAS* and a microdeletion involving exons 4-6 of the *GNAS* neighboring gene *STX16* in the patient and in his asymptomatic brother. The results suggest that various neuromuscular features probably associated with hypocalcemia can be the first symptoms of PHP-Ib, and that MS-MLPA serves as a powerful tool for screening of GNAS abnormalities in patients with atypical manifestations.

Key words: PHP-Ib, Neuromuscular symptoms, Hypocalcemia, *STX16*, MS-MLPA

PSEUDOHYPOPARATHYROIDISM (PHP; MIM 103580) is a genetically heterogeneous condition characterized by hypocalcemia and hyperphosphatemia resulting from end-organ resistance to PTH [1]. PHP is classified into 2 subtypes, PHP-Ia and -Ib, according to the molecular causes and clinical features of the patients [1]. PHP-Ia results from loss-of-function mutations in the maternally derived *GNAS* gene that encodes the stimulatory G protein α-subunit [1]. Patients with PHP-Ia manifest multiple hormone resistance and characteristic physical stigmata such as short stature, obesity, round face, brachydactyly, subcutaneous ossification, and mild to moderate mental retardation, which are collectively referred to as Albright's hereditary osteodystrophy (AHO) [1, 2].

PHP-Ib is caused by imprinting defects of the maternally derived *GNAS* allele; patients with this condi-

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tion show hypomethylation at one or more of the 4 differentially methylated regions (DMRs) of *GNAS* [3-7]. Genetic causes of PHP-Ib include cryptic deletions within the genes neighboring *GNAS*, *STX16* and *NESP55*, and epimutation of *GNAS* DMRs [4, 5]. Patients with PHP-Ib manifest PTH resistance without AHO [1]. These patients are usually identified by hypocalcemia-associated neuromuscular irritability, such as tetany, generalized convulsions, and/or muscle cramps, although a substantial fraction of the patients remain asymptomatic and are identified only by familial studies [6, 7].

Previous studies of patients with primary hypoparathyroidism have shown that hypocalcemia can be associated with various types of neuromuscular symptoms [8, 9]. However, such clinical features have been rarely described in patients with PHP-Ib [10]. Here, we report a Japanese patient with familial PHP-Ib due to an intragenic deletion of *STX16*, who presented with unique neuromuscular symptoms.

Methods

Case report

This male patient was born as the third child to non-consanguineous Japanese parents at 39 weeks of gestation, after an uncomplicated pregnancy and delivery. His birth weight was 3482 g (+1.1 SD) and length 50 cm (+0.7 SD). Neonatal screening tests were normal. His postnatal growth and development were uneventful.

From the age of 6 years, he had general fatigue. At 12 years of age, he was seen by a local doctor because of general fatigue, gait disturbance, and myalgia in the lower legs. He was suspected to have congenital myopathy, and was referred to our clinic for further investigation. His height and weight at the time of examination were 161.4 cm (+1.1 SD) and 42.4 kg (-0.2 SD), respectively. Physical examinations revealed muscular atrophies with weakness in the lower legs, a shortening of the bilateral Achilles' tendons and absence of deep tendon reflexes. He showed a high stepping gait with markedly reduced strength of dorsiflexors of the ankles. Sense of touch and temperature was normal. The Chvostek's sign was positive, while the Trousseau's sign was negative. He had neither AHO stigmata nor episodes of tetany or convulsions. Laboratory examinations revealed hypocalcemia, hyperphosphatemia, and an elevated serum intact PTH level, together with decreased urinary calcium excretions (Table 1). Serum creatine kinase (CK) level was markedly elevated. An Ellsworth-Howard test showed impaired responses of both urinary phosphaturic and cyclic AMP levels (Table 1). The TSH level was slightly elevated, while free T4 and gonadotropin levels were within the normal range. The serum 1,25-dihydroxy vitamin D (1,25(OH)2D) level was mildly elevated. Head computerized tomography (CT) delineated symmetric calcifications of the basal ganglia and thalami, and subcortical calcification of the right middle frontal gyrus. Dual-energy X-ray absorptiometry (DEXA) revealed decreased bone mineral density at the lumbar spine (L2-L4) (0.640 g/cm^2, -2.9 SD). Based on these data, we diagnosed him as having PHP-Ib with neuromuscular symptoms. After 1 month of treatment with alfacalcidol (1.5 μg/day) and calcium lactate (3.0 g/day), his general fatigue, gait disturbance, and myalgia were markedly improved.

The 15-year-old brother of the patient manifested no clinically discernible phenotype; the brother had no gait disturbance or muscle weakness. Furthermore, physical examinations revealed neither muscular atrophy nor neurologic abnormalities. However, laboratory examinations detected an elevated serum intact PTH level, although serum calcium level was within the normal range (Table 1). Thus, the brother was also suspected as having PHP-Ib. The brother manifested mildly elevated serum 1,25(OH)2D level.

The 50-year-old father and 17-year-old sister were clinically normal. The mother, deceased at 49 years of age of an unknown cause, allegedly had no clinical symptoms indicative of PHP. Endocrine studies revealed no abnormalities in the father, sister, or mother (Table 1).

Molecular analyses

This study was approved by the Institutional Review Board Committee at the National Center for Child Health. After obtaining written informed consent, we extracted genomic DNA from leukocytes of the patient and his brother and father.

We examined mutations in the coding region of *GNAS* by direct sequencing, and copy number alternations and methylation defects in the *GNAS*-flanking region by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), using a commercially available probe mix (SALSA MLPA kit, ME031-A1) (MRC-Holland, Amsterdam, The Netherlands). To confirm the results of MS-MLPA, we performed PCR analyses using forward and reverse

Table 1 Laboratory findings of the patient and his family members

	Patient	Brother	Father	Mother	Sister	Reference range
Age at the examinations (years)	12	15	50	43	17	
Height (cm) (SDS)	161.4 (+1.1)	171 (+0.1)	N.A.	N.A.	N.A.	
Weight (kg) (SDS)	42.4 (-0.2)	53 (-0.9)	N.A.	N.A.	N.A.	
<Blood>						
Intact PTH (pg/mL)	**430**	**254**	26	44	26	10-65
Calcium (mg/dL)	**6.4**	8.9	9.3	8.7	9.2	8.5-10.2
Phosphate (mg/dL)	**9.1**	**5.2**	2.9	3.8	3.4	2.4-4.3
Magnesium (mg/dL)	1.8	2.0	N.A.	N.A.	N.A.	1.8-2.5
Na (mEq/l)	142	140	N.A.	N.A.	N.A.	135-147
K (mEq/l)	4.1	4.0	N.A.	N.A.	N.A.	3.6-5.0
Creatinine (mg/dL)	0.6	0.7	N.A.	N.A.	N.A.	0.4-1.1
Alb (g/dL)	4.9	4.5	N.A.	N.A.	N.A.	3.9-5.1
CK (IU/L)	**741**	136	N.A.	N.A.	N.A.	0-170
ALP (IU/L)	**1809** (388-1190)[a]	648 (225-680)[a]	N.A.	N.A.	N.A.	
1,25(OH)2D (pg/mL)	**69**	**79**	N.A.	N.A.	N.A.	20-60
TSH (mU/L)	**5.6**	4.1	N.A.	N.A.	N.A.	0.5-5.0
Free T4 (ng/dL)	1.0	1.0	N.A.	N.A.	N.A.	0.9-1.6
<Urine>						
Calcium/Creatinine ratio	**0.004**	**0.008**	N.A.	N.A.	N.A.	0.08-0.20
%TRP	**99.6**	**99.6**	N.A.	N.A.	N.A.	89.6-93.6
<Ellsworth-Howard test>						
Urinary phosphate (mg/2 hrs)[b]	**8.33**	N.A.	N.A.	N.A.	N.A.	≥30
Urinary cAMP (μmol/hr)[c]	**0.029**	N.A.	N.A.	N.A.	N.A.	≥1.0

The conversion factors to the international system of units (SI unit) are as follows: intact PTH 1.0 (ng/liter), serum calcium 0.25 (mmol/liter), serum phosphate 0.3229 (mmol/liter) serum magensium 0.411 (mmol/liter), serum sodium 1.0 (mmol/liter), serum potassium 1.0 (mmol/liter), serum creatine 88.4 (μmol/liter), serum albumin 10 (g/liter), serum 1,25(OH)2D 2.6 (pmol/liter), serum Free T4 12.9 (pmol/liter). Hormone values have been evaluated by the age- and sex-matched Japanese reference data; abnormal data are in bold.

[a] The values in parentheses indicate the age-and sex-matched reference laboratory data.

[b] Urinary phosphate denotes the increment of 2 hours urinary excretion of phosphate after injection of human PTH (100 unit).

[c] Urinary cAMP denote the increment of 1 hour urinary cAMP excretion after injection of human PTH (100 unit).

N.A., not analysed; CK, creatine kinase; 1,25(OH)2D, 1,25-dihydroxy vitamin D; %TRP, % tubular reabsorption of phosphate

primers that hybridize to introns 3 and 6 of *STX16*, respectively [4].

Results

Direct sequence analysis for the patient identified no mutation in the coding region of *GNAS*. However, MS-MLPA revealed decreased peak heights of probes that correspond to exons 5 and 6 of *STX16*, indicating a heterozygous deletion within *STX16*. In addition, MS-MLPA indicated hypomethylation at *GNAS* exon A/B and a normal methylation pattern of the other 3 *GNAS* DMRs (Fig. 1A, B). Subsequent PCR analyses showed the presence of a heterozygous 3 kb deletion involving exons 4-6 of *STX16* (STX16Δexons 4-6) (Fig. 1C). The microdeletion and methylation defect were also observed in the brother, but not in the father. DNA samples of the mother and the sister were not available for genetic analyses.

Discussion

We report here a Japanese patient with PHP-Ib, who was identified by general fatigue, gait disturbance, and myalgia in the lower legs. He showed muscular atrophies in the lower legs, a shortening of the bilateral Achilles' tendons, absence of deep tendon reflexes, and an elevated serum CK value. Such clinical features are indicative of neuromuscular symptoms, although a detailed neurological workup was not performed for

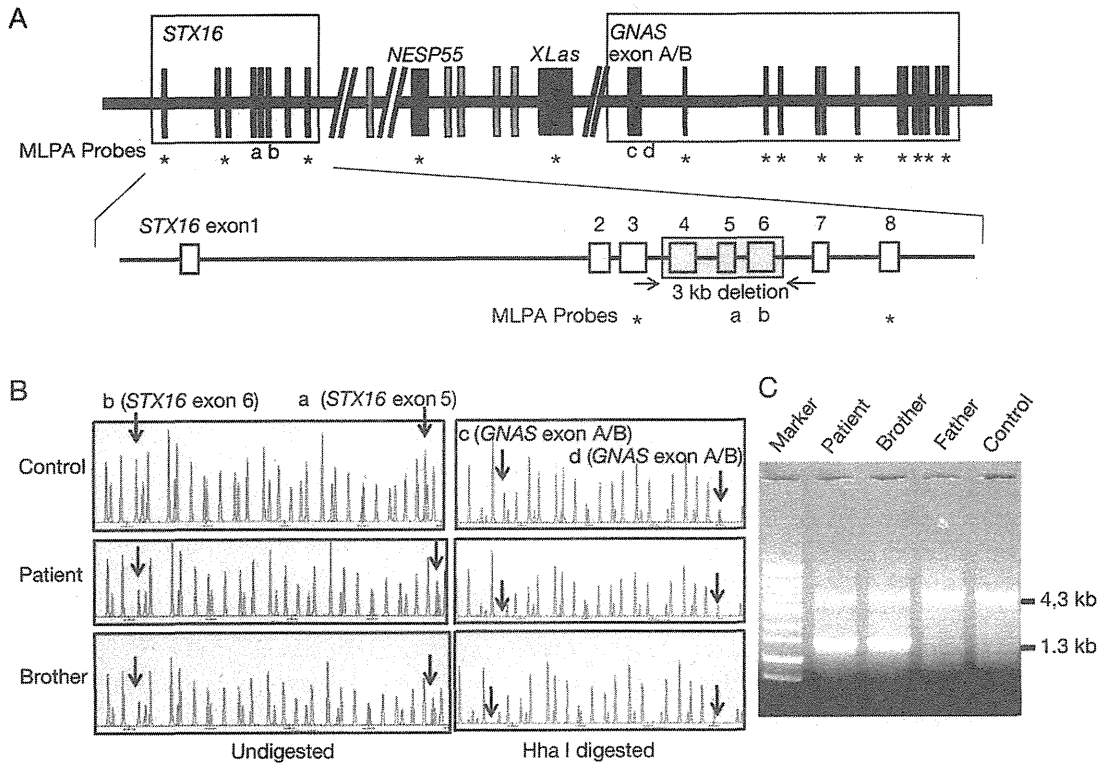

Fig. 1 Molecular analysis of the patient and his family members.

A, Schematic representation of the genomic region around *GNAS*. Upper panel: The loci examined by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) are indicated by letters (a-d) and asterisks. Lower panel: Microdeletion identified in the patient and his brother. Horizontal arrows indicate the binding sites of the primers used for PCR analysis.

B. Representative results of MS-MLPA. Left panel: Decreased peak heights with probes a and b in the patient and his brother indicate heterozygous deletion involving exons 5 and 6 of *STX16*. Right panel: Absence of peaks with probes c and d indicate hypomethylation of *GNAS* exon A/B.

C, PCR analysis using a primer pair flanking the deletion. Both the 4.3 kb (wild-type) and 1.3 kb (STX16Δexons4-6) products were amplified from the patient and his brother, while only the 4.3 kb product was obtained from the father and the control individual.

this patient. In this regard, it is noteworthy that peripheral neuropathy and metabolic myopathy have been reported in patients with primary hypoparathyroidism [8, 9], whereas such symptoms have not been described in patients with PHP, except for mildly elevated blood CK and lactate dehydrogenase (LDH) levels in a single case of PHP-Ia [10]. Moreover, *in vitro* experiments showed that calcium concentration affects excitability at neuromuscular junctions [11]. Thus, the neuromuscular symptoms of our patient are likely to be associated with hypocalcemia. A significant improvement in the clinical features of the patient after 1 month of treatment with alfacalcidol and calcium supports this hypothesis. However, we cannot exclude the possibility that other factors such as vitamin D deficiency may also have played a role in the development of these features. Indeed, slightly elevated serum levels of ALP and 1,25(OH)2D in the patient are consistent with mild vitamin D deficiency [12]. On the other hand, since serum 1,25(OH)2D levels were similarly elevated in the patient and his asymptomatic brother, phenotypic variation in this family can not be explained by vitamin D deficiency. These results indicate that neuromuscular features probably associated with hypocalcemia can be the first symptoms of PHP-Ib. Nevertheless, this notion is based on observations of a single case, and

requires further investigations.

Both the patient and his brother carried a heterozygous STX16Δexons4-6. Although DNA samples of the mother were not available for genetic analyses, the absence of the deletion in the father indicated the maternal inheritance of the deletion. It has been shown that maternally inherited STX16Δexons4-6 (STX16Δexons4-6mat) is associated with hypomethylation at *GNAS* exon A/B, whereas *GNAS* epimutations are usually accompanied by methylation defects not only at exon A/B but also at other *GNAS* DMRs [3, 7]. These results suggest that the 3 kb region around exon 4-6 of *STX16* contains a cis-acting element that regulates methylation status at *GNAS* exon A/B. Consistent with this, our patient and his brother had methylation defects exclusively at exon A/B. Further studies are necessary to clarify the mechanism by which a DNA element >200 kb from *GNAS* controls the methylation status at exon A/B.

Clinical severities of patients with PHP-Ib are known to be variable [6, 7]. Notably, Linglart *et al.* have shown that STX16Δexons4-6mat is often associated with a mild phenotype. They found that about 40% of patients carrying this microdeletion remained asymptomatic, and more than 50% of asymptomatic individuals had normocalcemia at the time of diagnosis [7]. Consistent with this, our patient and his brother lacked typical PHP-Ib features such as tetany, generalized convulsions, or muscle cramps. Furthermore, the brother had normocalcemia. These results suggest that physical examinations and measurement of serum calcium levels are not sufficient to identify patients with PHP-Ib, and that genetic analyses or detailed endocrine evaluations, such as measurement of intact PTH levels and an Ellsworth-Howard test, are necessary for patients with atypical manifestations. In this context, although STX16Δexons4-6mat is the most frequent genetic cause of familial PHP-Ib [7], microdeletions affecting *NESP55* as well as epimutations of *GNAS* DMR also account for etiology of PHP-Ib [5, 7]. Since MS-MLPA is capable of detecting both copy number abnormalities and methylation defects in the *GNAS*-flanking region in a single assay, this method should be particularly useful for the molecular diagnosis of PHP-Ib.

In summary, the present study provides that various neuromuscular features probably associated with hypocalcemia can be the first symptoms of PHP-Ib, and suggests that MS-MLPA serves as a powerful tool for screening of *GNAS* abnormalities in patients with atypical manifestations.

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Review Article

Molecular Bases and Phenotypic Determinants of Aromatase Excess Syndrome

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Aromatase excess syndrome (AEXS) is a rare autosomal dominant disorder characterized by gynecomastia. This condition is caused by overexpression of *CYP19A1* encoding aromatase, and three types of cryptic genomic rearrangement around *CYP19A1*, that is, duplications, deletions, and inversions, have been identified in AEXS. Duplications appear to have caused *CYP19A1* overexpression because of an increased number of physiological promoters, whereas deletions and inversions would have induced wide *CYP19A1* expression due to the formation of chimeric genes consisting of a noncoding exon(s) of a neighboring gene and *CYP19A1* coding exons. Genotype-phenotype analysis implies that phenotypic severity of AEXS is primarily determined by the expression pattern of *CYP19A1* and the chimeric genes and by the structural property of the fused exons with a promoter function (i.e., the presence or the absence of a natural translation start codon). These results provide novel information about molecular mechanisms of human genetic disorders and biological function of estrogens.

1. Introduction

Aromatase encoded by *CYP19A1* is a cytochrome P450 enzyme that plays a key role in estrogen biosynthesis [1]. It catalyzes the conversion of Δ^4-androstendione into estrone (E_1) and that of testosterone (T) into estradiol (E_2) in the placenta and ovary as well as in other tissues such as the fat, skin, bone, and brain [1].

Overexpression of *CYP19A1* causes a rare autosomal dominant disorder referred to as aromatase excess syndrome (AEXS, OMIM no. 139300) [2–8]. AEXS is characterized by pre- or peripubertal onset gynecomastia, gonadal dysfunction, advanced bone age from childhood to pubertal period, and short adult height in affected males [2–8]. In particular, gynecomastia is a salient feature in AEXS, and, therefore, this condition is also known as hereditary gynecomastia or familial gynecomastia [5]. Affected females may also show several clinical features such as macromastia, precocious puberty, irregular menses, and short adult height [5, 6, 8].

Recently, three types of cryptic genomic rearrangements around *CYP19A1* have been identified in 23 male patients with AEXS [2–4]. The results provide useful implications not only for the clarification of underlying mechanisms but also for the identification of phenotypic determinants. Here, we review the current knowledge about AEXS.

2. The Aromatase Gene (*CYP19A1*)

CYP19A1 encoding aromatase is located on 15q21.2 adjacent to *DMXL2* and *GLDN* (Figure 1) [3, 9]. It spans ~123 kb and consists of at least 11 noncoding exons 1 and nine coding exons 2–10 [9–12]. Each exon 1 is accompanied by a tissue-specific promoter and is spliced alternatively onto a common splice acceptor site at exon 2, although some transcripts are known to contain two of the exons 1 probably due to a splice error [9–11]. Transcription of *CYP19A1* appears to be tightly regulated by alternative usage of the multiple

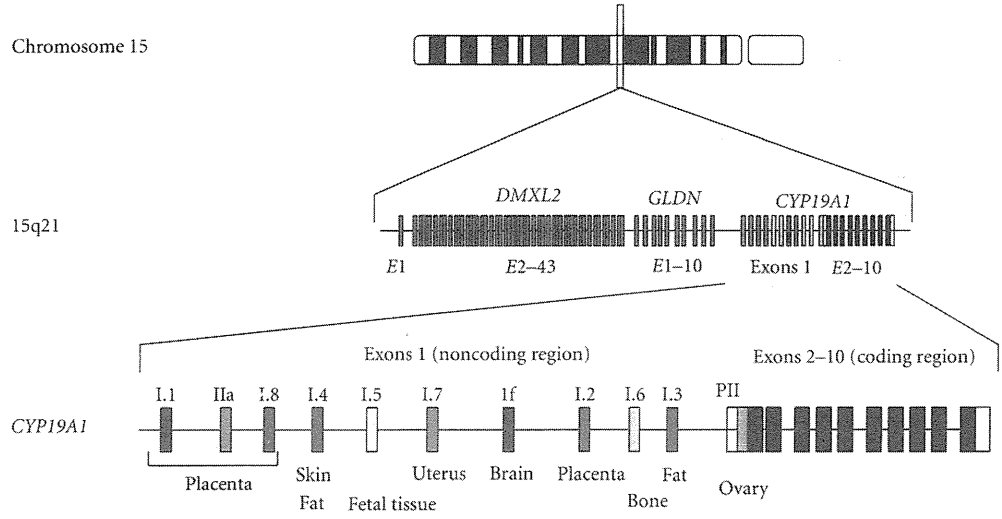

FIGURE 1: Simplified schematic representation indicating the genomic structure of *CYP19A1*. *CYP19A1* is located on 15q21.2 adjacent to *DMXL2* and *GLDN* and consists of at least 11 noncoding exons 1 and nine coding exons 2–10 [9, 10]. Each exon 1 is accompanied by a tissue-specific promoter and is spliced alternatively onto a common splice acceptor site at exon 2 [9–13].

promoters [9–13]. Actually, *CYP19A1* is strongly expressed in the placenta and moderately expressed in the ovary, whereas it is only weakly expressed in a rather limited number of tissues including skin, fat, and hypothalamus [4, 13]. Of the 11 noncoding exons 1, exon I.4 seems to play a critical role in the regulation of estrogen biosynthesis in males, because this exon contains the major promoter for extragonadal tissues [9, 10].

3. Molecular Bases of AEXS

A family with dominantly transmitted gynecomastia of prepubertal onset was first described in 1962 by Wallach and Garcia [14]. After this initial report, several cases have been described [5–8, 15]. Laboratory examinations of the affected males revealed markedly elevated serum estrogen values and estrogen/androgen ratios and significantly increased aromatase activity in fibroblasts and lymphocytes [5–8, 15]. Linkage analyses in two families indicated a close association between *CYP19A1*-flanking polymorphic markers and the disease phenotype [5, 6]. Thus, the condition was assumed to be caused by gain-of-function mutations of *CYP19A1*, and, therefore, the name of AEXS was coined for this condition [7, 8]. However, since direct sequencing and Southern blotting analysis failed to detect mutations or copy number abnormalities in the coding region of *CYP19A1* [5, 6], the molecular basis of this entity remained elusive until recently.

In 2003, Shozu et al. reported a father-son pair and a sporadic case with AEXS in whom they identified heterozygous chromosomal inversions of the chromosome 15 [2]. Subsequently, Demura et al. performed detailed molecular studies for these cases and additional two cases and characterized four types of inversions affecting the 5′ region of *CYP19A1* [3]. Each inversion has resulted in the formation of a chimeric gene consisting of *CYP19A1* coding exons and exon 1 of the widely expressed neighboring genes, that is, *CGNL1*, *TMOD3*, *MAPK6*, and *TLN2*. These data imply that overexpression of *CYP19A1* in the inversion-positive cases are caused by cryptic usage of constitutively active promoters. Consistent with this, *in silico* analysis revealed the presence of promoter-compatible sequences around exon 1 of *CGN1*, *TMOD3*, and *MAPK6* in multiple cell types, although such sequences remain to be identified for noncoding exons of *TLN2* [4].

We recently studied 18 males from six families with AEXS (families A–F) and identified three types of heterozygous cryptic genomic rearrangements in the upstream region of the *CYP19A1* coding exons (Figure 2) [4]. In families A and B, we identified the same 79,156 bp tandem duplication encompassing seven of the 11 noncoding exons 1 of *CYP19A1*. Notably, this duplication includes exon I.4 that functions as a major promoter for extragonadal tissues such as fat and skin; therefore, *CYP19A1* overexpression in these families would be explained by increasing the number of this promoter. Indeed, RT-PCR analysis detected a splice variant consisting of exon I.4 at the 5′ side and exon I.8 at the 3′ side in lymphoblastoid cell lines and skin fibroblasts of the patients, indicating that the duplicated exon I.4 at the distal nonphysiological position actually functions as transcription start sites. In family C, we identified a 211,631 bp deletion affecting exons 2–43 of *DMXL2* and exons 5–10 of *GLDN*. This deletion appears to have caused *CYP19A1* overexpression because of cryptic usage of *DMXL2* exon 1 as an extra transcription start site for *CYP19A1*. Indeed, RT-PCR revealed the presence of chimeric mRNA clones consisting of *DMXL2* exon 1 and *CYP19A1* exon 2, supporting the notion that aberrant splicing has occurred between these two exons. Such *DMXL2/CYP19A1* chimeric mRNA accounted for 2–5% of *CYP19A1*-containing transcripts from skin fibroblasts. In families D–F, we identified

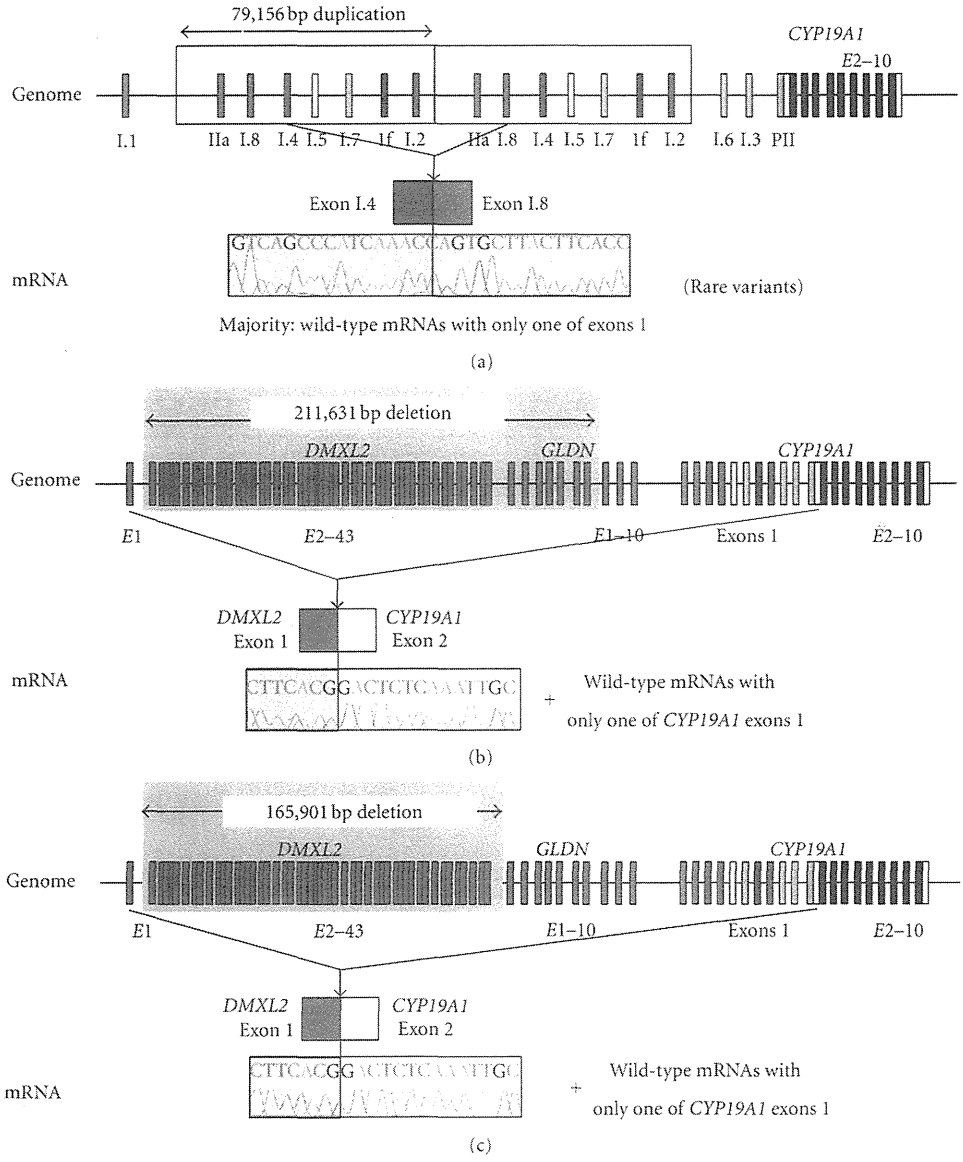

FIGURE 2: Schematic representation of duplications and deletions identified in patients with AEXS. (a) the tandem duplication of families A and B [4]. Genome: the duplication (yellow boxes) includes seven of the 11 noncoding exons 1 of *CYP19A1*. mRNA: the sequence of a rare transcript is shown. The 3′-end of exon I.4 is connected with the 5′-end of exon I.8. (b) The deletion of family C [4]. Genome: the deletion (a gray area) includes exons 2–43 of *DMXL2* and exons 5–10 of *GLDN*. mRNA: The sequence of a rare chimeric gene transcript is shown. *DMXL2* exon 1 consisting of a noncoding region and a coding region is spliced onto the common acceptor site of *CYP19A1* exon 2. (c) The deletion of families D–F [4]. Genome: the deletion (a gray area) includes exons 2–43 of *DMXL2*. mRNA: the sequence of a rare chimeric gene transcript is delineated. The mRNA structure is the same as that detected in family C.

an identical 165,901 bp deletion including exons 2–43 of *DMXL2*. RT-PCR identified the same chimeric mRNA as that detected in family C.

Collectively, three types of genomic rearrangements on 15q21 have been identified in AEXS to date, namely, inversion type (four subtypes), duplication type, and deletion type (two subtypes) (Figure 3(a)) [2–4]. In this regard, sequence analyses for the breakpoints have indicated that (1) inversion types are formed by a repeat sequence-mediated nonallelic intrachromosomal or interchromosomal recombination or by a replication-based mechanism of fork stalling and template switching (FoSTeS) that occurs in the absence of repeat sequences and is often associated with microhomology [16], (2) duplication type is generated by FoSTeS, and (3) deletions are produced by nonhomologous end joining that takes place between nonhomologous sequences and is frequently accompanied by an insertion of a short segment at the fusion point or by a nonallelic recombination [16].

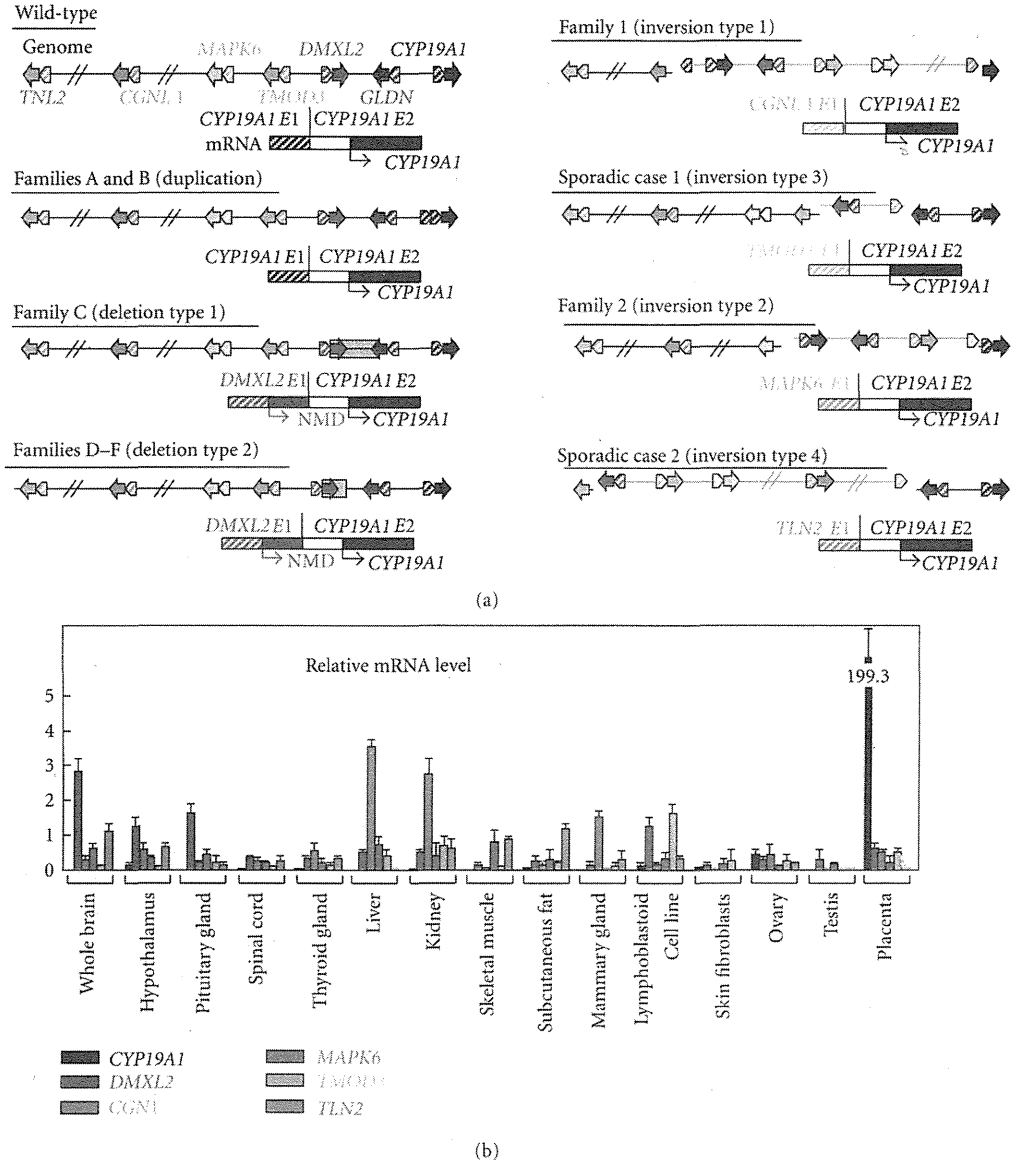

FIGURE 3: Structural and functional properties of the fused exons. (a) Schematic representation of the rearranged genome and mRNA structures. The white and the black boxes of *CYP19A1* exon 2 show untranslated region and coding region, respectively. For genome, the striped and the painted arrows indicate noncoding and coding exons, respectively (5′ → 3′). The inverted genomic regions are delineated in blue lines. For mRNA, colored striped boxes represent noncoding regions of each gene. The *DMXL2-CYP19A1* chimeric mRNA has two translation initiation codons and therefore is destined to produce not only *CYP19A1* protein but also a 47 amino acid protein which is predicted to undergo nonsense-mediated mRNA decay (NMD). The deletion and the inversion types are associated with heterozygous impairment of neighboring genes (deletion or disconnection between noncoding exon(s) and the following coding exons). The inversion subtype 1 is accompanied by inversion of eight of the 11 *CYP19A1* exons 1, and the inversion subtype 2 is associated with inversion of the placenta-specific *CYP19A1* exon I.1. (b) Expression patterns of *CYP19A1* and the five neighboring genes involved in the chimeric gene formation [4]. Relative mRNA levels against *TBP* in normal human tissues are shown.

Thus, it appears that genomic sequence around *CYP19A1* harbors particular motifs that are vulnerable to replication- and recombination-mediated errors. The results provide novel mechanisms of gain-of-function mutations leading to human diseases.

4. Clinical Features of AEXS

To date, a total of 23 male cases from 10 families have been reported to have molecularly confirmed AEXS (Table 1, Figure 3(a)) [2–4]. They exhibited pre- or peripubertal onset

TABLE 1: Summary of clinical studies in male patients with aromatase excess syndrome (modified from [4]).

(a)

Family			Family A			Family B		Family C					Family D				Family E	
Mutation types			Duplication			Duplication		Deletion					Deletion				Deletion	
The promoter involved in *CYP19A1* overexpression			*CYP19A1*			*CYP19A1*		*CYP19A1*					*DMXL2*				*DMXL2*	
Case		Case 1	Case 2		Case 3		Case 4	Case 5		Case 6		Case 7	Case 8		Case 9		Case 10	
Age at examination (year)		66	15		20		15	15		13		42	9		12		13	
<Phenotypic findings>																		
Gynecomastia (tanner breast stage)		**2**	**2**		**2**		**3**	**4**		**4**		**4**	**3**		**4**		**4**	
Onset of gynecomastia (year)		**13**	**13**		**10**		**11**	**12**		**11**		**11**	**7**		**9**		**10**	
Mastectomy (year)		No	**Yes (15)**		No		**Yes (15)**	**Yes (15)**		**Yes (13)**		No	No		**Yes (12)**		**Yes (13)**	
Testis (ml)		N.E.	**12**		**12**		**12**	**12**		12		N.E.	3		12		20	
Pubic hair (tanner stage)		N.E.	2-3		4		5	4		3		N.E.	1		3		4	
Facial hair		Normal	Scarce		**Scarce**		Normal	Absent		Absent		N.E.	Absent		Absent		Absent	
Height (SDS)[a]		−1.2	−0.3		+0.4		+0.8	−2.0		−1.0		−1.6	**+2.7**		±0		+1.8	
Bone age (year)[b]		N.E.	N.E.		N.E.		16.0	16.0		13.5		N.E.	**13.0**		**15.0**		**17.0**	
Fertility (spermatogenesis)		Yes	?		(Yes)[h]		?	?		?		Yes	?		?		?	
<Endocrine findings>[c]		B	B	S	B	S	B S	B	S	B	S	B	B	S	B	S	B	S
<At Dx>	Stimulus																	
LH (mIU/mL)	GnRH[e]	3.8	2.3	**14.3**	2.1	**17.0**	2.4 29.4	1.9	40.6	1.8	69.2		1.1	11.5	**0.6**	39.5	6.7	14.8
LH (mIU/mL)	GnRH (after priming)[f]		1.8	**9.5**	**1.3**	**10.7**												
FSH (mIU/mL)	GnRH[e]	**1.7**	**3.1**	**5.3**	**<0.5**	**1.2**	**0.9 2.4**	**1.4**	**4.2**	**2.0**	**7.8**		**3.2**	**6.6**	**0.6**	**2.9**	**0.7**	**1.0**
FSH (mIU/mL)	GnRH (after priming)[f]		**2.6**	**3.2**	**<0.5**	**0.9**												
Prolactin (ng/ml)			4.3		5.3			8.2		9.1			11.3		18.8			
Δ^4A (ng/mL)		**0.5**			**1.1**		**1.2**					**0.6**			**0.7**		**2.4**	2.9
T (ng/mL)	hCG[g]	2.9	**1.6**		**2.2**		4.0	**2.6**	7.2	**1.4**	7.9		**0.6**	3.6	**2.4**		3.2	9.7
DHT (ng/mL)		0.4			0.2												0.4	1.2
Inhibin B (pg/mL)		**61.6**			**74.6**		**83.5**	**75.2**										
E_1 (pg/mL)		157			120		124					57			63		53	
E_2 (pg/mL)		29	15		22		59	56		38		24	19		25		58	
E_2/T ratio ($\times 10^3$)		10.0	9.4		10.0		14.8	21.5		27.1			31.7		10.4		18.1	

(b)

Family					Family F						Family G		Family H		Sporadic	
Mutation types					Deletion						Inversion		Inversion		Inversion	
The promoter involved in *CYP19A1* overexpression					*DMXL2*						*CGNL1*		*MAPK6*		*TMOD3*	*TLN2*
Case		Case 11	Case 12	Case 13	Case 14	Case 15	Case 16	Case 17	Case 18	Case 19	Case 20		Case 21[j]		Case 22	Case 23
Age at examination (year)		69	35	44	45	9	8	13	10	35	7		13		17	36
<Phenotypic findings>																
Gynecomastia (tanner breast stage)		**Yes**[i]	**Yes**[i]	**Yes**[i]	**Yes**[i]	**2**	**3**	**3**	**3**	**Yes**	**3**		**5**		N.E.	**Yes**
Onset of gynecomastia (year)		?	?	?	?	**8**	**8**	**11**	**10**	**5**	**5**		**8**		**7**	?
Mastectomy (year)		**Yes**[i]	**Yes**[i]	**Yes**[i]	**Yes**[i]	No	No	**Yes (?)**	**Yes (?)**	**Yes (16)**	No		**Yes (?)**		**Yes (?)**	**Yes (19)**
Testis (ml)		N.E.	N.E.	N.E.	N.E.	2	1.5	**2**	2	N.E.	N.E.		N.E.		Normal	N.E.
Pubic hair (tanner stage)		N.E.	N.E.	N.E.	N.E.	1	1	2	1	Normal	1		**2-3** (at 21.0)		N.E.	N.E.
Facial hair		N.E.	N.E.	N.E.	N.E.	Absent	Absent	Absent	Absent	**Absent**	Absent		N.E.		**Scarce**	N.E.
Height (SDS)[a]		N.E.	~ −1.5	~ −1.5	~ −1.5	+1.4	N.E.	**+2.0**	**+2.4**	**Short**	>**+2.5**		−1.6 (at 21.0)		**Short**	N.E.
Bone age (year)[b]		N.E.	N.E.	N.E.	N.E.	**12.5**	**13.0**	**15.0**	**14.5 (at 12.5)**	N.E.	**13.0 (at 5.5)**		**17.0**		N.E.	N.E.
Fertility (spermatogenesis)		Yes	Yes	Yes	Yes	?	?	?	?	Yes	?		?		?	?
<Endocrine findings>[c]		B	B	B	B	B	B	B	S	B	B	B	B	S	B	
<At Dx>	Stimulus															
LH (mIU/mL)	GnRH[e]	**0.2**	3.5	1.7	3.0	0.2	<0.1	2.6	6.3	1.5	1.7	0.1	2.6	**10.0**	4.3	
LH (mIU/mL)	GnRH (after priming)[f]															
FSH (mIU/mL)	GnRH[e]	**1.4**	**2.3**	**0.8**	**0.8**	1.4	0.5	**0.8**	**1.2**	**1.2**	**1.5**	0.3	**<0.1**	**<0.1**	**2.7**	
FSH (mIU/mL)	GnRH (after priming)[f]															
Prolactin (ng/ml)																
Δ^4A (ng/mL)		1.4	**0.4**	1.7	**0.5**	0.3	<0.3	**0.9**	1.5	1.3	**0.8**	0.3	2.4	**0.9**		
T (ng/mL)	hCG[g]	**2.6**	**2.5**	**2.1**	**2.5**	<0.1	<0.1	**2.7**	9.2	**2.7**	3.2	<0.1	**1.2**	3.8	**2.3**	
DHT (ng/mL)													0.2	0.5		
Inhibin B (pg/mL)																
E_1 (pg/mL)		32	34	59	34	26	41	77		86	903	119	544		556	
E_2 (pg/mL)		10	19	24	31	11	7	25		40	223	15	178		392	
E_2/T ratio ($\times 10^3$)		3.8	7.6	11.4	12.4			9.3		14.8	69.6		148.3		170.4	

SDS: standard deviation score; Dx: diagnosis; Tx: therapy; LH: luteinizing hormone; FSH: follicle stimulating hormone; Δ^4A: androstenedione; T: testosterone; DHT: dihydrotestosterone; E_1: estrone; E_2: estradiol; GnRH: gonadotropin-releasing hormone; hCG: human chorionic gonadotropin; N.E.: not examined; B: basal; and S: stimulated.
Abnormal clinical findings are boldfaced.
Abnormally low hormone values are boldfaced, and abnormally high hormone values are underlined.
[a]Evaluated by age- and ethnicity-matched growth references; heights ≥+2.0 SD or below ≤ −2.0 SD were regarded as abnormal.
[b]Assessed by the Tanner-Whitehouse 2 method standardized for Japanese or by the Greulich-Pyle method for Caucasians; bone age was assessed as advanced when it was accelerated a year or more.
[c]Evaluated by age-matched male reference data, except for inhibin B and E_1 that have been compared with data from 19 adult males.
[d]Treated with aromatase inhibitors (anastrozole).
[e]GnRH 100 μg/m^2 (max. 100 μg) bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 minutes.
[f]GnRH test after priming with GnRH 100 μg i.m. for 5 consecutive days.
[g]hCG 3000 IU/m^2 (max 5000 IU) i.m. for 3 consecutive days; blood sampling on days 1 and 4.
[h]Although Case 3 has not yet fathered a child, he has normal spermatogenesis with semen volume of 2.5 ml (reference value: >2 ml), sperm count of 105 × 10^6/ml (>20 × 10^6/ml), total sperm count of 262.5 × 10^6 (>40 × 10^6), motile cells of 70% (>50%), and normal morphological sperms 77% (>30%).
[i]These four patients allegedly had gynecomastia that required mastectomy (age unknown).
[j]The sister has macromastia, large uterus, and irregular menses; the parental phenotype has not been described.
The conversion factor to the SI unit: LH 1.0 (IU/L), FSH 1.0 (IU/L), E_1 3.699 (pmol/L), E_2 3.671 (pmol/L), Δ^4A 3.492 (nmol/L), and T 3.467 (nmol/L).

gynecomastia, small testes with fairly preserved masculinization, obvious or relative tall stature in childhood and grossly normal or apparent short stature in adulthood, and age-appropriate or variably advanced bone ages. Blood endocrine studies revealed markedly elevated E_1 values and E_2/T ratios in all cases examined and normal or variably elevated E_2 values. In addition, Δ^4-androstenedione, T, and dihydrotestosterone values were low or normal, and human chorionic gonadotropin (hCG) test indicated normal T responses. Notably, LH values were grossly normal at the baseline and variably responded to GnRH stimulation, whereas FSH values were low at the baseline and poorly responded to GnRH stimulation even after preceding GnRH priming, in all cases examined.

The severity of such clinical phenotypes is primarily dependent on the underlying mechanisms (Table 1). They are obviously mild in the duplication type, moderate in the deletion type, and severe in the inversion type, except for serum FSH values that remain suppressed irrespective of the underlying mechanisms. Likewise, gynecomastia has been reported to be ameliorated with 1 mg/day of aromatase inhibitor (anastrozole) in the duplication and the deletion types and with 2–4 mg/day of anastrozole in the inversion type [4].

5. Expression Pattern of *CYP19A1* and the Chimeric Genes as One Phenotypic Determinant

Phenotypic severity is much milder in the duplication type than in the deletion and the inversion types. This would be explained by the tissue expression pattern of *CYP19A1* and the chimeric genes. Indeed, RT-PCR analysis using normal human tissue samples revealed that *CYP19A1* is expressed only in a limited number of tissues such as placenta, ovary, skin, and fat, while the five genes involved in the formation of chimeric genes are widely expressed with some degree of variation (Figure 3(b)). Therefore, it is likely that the duplication types would simply increase *CYP19A1* transcription in native *CYP19A1*-expressing tissues, whereas the deletion and the inversion types lead to *CYP19A1* overexpression in a range of tissues, because expression patterns of chimeric genes are predicted to follow those of the original genes. Furthermore, it is also likely that the native *CYP19A1* promoter is subject to negative feedback by elevated estrogens [17], whereas such negative feedback effect by estrogen is weak or even absent for the chimeric genes in the deletion and the inversion types.

6. Structural Property of the Fused Exons as Another Phenotypic Determinant

Phenotypic severity is also milder in the deletion type than in the inversion types, despite a similar wide expression pattern of genes involved in the chimeric gene formation (Table 1, Figure 3(b)). In this context, it is noteworthy that a translation start codon and a following coding region are present on exon 1 of *DMXL2* of the deletion type but not on exons 1 of the chimeric genes of the inversion types (Figure 3(a)). Thus, it is likely that *DMXL2/CYP19A1* chimeric mRNAs transcribed by the *DMXL2* promoter preferentially recognize the natural start codon on *DMXL2* exon 1 and undergo nonsense-mediated mRNA decay and that rather exceptional chimeric mRNAs, which recognize the start codon on *CYP19A1* exon 2, are transcribed into CYP19A1 protein. By contrast, such a phenomenon would not be postulated for the inversion-mediated chimeric mRNAs. Consistent with this, it has been shown that the *DMXL2/CYP19A1* chimeric mRNA is present only in 2–5% of *CYP19A1*-containing transcripts from skin fibroblasts, whereas the *CGNL1/CYP19A1* chimeric mRNA and the *TMOD3/CYP19A1* chimeric mRNA account for 89–100% and 80% of transcripts from skin fibroblasts, respectively [2, 4].

In addition, the genomic structure caused by the rearrangements would affect efficiency of splicing between noncoding exon(s) of neighboring genes and *CYP19A1* exon 2. For example, in the inversion subtype 1, the physical distance between *CGNL1* exon 1 and *CYP19A1* exon 2 is short, and, while a splice competition may be possible between exon 1 of neighboring genes and original *CYP19A1* exons 1, eight of 11 *CYP19A1* exons 1 including exon I.4 have been disconnected from *CYP19A1* coding exons by inversion (Figure 3(a)). This may also enhance the splicing efficiency between *CGNL1* exon 1 and *CYP19A1* exon 2 and thereby lead to relatively severe overexpression of the *CGNL1-CYP19A1* chimeric gene, although this hypothesis would not be applicable for other chimeric genes.

7. Implication for the Hypothalamus-Pituitary-Gonadal Axis Function

It is notable that a similar degree of FSH-dominant hypogonadotropic hypogonadism is observed in the three types, although E_1 and E_2 values and E_2/T ratios are much higher in the inversion type than in the duplication and deletion types (Table 1). In particular, FSH was severely suppressed even after GnRH priming in the duplication type [4]. This implies that a relatively mild excess of circulatory estrogens can exert a strong negative feedback effect on FSH secretion primarily at the pituitary. This would be consistent with the results of animal studies that show strong inhibitory effect of E_2 on transcription of FSH beta-subunit gene in the pituitary cells and almost negligible effect on synthesis of LH beta-subunit and secretion of LH [18, 19]. In this regard, while T responses to hCG stimulation are normal in the duplication and the deletion types and somewhat low in the inversion type, this would be consistent with fairly preserved LH secretion in the three types and markedly increased estrogen values in the inversion type. In addition, whereas fertility and spermatogenesis are normally preserved in the three types, this would be explained by the FSH-dominant hypogonadotropic hypogonadism, because FSH plays only a minor role in male fertility (spermatogenesis) [20].

8. Conclusions

Current studies argue that AEXS is caused by overexpression of *CYP19A1* due to three different types of cryptic genomic rearrangements including duplications, deletions, and inversions. It seems that transcriptional activity and structural property of the fused promoter constitutes the underlying factor for the clinical variability in most features of AEXS except for FSH-dominant hypogonadotropic hypogonadism. Thus, AEXS represents a novel model for gain-of-function mutation leading to human genetic disorders.

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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 (4) *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 resides 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											

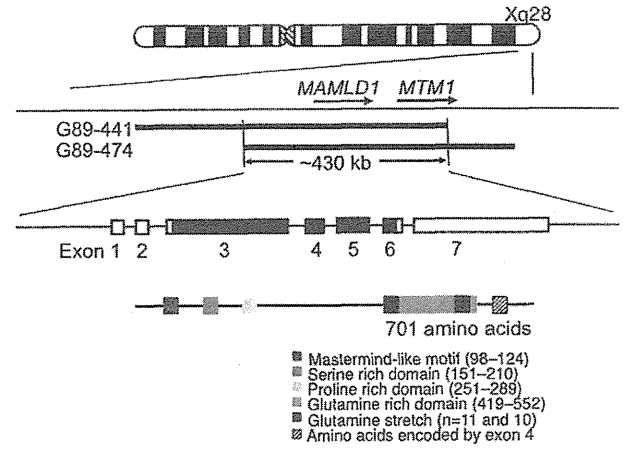

Figure 1 Positional cloning and the structure of *MAMLD1*. *MAMLD1* (*CXORF6*) has been isolated from a ~430 kb region commonly deleted in two patients with 46,XY DSD and myotubular myopathy (G89–441 and G89–474).[2] The horizontal bars indicate the deleted segments that involve *MAMLD1* and *MTM1* for 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 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.

The three nonsense mutations satisfy the conditions for the occurrence of nonsense mediated mRNA decay (NMD).[8] Consistent with this, RT-PCR 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 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 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 41 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.

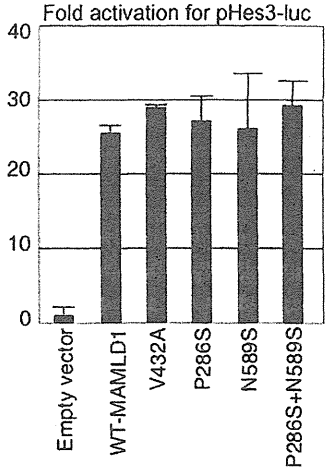

Figure 2 Functional studies for *MAMLD1* substitutions. The p.V432A, p.P286S, p.N589S, and p.P286S–p.N589S (S–S haplotype) have normal transactivating activities for the promoter of a non-canonical Notch target Hes3.

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* expression pattern in the fetal 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 *Mamld1* expression in the 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

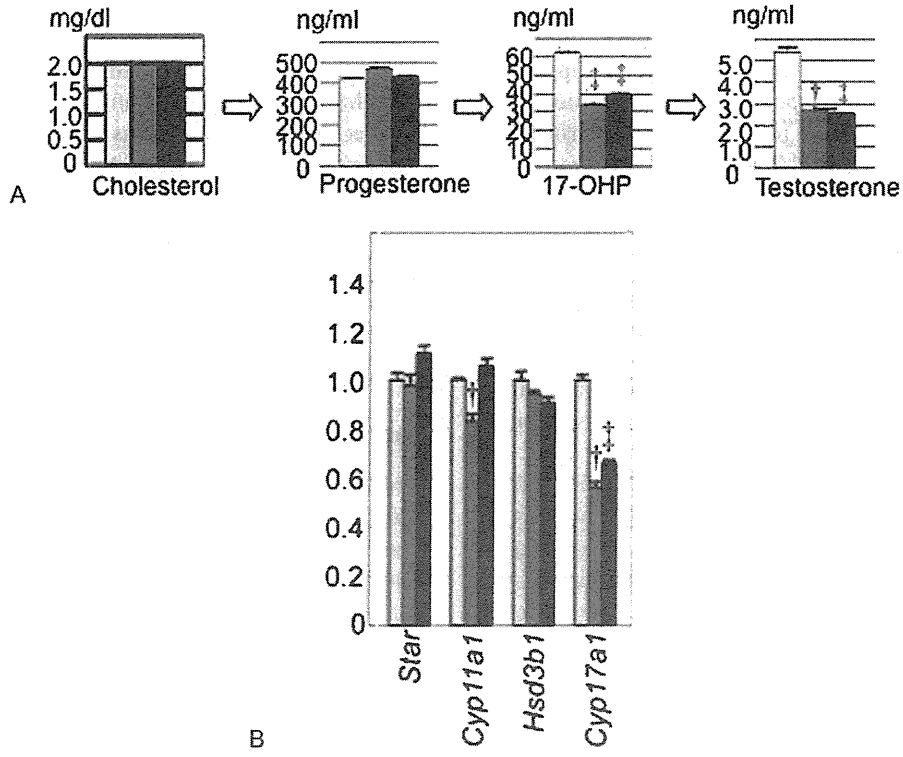

Figure 3 Representative data of the *Mamld1* knockdown experiments using MLTCs. Endogenous *Mamld1* expression has been markedly reduced to 10–15% by knockdown with si-RNAs. Shown are steroid metabolite concentrations in culture media and endogenous *Mamld1* expression levels in MLTCs. The white bars 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 *Mamld1* 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 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).[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