F．健康危険情報

なし

G．研究発表

H．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得

なし

2．実用新案登録

なし

3．その他

厚生労働科学研究費補助金（子ども家庭総合研究事業）
研究分担報告書

小児先天性疾患遺伝子診断チップの開発と標準的遺伝子診断法の確立

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研究要旨：当該年度においては、以下のことを行った。（1）遺伝子診断チップの作製とその応用、（2）インプリンティング疾患の迅速診断法の開発とその応用、（3）成育疾患遺伝子医療システムと命名したインターネット上における情報発信拠点の整備、（4）関連学会における遺伝子診断委員会、遺伝子診断予備委員会、希少疾患研究予備委員会の設置。（5）会席コストを賄うためのNPO法人オーファンネットジャパンとの連携。これらの活動は、維持可能な遺伝子診断体制の整備に大きく貢献すると考えられる。

A．研究目的

本年度における研究目的は、以下の通りである。（1）遺伝的異質性に富む疾患を対象として、関連する遺伝子を網羅的に解析できる遺伝子診断チップを開発する。（2）インプリンティング疾患の迅速診断法を開発する。（3）全国の小児内分泌医師、小児遺伝医師、小児泌尿器医師を対象として、臨床診断および遺伝子診断のディスカッションができるインターネットサイトを構築する。（4）関連学会との連携を深める。（5）解析コストを賄うために継続可能な経済的基盤整備を目指す。

B．研究方法

遺伝子診断チップの開発においては、遺伝的異質性に富む疾患のなかで、易腫瘍発症製を有する先天奇形症候群であり、かつ、変異のほぼ全てがミスセンスタイプであり、遺伝子診断チップで診断できない欠失・挿入変異ではないことが判明している疾患を対象とする。インプリンティング疾患の迅速診断法では、インプリンティング遺伝子の発現パターンと密接に関連するメチル化可変領域(DMR)の定量的判定法を開発する。インターネットサイトの構築においては、セキュリティ、利便性、将来の臨床および研究への波及効果を勘案しながら、成育医療センター内に設置する。関連学会との連携では、遺伝子診断にむけた学会を通したシステムを作製する。経済的基盤整備では、全国の病院を対象とできるNPO法人と連携する。

（倫理面への配慮）

本研究の遂行にあたっては、ヒトゲノム・遺伝子解析研究に関する倫理指針（平成13年3月29日文部科学省・厚生労働省・経済産業省告示第1号）に従っている。検体の収集を含めた研究計画については、国立成育医療センター、および各検体の収集施設において予め倫理委員会の承認を得ている。検体は、書面によるインフォームド・コンセントを取得後に収集している。

C．研究結果

1．遺伝子診断チップの開発：易腫瘍発症製を有する先天奇形症候群であるヌーナン症候群およびその類縁疾患を対象として、遺伝子診断チップを作製した（図1）。これを使って、PTPN11変異が診断できることを複数の患者において確認した。

2．インプリンティング疾患迅速診断法の開発：現在判明しているインプリンティング領域から、メチル化可変領域（DMR）を同定し、27のDMRにおいてBio-COBRAという方法を用いて迅速診断法を開発した。そして、様々な疾患を解析する過程で、世界初の全染色体母親性ダイソミー患者（図1）と、世界で6例目となる全染色体父親性ダイソミー患者を同定した。

3．インターネットサイトの構築：成育疾患遺伝子医療システムと命名した全国の小児内分泌医師、小児遺伝医師、小児泌尿器医師を対象として、臨床診断および遺伝子診断のディスカッションができるインターネットサイトを成育医療センター内に設置した（図2）。これにより、ネットワークシステム構築の基盤が確立した。

4．関連学会との連携：小児遺伝学会と連携して遺伝子診断委員会を、小児内分泌学会と連携して性分化委員会、遺伝子診断予備委員会、希少疾患研究予備委員会を設置した。これにより、学会と協調して臨床

的遺伝子診断を進める基盤が整備された。
5．経済的基盤の整備：解析コスト負担の窓口として、本年８月から稼動しているNPO法人オーファンネットジャパンと連携することを確認した。

D．考察

遺伝子診断チップの作製およびインプリンティング疾患の迅速診断法の開発は、包括的遺伝子解析を可能とすると共に、新規遺伝子同定など、厚生労働行政のみならず医学的にも大きな発展が期待できるものである。成育疾患遺伝子医療システムの構築は、臨床診断と遺伝子診断に大きく寄与し、前駆この患者および医師に有用な情報を発信する拠点となる。そして、関連学会において遺伝子診断委員会、遺伝子診断予備委員会、希少疾患研究予備委員会を設置しえたことは、全国規模の遺伝子診断普及に大きく貢献すると期待される。NPO法人オーファンネットジャパンとの連携は、維持可能な遺伝子診断体制の整備に必須であると考えられる。

E．結論

全国規模の遺伝子診断を実施する基盤整備が進展した。

F．研究発表

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G．知的財産権の出願・登録状況
本年度は該当なし

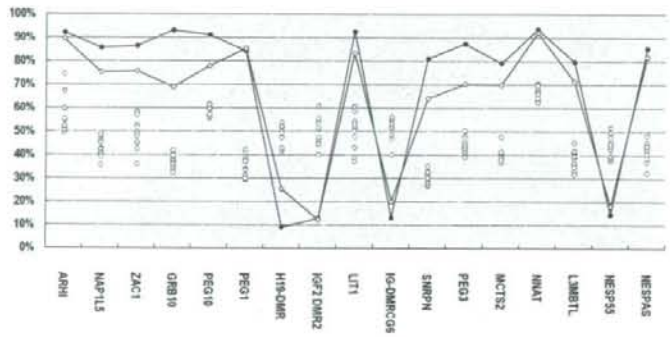

図１．世界初の全染色体母親性ダイソミー患者の結果。全てのメチル化可変領域において母親性ダイソミーに一致するデータとなっている。

図2. 成育疾患遺伝子医療システムのホームページ

研究成果刊行に関する一覧表

別紙4

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研究成果の刊行物・別刷り

nature genetics

Deletions and epimutations affecting the human 14q32.2 imprinted region in individuals with paternal and maternal upd(14)-like phenotypes

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Human chromosome 14q32.2 carries a cluster of imprinted genes including paternally expressed genes (PEGs) such as *DLK1* and *RTL1* and maternally expressed genes (MEGs) such as *MEG3* (also known as *GTL2*), *RTL1as* (*RTL1* antisense) and *MEG8* (refs. 1,2), together with the intergenic differentially methylated region (IG-DMR) and the *MEG3*-DMR[3–5]. Consistent with this, paternal and maternal uniparental disomy for chromosome 14 (upd(14)pat and upd(14)mat) cause distinct phenotypes[6,7]. We studied eight individuals (cases 1–8) with a upd(14)pat-like phenotype and three individuals (cases 9–11) with a upd(14)mat-like phenotype in the absence of upd(14) and identified various deletions and epimutations affecting the imprinted region. The results, together with recent mouse data[4,8–10], imply that the IG-DMR has an important *cis*-acting regulatory function on the maternally inherited chromosome and that excessive *RTL1* expression and decreased *DLK1* and *RTL1* expression are relevant to upd(14)pat-like and upd(14)mat-like phenotypes, respectively.

Upd(14)pat results in a unique phenotype characterized by facial abnormality, a small, bell-shaped thorax, abdominal wall defects and polyhydramnios[6], and upd(14)mat leads to clinically discernible features such as pre- and postnatal growth failure and early onset of puberty[7]. We identified five individuals with a typical upd(14)pat phenotype (cases 1, 2 and 6–8) and three individuals with a relatively mild upd(14)pat-like phenotype (cases 3–5); we also identified three individuals with a upd(14)mat-like phenotype (cases 9–11), among whom case 11 had severely compromised adult height (**Table 1** and **Supplementary Tables 1–3** online). Cases 1–8 were identified because of the presence of a bell-shaped thorax in the neonatal period (**Supplementary Fig. 1** online), and cases 9–11 were ascertained through familial studies of cases 1–8. Thus, cases 1 and 2 and cases 9 and 10 were identified in the same family (family A), as were case 3 and case 11 (family B) (**Fig. 1**). The remaining cases, 4–8, were sporadic. All karyotypes were normal except for 46,XY,r(14)(p11q32.2) in case 5, and upd(14) was excluded in all cases by microsatellite analysis (**Supplementary Table 4** online).

We examined the 14q32.2 imprinted region (**Fig. 2**) using leukocyte genomic DNA and lymphocyte metaphase spreads of cases 2–11 (case 1 was deceased) and their family members who were willing to participate in this study. We also analyzed blood samples of control subjects and previously reported upd(14)pat and upd(14) mat cases[6,11].

We first determined the DMRs to be examined in this study (**Supplementary Fig. 2a** online). For the IG-DMR[4], *in silico* analysis followed by bisulfite sequencing revealed two DMRs, which we

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Table 1 Summary of clinical and molecular findings

	Cases 1[a] & 2	Case 3	Case 4	Case 5	Cases 6–8	Cases 9 & 10	Case 11
Upd(14)pat-like phenotype	+ (typical)	+ (mild)	+ (mild)	+ (mild)	+ (typical)		
Upd(14)mat-like phenotype						+	+ (severe)[b]
IG-DMR	Deleted	Deleted	Deleted	Deleted	Epimutated[c]	Deleted	Deleted
MEG3-DMR	Deleted	Deleted	Deleted	Deleted	Epimutated[c]	Deleted	Deleted
Deleted PEGs	*DLK1*	*DLK1*	*RTL1*	*DLK1*	None	*DLK1*	*DLK1*
		RTL1		*RTL1*			*RTL1*
				DIO3			
Deleted MEGs	*MEG3*	*MEG3*	*MEG3*	*MEG3*	None	*MEG3*	*MEG3*
		RTL1as	*RTL1as*	*RTL1as*			*RTL1as*
		MEG8	*MEG8*	*MEG8*			*MEG8*
Parental origin[d]	Maternal	Maternal	Maternal	Maternal	Maternal	Paternal	Paternal

Detailed clinical features of cases 1–8 and upd(14)pat cases are described in **Supplementary Table 1**, and those of cases 9–11 and upd(14)mat cases are described in **Supplementary Table 2**. Phenotypic assessment is summarized in **Supplementary Table 3**. Chest roentgenograms of cases 1–8 are shown in **Supplementary Fig. 1**. [a]Case 1, though not studied, presumably has the same deletion as case 2. [b]Adult height is severely compromised in case 11. [c]Hypermethylation of the normally hypomethylated allele of maternal origin (**Fig. 3**). [d]Parental origin of chromosomes with deletions or epimutations. In cases 7, 10 and 11, although parental genotyping data are not informative or available, methylation and FISH analyses indicate hypermethylation of the normally hypomethylated allele of maternal origin in case 7 and loss of the normally hypermethylated allele of paternal origin in cases 10 and 11 (**Fig. 3** and **Fig. 4**).

designated CG4 and CG6. For the *MEG3*-DMR, we confirmed the previously reported DMR[5] (hereafter designated CG7) through bisulfite sequencing and PCR amplification with methylated and unmethylated allele-specific primers.

We then carried out methylation analysis, which showed that the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) were severely hypermethylated in cases 2–8, to an extent comparable to that found in the upd(14)pat case, whereas they were grossly hypomethylated in cases 9–11, to a degree similar to that identified in the upd(14)mat case (**Fig. 3** and **Supplementary Fig. 2b**). Notably, we confirmed hypermethylation of normally hypomethylated CG4 clones of maternal origin by informative SNP typing data in cases 6 and 8. We carried out FISH analysis with two long and accurate (LA)-PCR products covering the IG-DMR and the *MEG3*-DMR, and we found familial heterozygous microdeletions in cases from families A and B and a *de novo* heterozygous microdeletion in case 4 (**Fig. 4**). This deletion was also detected in case 5, the individual with the r(14) chromosome, but it was absent in cases 6–8.

Subsequently, we carried out genotyping analysis for 200 loci, showing lack of common alleles for multiple loci between cases 2 and 9 and between cases 9 and 10 in family A, between case 3 and case 11 in family B, between case 4 and the mother, and between case 5 and the mother (**Supplementary Table 4**). We carried out sequencing analyses for LA-PCR products obtained with primers flanking the deleted loci and identified a 108,768-bp deletion involving *DLK1* and *MEG3* in cases 2, 9 and 10 of family A (case 1 presumably had the same deletion), a 411,354-bp deletion involving *WDR25*, *BEGAIN*, *DLK1*, *MEG3*, *RTL1*, *RTL1as* and *MEG8* in cases 3 and 11 of family B, and a 474,550-bp deletion involving *MEG3*, *RTL1*, *RTL1as* and *MEG8* in case 4; in case 5, we carried out FISH analyses with nine BAC probes covering the imprinted region and identified a ~6.5-Mb deletion involving the whole imprinted region (**Fig. 2** and **Supplementary Fig. 3** online). In cases 6–8, we identified neither tiny deletion nor sequence variation around the *DLK1-MEG3* region, including the DMRs and the putative CTCF binding sites[12], by extensive analyses (**Supplementary Fig. 3**), and we found normal methylation patterns for DMRs at the *MEST* promoter[13], the *IGF2-H19* domain[14] and the *SNRPN* promoter[15]. Thus, we determined that cases 6–8 have epimutations affecting the 14q32.2 imprinted region. Although we attempted to examine the expression dosage of the imprinted genes on 14q32.2, expression was absent or extremely faint in leukocytes. The above molecular data from leukocytes are summarized in **Table 1**.

We further examined placental samples, because virtually all the imprinted genes studied to date are expressed in the placenta[16,17]. Using expression analyses combined with SNP genotypings, we confirmed monoallelic paternal expression of *DLK1* and maternal expression of *MEG3* in normal fresh placentas (**Supplementary Fig. 2c**). RT-PCR analyses showed positive PEGs expression and negative MEGs expression in formalin-treated and paraffin-embedded placental samples of cases 2 and 8 and the upd(14)pat case, with *RTL1* expression being obviously elevated in the three placentas compared to a similarly treated control placenta (**Fig. 5a**). Histological examinations showed proliferation of dilated and congested chorionic villi in the three placentas (**Fig. 5b**). Furthermore, CG4 was hypermethylated in the placentas of cases 2 and 8 and the upd(14)pat case and was delineated as the DMR in the control placenta, whereas CG7 was rather hypomethylated in the four placentas and did not show the DMR-compatible methylation patterns (**Supplementary Fig. 2c**).

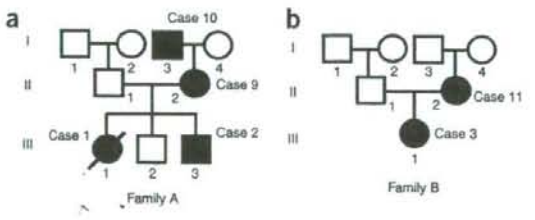

Figure 1 The pedigrees of two families. (**a**) Family A. Case 1 (III-1) and case 2 (III-3) show typical upd(14)pat phenotype (black), whereas case 9 (II-2) and case 10 (I-3) manifest upd(14)mat-like phenotype with mild to moderate short stature (gray). The remaining five family members have normal phenotype. (**b**) Family B. Case 3 (III-1) shows relatively mild upd(14)pat-like phenotype (black), and case 11 has upd(14)mat-like phenotype with marked short stature (gray). The remaining five family members, including the maternal grandparents, have normal phenotype (stature, ± 0 s.d. in the maternal grandfather and –0.8 s.d. in the maternal grandmother). The maternal grandparents refused to take part in molecular studies.

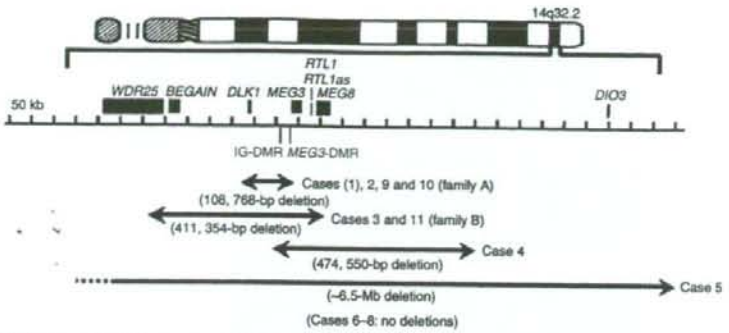

Figure 2 The regional physical map of the human chromosome 14q32.2 imprinted region and the four deletion intervals identified in this study. PEGs are shown in blue and MEGs are shown in red, although it remains to be clarified whether *DIO3* is a PEG; mouse *Dio3* is known to be preferentially but not exclusively expressed from a paternally derived chromosome in embryos[25]. *DAT*[2] (not shown) may also be a PEG in the human, and *Mirg* (*Meg9*)[18] resides between *Rian* (*Meg8*) and *Dio3* in the mouse. *WDR25* and *BEGAIN* seem to be biparentally expressed genes. The two DMRs are depicted in green. The electrochromatograms indicating the fusion points of the three microdeletions are shown in **Supplementary Figure 3**, together with the physical maps representing the deleted regions. The FISH findings identifying the 14q32.2 breakpoint of the r(14) chromosome in case 5 are also shown in **Supplementary Figure 3**. Case 1 of family A was not studied, and no deletion has been identified in cases 6–8.

The human 14q32.2 imprinted region is highly conserved on the distal part of mouse chromosome 12 (ref. 18), in which the germline-derived IG-DMR functions as a *cis*-acting regulator for the imprinted region of maternal origin[4,19]. Targeted deletion of the IG-DMR (ΔIG-DMR) causes paternalization of a maternally derived imprinted region and a unique phenotype comparable to that of paternal uniparental disomy for chromosome 12 (PatDi(12)) in embryos, with ~4.5 times of *Rtl1* expression and ~2 times of *Dlk1* and *Dio3* expression as well as nearly absent expression of MEGs in this imprinted region[4,20,21]. The markedly increased *Rtl1* expression is ascribed to the cumulative effect of the activation of the usually silent maternally derived *Rtl1* and the loss of the microRNA-containing *Rtl1as*, which acts as a repressor for *Rtl1* (refs. 4,9,10). The doubled *Dlk1* and *Dio3* expression is simply a result of the activation of PEGs of maternal origin[4]. The absent MEGs expression is associated with hypermethylation of the *Gtl2*-DMR[4], and this is consistent with the notion that the *Gtl2*-DMR can stay hypomethylated only in the presence of the hypomethylated IG-DMR[4,19]. By contrast, the ΔIG-DMR has no imprinting or clinical effect after paternal transmission[4].

The upd(14)pat-like phenotypes in cases 1–8 are explained by assuming that the human IG-DMR and *RTL1as* have similar functions to the mouse IG-DMR and *Rtl1as*. Indeed, the placental expression data (**Fig. 5a**) are consistent with the deletions and epimutations (hypermethylation) involving the maternally inherited IG-DMR described here, or another yet unidentified IG-DMR(s), resulting in a maternal to paternal epigenotypic alteration with augmented *RTL1* expression. In this case, the excessive *RTL1* expression seems to have a major role in the development of the upd(14)pat-like phenotype, because it is predicted from the mouse data[4,8–10] that cases 3–5, with mild upd(14)pat-like phenotypes, should have moderately increased *RTL1* expression as a result of the presence of a single active copy of *RTL1* in the absence of functional *RTL1as* and that the remaining cases, with typical upd(14)pat phenotypes, should have markedly elevated *RTL1* expression, as in the upd(14)pat cases with two active copies of *RTL1* in the absence of functional *RTL1as* (**Supplementary Table 3**). In support of this, the mouse *Rtl1* is expressed not only in the placenta but also in various fetal tissues including ribs and skeletal muscles[8], and mice with 2.5–3.0 times of *Rtl1* expression caused by maternally derived *Rtl1as* deletion have placental abnormalities[8] similar to those of individuals with the upd(14)pat-like phenotype. By contrast, the typical upd(14)pat phenotype of cases 1 and 2 and the phenotypic similarity between cases 3 and 4 would argue against a

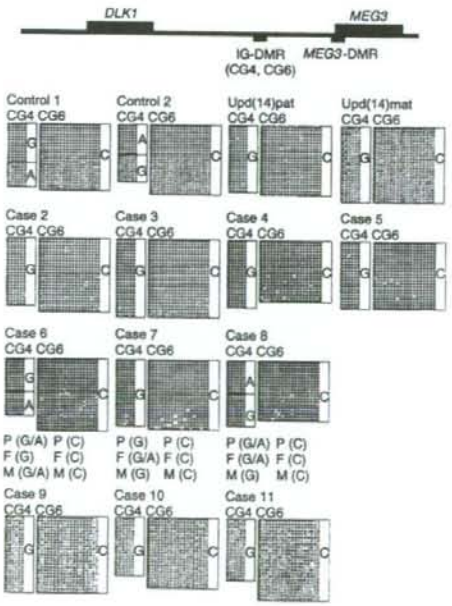

Figure 3 Methylation patterns of the IG-DMR (CG4 and CG6) (those of the *MEG3*-DMR (CG7) are shown in **Supplementary Fig. 2b**). The upper ideogram represents the positions of the IG-DMR and the *MEG3*-DMR. Bisulfite sequencing was done for CG4 and CG6. Each line indicates a single clone, and each circle denotes a CpG island; filled and open circles represent methylated and unmethylated cytosines, respectively. CG4 and CG6 are differentially methylated in control subjects, severely hypermethylated in the upd(14)pat case and cases 2–8 with a upd(14)pat-like phenotype, and grossly hypomethylated in the upd(14)mat case and cases 9–11 with a upd(14)mat-like phenotype. Furthermore, the CG4 SNP typing data (*rs12437020*) are consistent with the parental origin–dependent methylation patterns in the control subjects and show hypermethylation of the maternally derived clones associated with the 'A' allele as well as the paternally derived clones associated with the 'G' allele in case 6 and that of the maternally derived clones associated with the 'G' allele as well as the paternally derived clones associated with the 'A' allele in case 8 (P, patient; F, father; M, mother). The CG6 SNP typing data (*rs10133627*) were not informative. In addition, normal differential methylation patterns were obtained in other examined individuals with a normal phenotype (the father and the maternal grandmother in family A, the father in family B and the parents of cases 4–8) (not shown).

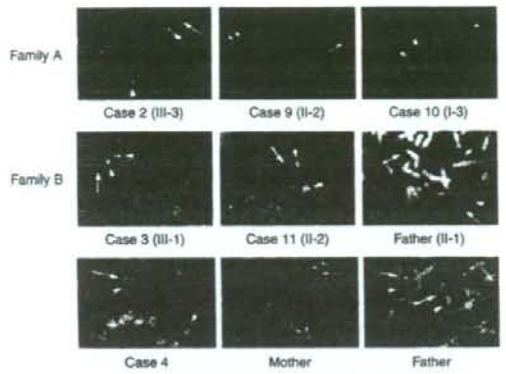

Figure 4 FISH results for the IG-DMR. The red signals (arrows) have been detected by a 5,104-bp LA-PCR product encompassing the CG1–CG6 region (**Fig. 3** and **Supplementary Fig. 2a**), and the green signals (arrowheads) have been identified by an RP11-566I2 probe for 14q12 used as an internal control. Familial heterozygous deletions are identified in cases 2, 9 and 10 of family A and in cases 3 and 11 of family B, and a *de novo* heterozygous deletion is detected in case 4. We also did FISH analyses with a 5,182-bp product encompassing the CG7–CG9 region, showing the same results (not shown).

major role of doubled *DLK1* dosage in the development of upd(14)pat-like phenotype (**Supplementary Table 3**).

The upd(14)mat-like phenotypes of cases 9–11 most likely reflect loss of functional PEGs because it is predicted from the mouse data[4] that deletions involving the paternally derived IG-DMR should not perturb the imprinting status. In this case, loss of active *DLK1* and *RTL1* seems to constitute additive underlying major factors for the development of the upd(14)mat-like phenotype, because a upd(14)mat-like phenotype is common to cases 9–11, who lack active *DLK1*, and growth is more severely compromised in case 11, with additional loss of active *RTL1* (**Supplementary Table 3**). In support of this, the paternally derived *Dlk1* mutation has been previously shown to result in several upd(14)mat-like features, such as pre- and postnatal growth deficiency and obesity and facial abnormalities in mice[22], and the paternally inherited *Rtl1* deletion has been shown to cause mild growth deficiency in mice[8], with the degree of growth failure being ~80% in the two types of knockout mice and ~60% in the MatDi(12) mice lacking functional copies of both *Dlk1* and *Rtl1* (ref. 20). Furthermore, hypomethylation of the paternally inherited DMR adjacent to CG7 has been observed in a single individual with upd(14)mat-like phenotype[23]. This represents a mirror image of epimutations leading to the upd(14)pat-like phenotype and implies that an epimutation is involved in the development of the upd(14)mat-like phenotype.

Although altered *DIO3* and MEGs expression may also be relevant to upd(14)pat/mat phenotypes, the clinical effects would remain minor, if any. Although the primary function of *DIO3* is inactivation of thyroid hormones[24], thyroid dysfunction was absent from cases 1–11 and upd(14)pat/mat cases (this may suggest partial imprinting of human *DIO3*, like that of mouse *Dio3* (ref. 25)) (**Supplementary Tables 1** and **2**). Similarly, although there has been no model mouse with null or doubled MEGs expression that did not show effects on PEGs, *Gtl2*^{lacZ} mice with dysregulated imprinting status caused by a transgene insertion have a normal phenotype with at least 60–80% reduction of all the MEGs[26].

Two findings are noteworthy in reference to the placental studies. First, the methylation profiles of CG4 were similar and those of CG7 were different between leukocytes and placentas of affected and control subjects, as observed in the corresponding regions of normal mice[21]. This implies that the placental imprinting regulation is influenced by the IG-DMR, but is independent of the *MEG3*-DMR, and is also subject to some mechanism(s) such as the control of chromatin conformation[27,28]. Second, phenotypic abnormalities and epigenotypic alterations were identified in the placentas of cases 2 and 8, which had impaired IG-DMRs, whereas they are absent or obscure in the placentas of ΔIG-DMR mice[21]. This suggests that altered gene expression dosage has an essential role in phenotypic development and that there is some difference in IG-DMR function between the human and the mouse placentas (see legend for **Supplementary Fig. 2**).

METHODS

Extraction of DNA and RNA samples. This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development, and written informed consent was obtained from each subject or his or her parent(s). For leukocytes and fresh placental tissues, we obtained genomic DNA with FlexiGene DNA Kit (Qiagen) and prepared RNA with RNeasy Plus Mini (Qiagen). For paraffin-embedded placental samples, we extracted genomic DNA and RNA with RecoverAll Total Nucleic Acids Isolation Kit (Ambion) using slices of 40 μm thick.

Primers. The primers used in this study are summarized in **Supplementary Table 5** online.

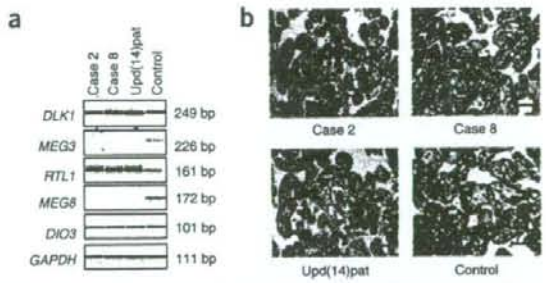

Figure 5 Examinations of placental samples. (**a**) RT-PCR analysis (35–40 cycles) using formalin-treated and paraffin-embedded placental samples of case 2 (30 weeks of gestational age), case 8 (35 weeks), the upd(14)pat case (32 weeks) and a normal subject (33 weeks). *DLK1*, *RTL1* and *DIO3* are expressed and *MEG3* and *MEG8* are not expressed in the placentas of case 2, case 8 and the upd(14)pat case, whereas all the PEGs and MEGs are identified in the control placenta. The results were consistent in RT-PCR experiments performed seven times, except for faint possible *MEG8* expression detected once in the placenta of case 2. Compared to the expression pattern in the control placenta, *RTL1* expression is notably elevated in the placentas of cases 2 and 8 and the upd(14)pat case, and *DLK1* expression is possibly increased in the placentas of case 8 and the upd(14)pat case, although precise quantification was impossible because of poor RNA quality. Although contamination from maternal leukocytes is likely present in these samples, this would not influence the expression pattern, because of absent expression of the imprinted genes in this cell type. (**b**) Histological findings (hematoxylin-eosin staining). The chorionic villi of cases 2 and 8 and the upd(14)pat case are notably proliferated and dilated with congestion, as compared with those of the control subject. The bars represent 100 μm. In addition, nonspecific chorangioma was observed in the placenta of case 2, and interspersed villous calcifications suggestive of degeneration was identified in the placentas of case 8 and the upd(14)pat case.

Genotyping and sequence deletion and variation analyses. Leukocyte genomic DNA was used for these studies. For microsatellite genotyping, we PCR-amplified a segment encompassing each locus with a fluorescently labeled forward primer and an unlabeled reverse primer and determined its size on an ABI PRISM 310 autosequencer using GeneScan software (Applied Biosystems). For polymorphism genotyping, a segment encompassing polymorphism(s) was PCR-amplified and subjected to direct sequencing on a Cequation (8000) autosequencer (Beckman Coulter). For the examination of a tiny deletion and a sequence variation around the *DLK1–MEG3* region, we obtained three LA-PCR products from cases 6–8 and a control subject and subjected them to fragment size comparisons after restriction enzyme digestions and to direct sequencing using serial forward primers (when clear electrochromatograms were not obtained, the corresponding sequences were also analyzed with reverse primers). The three LA-PCR products were a 5,104-bp product encompassing the CG1–CG6 region (also used as FISH probe 1 for the IG-DMR), a 3,142-bp product covering a putative CTCF binding site (A)[12] and a 5,182-bp product encompassing the CG7–CG9 region and six putative CTCF binding sites (B–G)[12] (also used as FISH probe 2 for the *MEG3*-DMR). The restriction enzymes used were *Bam*HI, *Kpn*I and *Nco*I for the 5,104-bp product, *Drd*I and *Taq*I for the 3,142-bp product and *Acl*I, *Eco*RI, *Bss*HII, *Kpn*I and *Sac*I for the 5,182-bp product.

Methylation analysis. Leukocyte and placental genomic DNA were treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research) that converts all the cytosines except for methylated cytosines at the CpG islands into uracils and subsequently thymines. We PCR-amplified the CG1–CG9 regions with primer sets that hybridize to both methylated and unmethylated alleles because of absent CpG dinucleotides within the primer sequences. Then, we subcloned the PCR products with the TOPO TA Cloning Kit (Invitrogen) and subjected multiple clones to direct sequencing on the Cequation (8000) autosequencer. When the PCR products contained SNPs, we also carried out genotyping. For CG7, PCR amplification was also done with a methylated allele-specific primer pair hybridizing to a region containing unconverted methylated cytosines and with an unmethylated allele-specific primer pair hybridizing to a region containing thymines converted from unmethylated cytosines, and the PCR products were subjected to gel electrophoresis, as reported previously[5]. For CG8, combined bisulfite restriction analysis (COBRA) was also carried out using a methylated allele-specific *Hga*I restriction site.

FISH analysis. Lymphocyte metaphase spreads were hybridized with probes for specific sequences at the 14q32.2 imprinted region, together with an RP11-566I2 probe for 14q12 used as an internal control. The FISH probes 1 and 2 were as described above, and FISH probes 3 (5,540 bp) and 4 (6,058 bp) were obtained by LA-PCR for the polymorphism-poor sequences at the *DLK1–MEG3* region. We purchased the nine BAC probes covering the imprinted region from BACPAC Resources Center at Children's Hospital Oakland Research Institute. The probes for specific sequences were labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the control probe was labeled with biotin and detected with avidin conjugated to fluorescein isothiocyanate.

Expression analysis. We analyzed leukocyte and placental RNA for the expression of PEGs and MEGs on the 14q32.2 region. For the imprinted genes other than *RTL1*, cDNA was synthesized from 1 μg of RNA using Superscript III Reverse Transcriptase (Invitrogen), and RT-PCR was done with 20 ng of total RNA using ExTaq (Takara). For *RTL1*, because the primers hybridizing to exon sequences amplify both *RTL1* and *RTL1as*, we used 3′-RACE. We synthesized cDNA from 1 μg of RNA using Superscript III Reverse Transcriptase with a long primer hybridizing to the poly(A) site and introducing the adaptor sequence, and, subsequently, we carried out RT-PCR for 50 ng of cDNA using KOD Dash (Toyobo) with a primer hybridizing to the adaptor sequence and another primer hybridizing to the exon sequence. The reactions were carried out in 200-μl tubes, and small amounts (1–2 μl) of the reaction solutions were loaded onto Gel-Dye Mix (Agilent) after adjusting the *GAPDH* dosage among examined samples.

To examine monoallelic expression in normal placentas, we subjected placental cDNA and genomic DNA and maternal genomic DNA to direct sequencing with primers designed to amplify regions including exonic SNPs (*rs2295660* for *DLK1*; *rs1884540* for *MEG3*; *rs6575805*, *rs11623267*, *rs35695758*, and *rs12884005* for *RTL1*; *rs11847631* and *rs7159526* for *MEG8*; and *rs11627443* and *rs945006* for *DIO3*).

GenBank accession codes. Genome: NC_000014 for *DLK1*, *MEG3*, *RTL1* and *DIO3*, and AL117190 for *MEG8*; mRNA: NM_003836 for *DLK1*, NR_002766 for *MEG3*, XM_370776 for *RTL1*, CA396130 for *MEG8*, and NM_001362 for *DIO3*.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Molecular analysis was performed by M.K., Y.S., M.I., F.K., M.O. and S.Y., placental sample collection and preparation by H.K., M.N., Y.T., K.M. and K. Ko., placental pathological examination by K.M., and blood sampling and phenotype assessment by G.N., T.T., M.N., Y.T., K.M., T.U., H.K., Y.K., H.O., K. Ku. and T.O. The study was designed and coordinated by F.I. and T.O. with later input from A.C.F.-S., and the results were interpreted and discussed by A.C.F.-S., F.I. and T.O. The paper was written by T.O.

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Mastermind-like Domain-containing 1 (*MAMLD1* or *CXorf6*) Transactivates the *Hes3* Promoter, Augments Testosterone Production, and Contains the SF1 Target Sequence*[S]

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Although chromosome X open reading frame 6 (*CXorf6*) has been shown to be a causative gene for hypospadias, its molecular function remains unknown. To clarify this, we first examined CXorf6 protein structure, identifying homology to mastermind-like 2 (MAML2) protein, which functions as a co-activator in canonical Notch signaling. Transactivation analysis for wild-type CXorf6 protein by luciferase assays showed that CXorf6 significantly transactivated the promoter of a noncanonical Notch target gene hairy/enhancer of split 3 (*Hes3*) without demonstrable DNA-binding capacity. Transactivation analysis was also performed for the previously described three apparently pathologic nonsense mutations, indicating that E124X and Q197X proteins had no transactivation function, whereas R653X protein retained a nearly normal transactivation function. Subcellular localization analysis revealed that wild-type and R653X proteins co-localized with MAML2 protein in nuclear bodies, whereas E124X and Q197X proteins were incapable of localizing to nuclear bodies. Thus, further studies were performed for R653X, revealing the occurrence of nonsense mediated mRNA decay *in vivo*. Next, transient knockdown of *CXorf6* was performed using small interfering RNA, showing reduced testosterone production in mouse Leydig tumor cells. Furthermore, steroidogenic factor 1 (SF1) protein bound to a specific sequence in the upstream of the *CXorf6* coding region and exerted a transactivation activity. These results suggest that *CXorf6* transactivates the *Hes3* promoter, augments testosterone production, and contains the SF1 target sequence, thereby providing the first clue to clarify the biological role of *CXorf6*. We designate *CXorf6* as *MAMLD1* (mastermind-like domain-containing 1) based on its characteristic structure.

Chromosome X open reading frame 6 (*CXorf6*)[2] was identified by Laporte *et al.* (1, 2) as a candidate gene for 46,XY disorders of sex development. It spans ~70 kb in genomic sequence and comprises at least seven exons. An open reading frame resides on exons 3–6 and produces two proteins of 701 and 660 amino acids because of in-frame alternative splicing with and without exon 4. PCR-based human cDNA library screening has revealed ubiquitous expression of both splice variants, with the exon 4 positive variant being the major form (3). To date, however, the products of *CXorf6* have been poorly characterized, although glutamine- and proline-rich domains have been identified on exon 3 (1).

We have recently shown that *CXorf6* is a causative gene for hypospadias (3), a common male external genital anomaly defined by the urethral opening on the ventral side of the penis and classified into several types on the basis of the anatomical location of the urethral meatus (4). This notion is based primarily on the identification of nonsense mutations in two maternally related half-brothers (E124X) and in two sporadic boys (Q197X and R653X) with penoscrotal hypospadias (3). Because the mouse homolog (G630014P10Rik, NM_001081354) is transiently expressed in fetal Sertoli and Leydig cells around the critical period for sex development, it is likely that the *CXorf6* mutations cause hypospadias primarily because of testicular dysfunction and the resultant compromised testosterone production around that period (3). Indeed, although various genetic and environmental factors have been implicated in the development of hypospadias, it has been widely accepted that hypospadias can be caused by impaired testosterone effects around the critical period for sex development (4). Furthermore, the mouse homolog is co-expressed with steroidogenic factor 1 (*SF1*; aliases, *AD4BP* and *NR5A1*) (3), which regulates the transcription of a vast array of genes involved in sex devel-

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[2] The abbreviations used are: CXorf6, chromosome X open reading frame 6; SF1, steroidogenic factor 1; MAML, mastermind-like; N-ICD, Notch intracellular domain; RBP-J, recombination signal binding protein-J; STAT, signal transducer and activator of transcription; siRNA, small interfering RNA; RFP, red fluorescent protein; GFP, green fluorescent protein; MLT, mouse Leydig tumor; hCG, human chorionic gonadotropin; CR, conserved region; EMSA, electrophoretic mobility shift assay; NMD, nonsense-mediated mRNA decay; RT, reverse transcriptase; CHX, cycloheximide; MAMLD1, mastermind-like domain-containing 1.