Figure 5

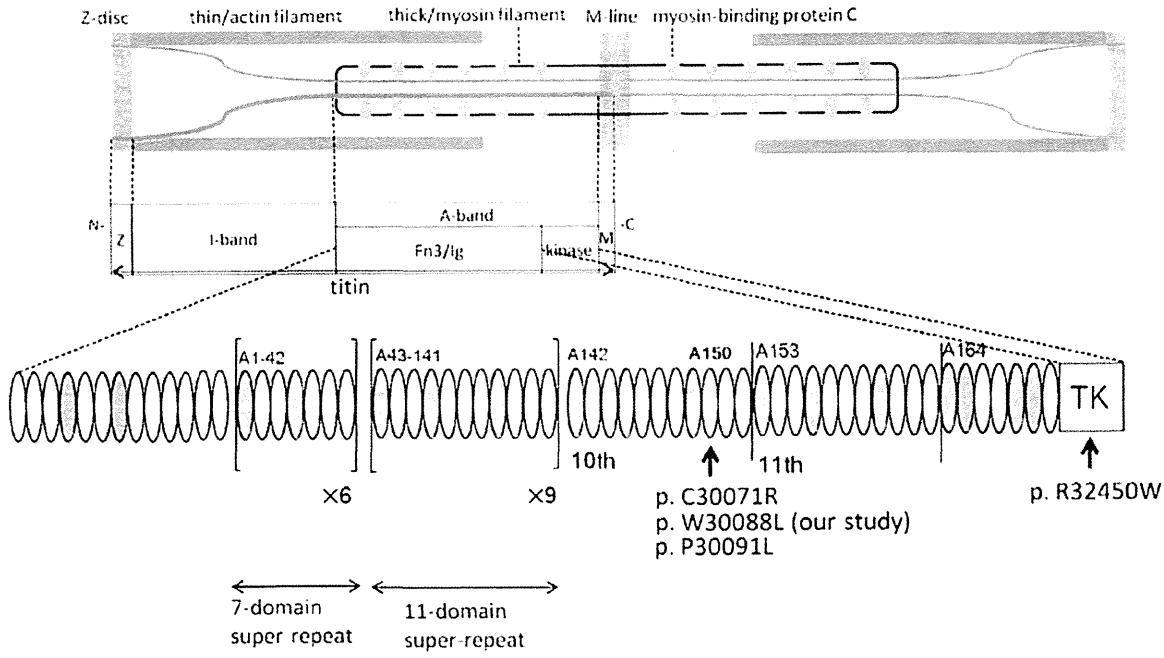

Table 1. Summary of variants detected by exome sequencing

Individual	A	B	C	D	E	H	I	segregated in 7 family members
Morbidity	affected	affected	unaffected	unaffected	affected	affected	unaffected	
Exonic, splicing	10089	10064	10079	10065	10230	10194	10216	64
Nonsynonymous, splicing, indel, nonsense	4987	5020	5055	5038	5143	5234	5200	32
Allele frequency not available	577	600	536	555	671	794	786	2

Supplementary Table 1. PCR primers for Sanger sequencing

Gene	Target position	Forward	Reverse
TTN	chr2: 179 410 777	5'-F-gctctgattcaacaggcaca-3'	5'-R-tactggcaaacgagcaactg-3'
IKBKB	chr8: 42 188 457	5'-F-ctcaattgtcctgatttcctcc-3'	5'-R-atcagtgcagcaggaaccac-3'

F: 5'- gtaaaacgacggccagt R: 5'- aggaaacagctatgacc

Supplementary Table 2. Summary of exome sequencing

Individual	A	B	C	D	E	H	I
Exome enrichment (target region)	SureSelect v4 (51 Mb)				SureSelect v2 (44 Mb)		
Total reads	208 765 930	234 409 236	203 069 726	214 983 066	268 432 814	311 054 980	436 108 596
Mapped reads	207 433 584	232 999 958	201 754 025	213 551 864	266 941 217	309 612 830	434 061 033
Reads without duplicate	103 635 209	144 338 950	101 210 836	137 835 318	111 761 210	125 930 633	145 943 484
Reads on target region	84 702 354	116 887 400	81 467 268	107 694 423	73 660 499	72 647 916	96 089 561
Mean depth of coverage on target region	141.52	195.02	136.06	179.29	117.66	131.65	153.19
% target region >= 50x	81.84	89.53	80.9	88.31	67.32	69.65	72.48

Table 2. Previously reported *TTN* mutations with skeletal and/or respiratory muscle involvement

Phenotype	LGMD	HMERF	our family	HMERF	HMERF	TMD	TMD	LGMD2J	TMD	TMD	TMD	TMD	TMD	early-onset myopathy with fatal cardiomyopathy	early-onset myopathy with fatal cardiomyopathy
Reported by	Vasli[16]	Ohlsson[14], Pfeffer[15]	Abe[5]	Vasli[16]	Edstrom[12], Nicolao[11],Lange[13]	Hackman[23]	Udd[20], Hackman[19]	Udd[25], Hackman[19]	Pollazzon[24]	Van den Bergh[22]	Seze[21], Hackman[19]	Hackman[23]	Hackman[23]	Carmignac[26]	Carmignac[26]
Mutation identified in	2012	2012	2012	2012	2005	2008	2002	2002	2010	2003	2002	2008	2008	2007	2007
Nucleotide (NM_001256850.1)	c.3100G>A, c.52024G>A	c.90315T>C	c.90263G>T	c.90272C>T	c.97348C>T	c.102724delT	102857_102867 del11ins11	102857_102867 del11ins11	c.102914A>C	c.102917T>A	c.102944T>C	c.102966delA	c.102967C>T	g.289385del ACCAAGTG	g 291297delA
Protein (NP_001243779.1)	p.V1034M, p.A17342T	p.C30071R	p.W30088L	p.P30091L	p.R32450W				p.H34305P	p.I34306N	p.L34315P		p.Q34323X		
Domain	I-band, A-band	A-band (Fn3)	A-band (Fn3)	A-band (Fn3)	A-band (Kinase)	M-line	M-line	M-line	M-line	M-line	M-line	M-line	M-line	M-line	M-line
Population	French	Swedish English	Japanese	Portuguese	Swedish	French	Finnish	Finnish	Italian	Belgian	French	Spanish	French	Sudanese	Moroccan
Inheritance	AR	AD	AD	AD	AD	AD	AD	AR	AD	AD	AD	AD	AD	consanguineous siblings	consanguineous siblings
Onset	35	33-71	27-45	46	20-50s	20-30s	35-55	20-30s	50-60s	47	45	40-50s	30s	neonatal	infant-early childhood
Skeletal muscles															
major	proximal UL and LL	TA,PL,EDL, ST	TA, ST	no	TA, neck flexor, proximals	TA,GA,HAM, pelvic	TA	all proximals	TA	TA	TA	TA	TA,HAM, pelvic	general muscle weakness and hypotonia	psoas,TA,GA, peroneus
minor		neck flexor	cervical, shoulder girdles, intercostals,			QF				EDL,peroneal, TP	GA,femoral, scapular	HAM,GA	GA, distal UL		QF,proximal UL, neck,facial, trunk flexor
spared			facial			proximal UL	facial,UL, proximals	facial		UL, proximal LL	facial	UL	proximal UL, QF		
Cardiac muscles	ND	no	no	ND	ND	ND	no	no	ND	ND	ND	ND	ND	DCM, onset: in the first decade	DCM, onset: 5-12yrs old
Respiratory failure	ND	yes, within 5-8yrs	yes, within 7yrs	isolated respiratory failure	yes, as first presentation	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Muscle pathologic features	ND	inclusion bodies(major) and RVs(minor)	cytoplasmic bodies(major) and RVs(minor)	cytoplasmic bodies	cytoplasmic bodies,positive for rhodamine-conjugated phalloidin	dystrophic pattern without vacuoles	nonspecific dystrophic change	nonspecific dystrophic change, loss of calpain-3	dystrophic pattern with RVs	nonspecific, RV	nonspecific	dystrophic pattern with RVs	nonspecific	minicore-like lesions and abundant central nuclei	minicore-like lesions and abundant central nuclei

Abbreviations: DCM, dilated cardiomyopathy; EDL, extensor digitorum longus; GA, gastrocnemius; HAM, hamstrings; LL, lower limb; ND, not described; no, no involvement; PL, peroneus longus; QF, quadriceps femoris; RV, rimmed vacuole; ST, semitendinosus; TA, tibialis anterior; TP, tibialis posterior; UL, upper limb

トピックス

生殖補助医療由来の先天性ゲノムインプリンティング異常症

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はじめに

近年，生殖補助医療（ART）の普及により，これまで非常に稀であったゲノムインプリンティング異常症の発生頻度の増加が世界各国で指摘されている［1，2］．このゲノムインプリンティング（遺伝子刷り込み）とは，特定の親由来の遺伝子発現（片親性発現）を示す現象で，生殖細胞形成過程におけるアレル特異的メチル化領域（DMR）のメチル化が刷り込みの本態と考えられている［3］．このメチル化には，卵子形成過程でメチル化を獲得する卵子型メチル化インプリントと精子形成過程でメチル化を獲得する精子型メチル化インプリントが存在する．卵子および精子で確立したそれぞれのメチル化インプリント（性特異的なメチル化状態）は，受精から着床初期に起きるリプログラミング（ゲノム全体の脱メチル化）の影響を受けず，そのメチル化は生涯安定して維持される．また，DMRにおけるメチル化は，始原生殖細胞においていったんリセット（インプリントの消去）され，その後，配偶子形成・成熟過程で性特異的にインプリントの獲得されることで再確立される．

DNAメチル化に代表されるエピジェネティックな修飾は，遺伝子配列の変化を伴わずに遺伝子機能を変換し，発生過程のゲノム機能を動的に制御している．初期胚におけるDNAメチル化は，可逆的な化学的な修飾であるため，生理的な環境とは異なる環境に暴露されると影響を受け，エピゲノム変異が起こることが動物実験で報告されている［4，5］．ヒトARTはインプリントが獲得される時期の配偶子を操作するため，排卵誘発，配偶子操作，培養液などの外的要因のメチル化への影響について懸念されている［6-9］．このため，DNAメチル化異常（エピゲノム変異）を伴うインプリンティング異常症（先天奇形症候群）の発症率が増加している理由と指摘する研究者が数多くみられる．本稿では，わが国のゲノムインプリンティング異常症5疾患：Beckwith-Wiedemann症候群（BWS），Angelman症候群（AS），Prader-Willi症候群（PWS），Silver-Russell症候群（SRS），新生児一過性糖尿病（TNDM）について，ARTとの関連性の実態把握と，その分子機構の特性について紹介する．

先天性インプリンティング異常症とARTの関連性

「厚生労働省難治性疾患克服研究事業」(2009)として，わが国の先天性インプリント異常症5疾患についてその患者数，病態等の把握のために，実態調査を行った．

調査対象施設総数3,158施設のうち，1,602施設から有効回答があり（有効回答率56.3%），報告患者総数は1,818人であった．その内訳は，BWSが70人，ASが123人，PWSが261人，SRSが42人，TNDMが25人である．回答のあった医療機関における患者数を階層毎に推計し，さらに抽出率と回答率から全患者数を推計した（表1）．BWS，SRSは最近増加傾向にあることが疑われた．ほとんどが孤発例で家族例は少なかった．また，不妊治療を受けたかどうか不明な患者が多いことが特徴で，十分な情報が得られていないことも判明した．しかし，不妊治療を受けていたことが明らかになったケースは，疾患別で比較した場合，PWS，BWS，AS，SRSの疾患は，それぞれ少なくとも1.5%，8.6%，1.6%，9.5%であった．平成17年度のIVF＋ICSIの出生児は年間10,338人で全出生児の0.86%であることから，SRSとBWSでは約10倍リスクが高い事が推測された．また，このほとんどの症例は体外受精（IVF）と顕微授精（ICSI）によるものであった．

ART出生児のインプリンティング異常症のメチル化インプリントの特徴

これまでの報告では，ARTと関連するインプリント異常症の発症原因がエピゲノム変異（メチル化異常）の

表1　先天性インプリント異常症とARTの関連性（平成21年度）

疾患	実患者数	推定患者数（95%信頼区間）	推定発症率	ART後の罹患児/患者総数
BWS	70	444（350-540）	1/287,000	6/70（8.6%）
AS	123	949（680-1220）	1/134,000	2/123（1.6%）
PWS	261	2070（1500-2640）	1/62,000	4/261（1.5%）
SRS	42	326（240-420）	1/392,000	4/42（9.5%）

階層毎に医療機関を推計し，抽出率と回答率から全患者数を推測した．IVF＋ICSIの出生児は年間10,338人で全出生児の0.86%（平成17年度）を考慮すると，SRSとBWSでは約10倍リスクが高いことが推測される．

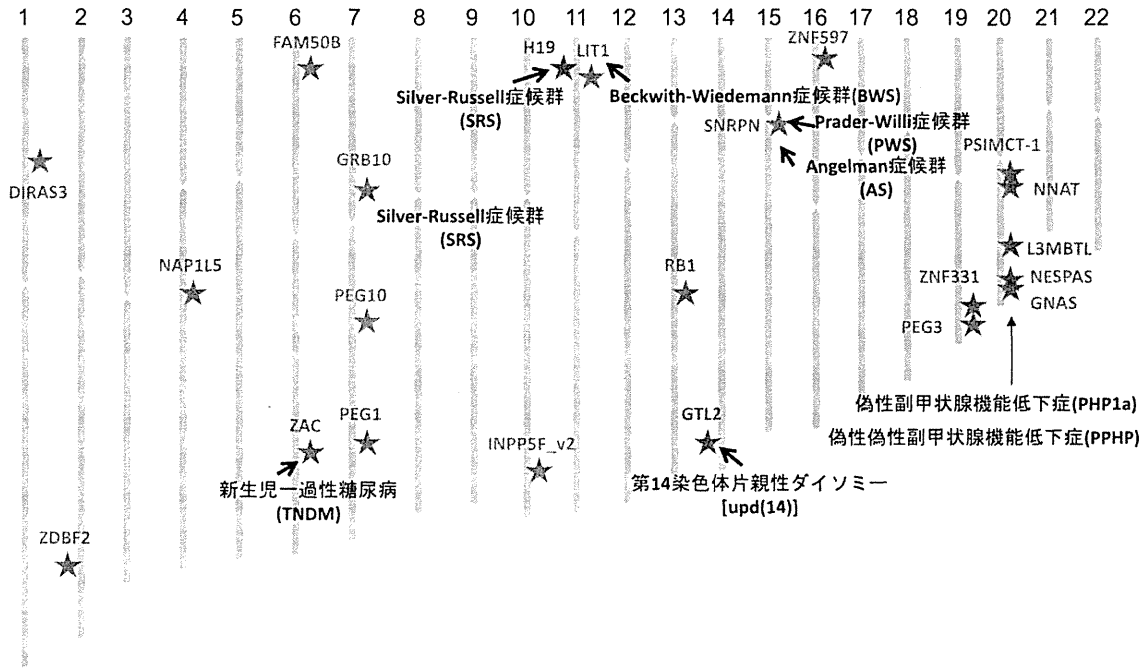


図1　ヒトメチル化インプリントマップと疾患
ヒトメチル化インプリント領域と関連する先天性疾患の染色体マップ．
★精子型メチル化インプリント（DMR）（3領域）．★卵子精子型メチル化インプリント（DMR）（19領域）．

頻度が圧倒的に高い．そのためDNAメチル化異常を呈するインプリンティング異常症を対象に，網羅的なメチル化インプリントの解析を行った．ヒトインプリントマップを作成し，DNA多型を含むBisulphite PCR Sequence法をデザインし，正確なメチル化解析を行った（図1）．ART後の罹患児と自然発症の患児にみられるメチル化異常のパターンについて比較分析し，その特徴から，発症機序について推測した．その結果，SRSの場合，ART治療を受けた患者では，5例中4例において，複数のインプリント領域で異常を認めた．さらに，4例は，精子型と卵子型DMRの両方に異常を認めた．また，同一症例で，高メチル化と低メチル化を示す症例が多く，またその程度は，完全型ではなく，細胞内のモザイクパターンを示す特徴にみられた．ART後のBWSはわずか1例の症例であるが，SRSの場合と同様の傾向がみられた（表2）．

これらの結果をまとめると，ART出生児の場合，複数のインプリント領域において複雑なメチル化異常のパターンが認められ，生殖細胞形成過程におけるメチル化の獲得の異常というより，むしろ　受精以降のメチル化の安定性維持機構の障害が疑われる．

ART後の罹患児の臨床症状の特徴

ART群と非ART群で，各疾患についてその臨床症状について比較した（表3）．

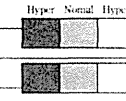
その結果，ART症例では疾患毎にいくつかの特徴が示されたが，特異性のあるものではなかった．また，網羅的なメチル化解析を行った症例に関しても，特徴的な差はみられなかった．しかし，症例数が少なく正確な評価ができなかったものかもしれない．今後，エピゲノム変異の頻度，程度，パターン分類を正確に行うことで，臨床症状の特異性が現れるかもしれない．近年，ART出生児で，診断のつかない症候群やBWS，SRS類似疾患などの報告が散見される．これらの症例では，これまでに報告のないエピゲノム変異が原因となっている可能性は否定できない．

おわりに

ARTの新技術は着実に進歩し，不妊症に重要な治療法として多大な恩恵をもたらし，国内では，年間数万人が出生している．しかしARTは配偶子を操作するため，ゲノムインプリンティングを引き起こす可能性が十分示

表2　ART後の先天性インプリント異常症患者のメチル化異常

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



SRS、BWSの場合、ART治療を受けた患者では、1）複数のインプリント領域での異常、2）精子型と卵子型DMRの両方に異常、3）高メチル化と低メチル化を示す症例が多い、4）完全型の異常ではなく、細胞内のモザイクパターンを示す、など複雑なメチル化異常を示す特徴がみられた。

唆される．インプリンティング異常，特にエピゲノム変異は，先天性疾患だけでなく，身体的，精神的発育・発達，性格，行動異常等に関連し，さらに，次世代の癌や成人性疾患の原因となりうる．わが国では，晩婚化，少子化により今後もART出生児が増加すると予想され，インプリンティング異常については，次世代社会の重要な課題として早急に実態を把握し，適切な対応が必要とされる．ARTを受ける患者が一般の人口統計と異なる特殊な集団であるため，単純にART治療がそのような危険を引き起こすのか，正確に評価することは難しい．しかし，分子生物学的解析による正確なリスク評価が十分ではなく，現時点で明確な結論は得ていない．今後長期追跡調査も含め，詳細な表現型やエピゲノム型の比較検討が必要と考える．

一方で，ARTを適正に実施するための制度が十分であるとはいえず，ARTをめぐり発生する倫理上，健康上のさまざまな問題に対して適切な対応ができていないのが現状である．さらに，ARTの多くは，医療保険が適用されていない自由診療であり，受診者は多額の費用を負担しなければならない．また，経済上の問題をクリアしてARTに臨んだとしても，薬物療法による副作用や，出生した児の健康に問題があるなどのリスクは避けることはできない．このような現状を打開し，不妊に悩む人々に対し，身体的，経済的負担の少ない，良質かつ

表3　臨床症状の相違点

BWS

	耳介の溝		眼間開離		巨舌症		ギョロ眼		咬合障害		臍ヘルニア		半身肥大症		停留睾丸	
全体	48/89	53.9	19/89	21.3	81/89	91.0	24/89	27.0	7/89	7.9	67/89	75.3	22/89	24.7	12/89	13.5
非ART	44/82	49.4	18/82	20.2	74/82	83.1	21/82	23.6	7/82	7.9	61/82	68.5	20/82	22.5	11/82	12.4
ART	4/7	57.1	1/7	14.3	7/7	100	3/7	42.9	0/7	0.0	6/7	85.7	2/7	28.6	1/7	14.3
	腎腫大		肝腫大													
全体	20/89	22.5	8/89	9.0												
非ART	18/82	20.2	7/82	7.9												
ART	2/7	28.6	1/7	14.3												

AS

	精神発達遅滞		難治性てんかん		色白		不眠症		小頭症		発語障害		下顎突出		操り人形様の歩行	
全体	133/146	91	69/146	47.2	101/146	69.1	65/146	44.5	54/146	36.9	129/146	88.3	91/146	62.3	71/146	48.6
非ART	133/144	91	68/144	46.5	101/144	69.1	64/144	43.8	53/144	36.3	127/144	86.9	89/144	60.9	69/144	47.2
ART	0/2	0	1/2	50.0	0/2	0	1/2	1/250.0	1/2	50.0	2/2	100.0	2/2	100.0	2/2	100.0

PWS

	筋緊低下		過食症		色白		精神発達遅滞		低身長		性器低形成		哺乳不良			
全体	259/297	87.2	155/297	52.1	215/297	72.3	249/297	84.0	186/297	62.6	125/297	42	236/297	79.4		
非ART	256/290	86.1	153/290	51.5	211/290	71.0	245/290	82.0	183/290	61.6	124/290	41.7	232/290	78.1		
ART	3/7	42.9	2/7	28.6	4/7	57.1	4/7	57.1	3/7	42.9	1/7	14.3	4/7	57.1		
	停留睾丸		小さな手足		特異な顔貌		魚様の三角の口									
全体	100/297	33.6	207/297	69.6	238/297	80.1	192/297	64.4								
非ART	99/290	33.3	204/290	68.6	234/290	78.7	188/290	63.2								
ART	1/7	14.3	3/7	42.9	4/7	57.1	4/7	57.1								

SRS

	身体非対称		成長障害		発汗		低身長		精神発達遅滞		指の奇形		相対的大頭を伴う逆三角形の特異的顔貌	
全体	37/54	68.5	49/54	90.7	11/54	20.3	54/54	100	17/54	31.4	29/54	53.7	54/54	100
非ART	30/47	55.5	45/47	95.7	10/47	18.5	50/47	92.5	17/47	31.4	26/47	48.1	51/47	94.4
ART	7/7	100	4/7	57.1	1/7	14.3	4/7	57.1	0/7	0.0	3/7	42.9	4/7	57.1

ART症例では疾患毎にいくつかの特徴が示されたが，特異的なものではなかった．しかし症例数を増やし，エピ変異の頻度，程度，パターン分類を正確に行うことで，臨床症状の特異性が現れるかもしれない．

適切なARTを実施するためには，医療保険の適用や公的な補助の対象として検討することはもちろん，現在のARTにおける技術的な問題をできるだけ排除し，短期間で成功率の高い，より安全な治療システムの構築が必要であると考える．

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Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression

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Within the vertebrate groups, only mammals are subject to a specialized epigenetic process termed genomic imprinting in which genes are preferentially expressed from one parental allele. Imprinted expression has been reported for >100 mouse genes and, for approximately one-quarter of these genes, the imprinted expression is specific to the placenta (or extraembryonic tissues). This seemingly placenta-specific imprinted expression has garnered much attention, as has the apparent lack of conserved imprinting between the human and mouse placenta. In this study, we used a novel approach to re-investigate the placenta-specific expression using embryo transfer and trophoblast stem cells. We analyzed 20 genes previously reported to show maternal allele-specific expression in the placenta, and only 8 genes were confirmed to be imprinted. Other genes were likely to be falsely identified as imprinted due to their relatively high expression in contaminating maternal cells. Next, we performed a genome-wide transcriptome assay and identified 133 and 955 candidate imprinted genes with paternal allele- and maternal allele-specific expression. Of those we analyzed in detail, 1/6 (*Gab1*) of the candidates for paternal allele-specific expression and only 1/269 (*Ano1*) candidates for maternal allele-specific expression were authentically imprinted genes. Imprinting of *Ano1* and *Gab1* was specific to the placenta and neither gene displayed allele-specific promoter DNA methylation. Imprinting of *ANO1*, but not *GAB1*, was conserved in the human placenta. Our findings impose a considerable revision of the current views of placental imprinting.

INTRODUCTION

Genomic imprinting is an essential mechanism in mammalian development that regulates the preferential expression of the paternally or maternally inherited allele of a subset of genes. Within vertebrate lineages, imprinting appears to be restricted to eutherian mammals and marsupials (1). Imprinting arose during mammalian evolution and could thus be linked to placental development and function (2,3). Various theories have been proposed to explain the biological and evolutional significance of this phenomenon (4,5).

One of the key defining features of eutherian mammals is the chorioallantoic placenta. This structure, which infiltrates the maternal uterus, has a pivotal role in embryonic growth and development through regulating the transport of nutrition, gas and waste between fetal and maternal blood (6,7). A large number of imprinted genes are expressed in the placenta and it has been proposed that some of these control the supply of

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nutrients to the fetus (8–11). In contrast, imprinted genes expressed in the embryo may determine nutritional demand by regulating the growth rate of fetal tissues (3). Importantly, low birth weight has implications for postnatal development and has been linked to the development of certain diseases later in life (12), highlighting the critical role of the placenta both in the neonatal period and, more perniciously, in the adult.

In the mouse, X chromosome inactivation does not occur randomly in extraembryonic lineages and genes subject to X-inactivation in female mice display tissue-specific imprinted expression in the placenta but are expressed mosaically in the embryo and adult (13,14). Similarly, there are ~30 autosomal genes, which have been reported to show imprinted expression only in the placenta (or extraembryonic tissues) (15–17). Remarkably, almost all of these genes specifically imprinted in the placenta are active on the maternal allele and repressed on the paternal allele.

The mechanisms for maintaining imprinted expression in the embryo may differ from those at work in the placenta as loss of the maintenance DNA methyltransferase, Dnmt1, results in the relaxation of imprinting of some genes preferentially in the embryo (18,19). For some genes with placenta-specific imprinted expression, imprinted gene expression depends on H3-K9 dimethylation (H3K9me2) and/or H3-K27 trimethylation (H3K27me3) and is impaired in the absence of G9a and EED, which may be a consequence of lineage-specific, temporal dependence on long non-coding RNAs (9,15,16). A comprehensive, whole genome analysis aimed at identifying genes with placenta-specific imprinted expression may provide a clearer picture regarding the requirement of imprinted gene expression in the placenta versus the embryo. However, the mouse placenta consists of contributions from both the mother and the fetus (6) raising the possibility that strategies aimed at identifying novel imprinted genes may be confounded by contaminating material. There are several potential sources of contamination as complete removal of the decidua from the placenta is difficult, maternal cells are also known to exist in the spongiotrophoblast and labyrinth layers (20–22) and the ectoplacental cone is already invaded by maternal blood at embryonic day (E) 6.5 (15).

To assess the importance of this issue, we re-investigated the imprinted status of genes previously reported to show placenta-specific imprinted expression first using an embryo transfer procedure to identify the maternal cell contribution and then using trophoblast stem (TS) cells grown in culture away from the maternal environment. In addition, we performed genome-wide screen to identify all the genes that might fall into this same category, either as contaminants or imprinted genes.

RESULTS

Imprinted gene expression in the placenta without maternal decidua

The expression level of 27 genes, previously reported to show placenta-specific maternal allele expression, was first determined by quantitative polymerase chain reaction (QPCR) in the maternal decidua of E13.5 placenta after dissection (Fig. 1A). Of the 27 genes examined, 6 (*Cntn3*, *Klrb1f*, *Art5*, *Cmah*, *Drd1a*, *Fbxo40*) were expressed at negligible levels in the placenta. Low expression of these genes was also confirmed in the whole transcriptome sequencing as described below. Ten of 21 genes with placenta-specific imprinted expression showed more than 10 times higher expression in the decidua than in the placenta (Fig. 1A). The preferential expression in the decidua was also confirmed using *in situ* hybridization for *Gatm*, *Tfpi2* and *Ampd3* (Fig. 1B).

In order to determine whether there was any remaining maternal contamination after surgical removal of the maternal decidua, we employed an embryo transfer strategy. C3H/HeJ (C3H) embryos were transferred into pseudopregnant C57BL/6 (B6) mice. Placentas derived from this mating strategy are composed of a C3H embryonic component and a B6 maternal component. E13.5 placentas were again collected and the decidua was carefully removed. After removal of the decidua, the remaining material was subjected to genomic DNA amplification over a polymorphism between the C3H and B6 strains spanning the *Gapdh* gene. This revealed that most of maternal cells were removed when dissecting away the deciduas (Fig. 2A).

Single nucleotide polymorphisms (SNPs) between C3H and B6 were used to examine the expression of three genes highly expressed in the decidua and previously reported to be imprinted (*Wt1*, *Gatm* and *Qpct*). When *Wt1* was amplified from genomic DNA obtained from the placenta after removal of the decidua, the peak of the B6 allele was near background level, consistent with the very small level of maternal contamination in this dissected material (Fig. 2B). However, analysis of the cDNA from dissected material revealed predominant expression of the *Wt1* B6 allele (Fig. 2B). A similar pattern was obtained with *Gatm* and *Qpct* (Supplementary Material, Fig. S1A). These data demonstrated that, even after the careful removal of the decidua, there was still sufficient maternal cell contamination to significantly impact expression studies.

The analyses of the placenta-specific imprinting using embryo transfer and TS cells

As described above, maternal cell contamination was a significant factor in the analysis of imprinted gene expression in the mouse placenta. We therefore set up an experiment to ask how many of the genes previously reported to show maternal allele expression in the placenta might have been falsely identified. To distinguish between maternal allele-specific expression and maternal contamination, embryos obtained by crossing Japanese fancy 1 (JF1) females and B6 males ([JF1xB6]F1) were transferred to pseudopregnant B6 recipients. Placentas derived from this embryo transfer experiment were composed of [JF1xB6]F1 embryonic cells and B6 maternal cells. Genes expressed from the maternal allele would carry JF1 SNPs, while genes expressed in contaminating maternal cells would have the B6 SNPs. Genes expressed from the paternal allele also carry B6 SNPs. E13.5 placentas were collected, the decidua was carefully removed as before and the allelic expression was determined. We could confirm that 6 out of

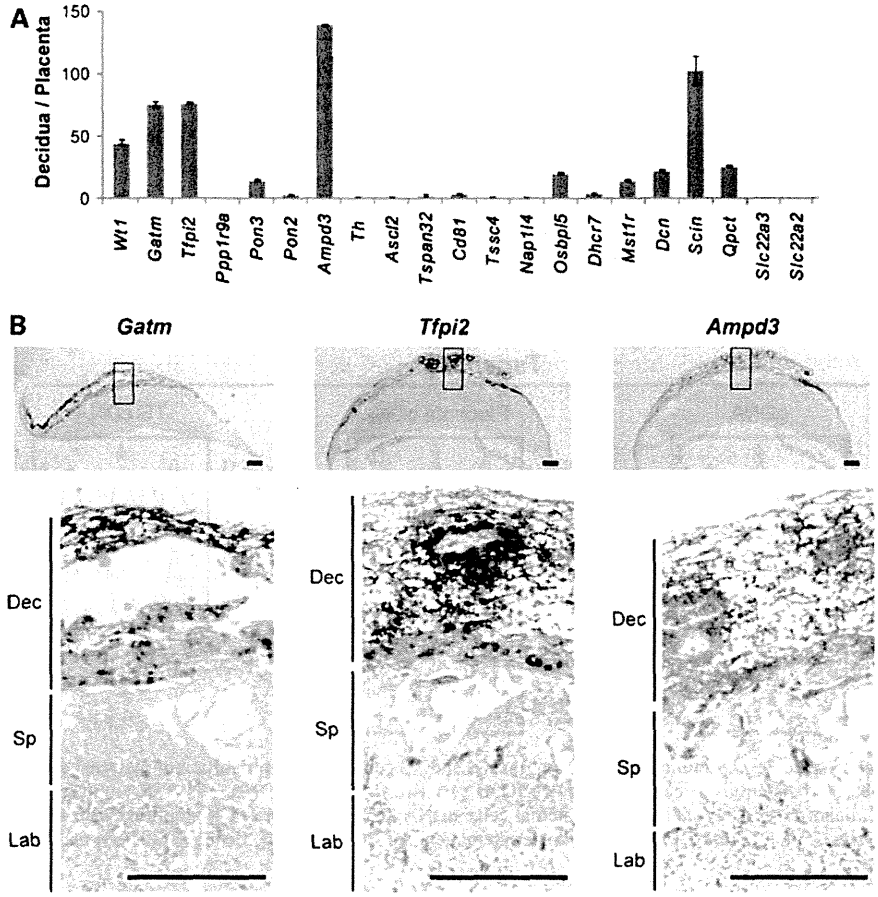

Figure 1. Expression of genes with placenta-specific imprinted expression in the decidua. (**A**) Real-time RT–PCR analysis of genes with placenta-specific imprinted expression in the decidua. Messenger RNA levels in the decidua were normalized to those observed in the placenta from which the decidua was dissected. The bars indicate the means ± standard deviation (SD) from two replicates. (**B**) Expression analysis of *Gatm*, *Tfpi2* and *Ampd3* in sagittal sections of E13.5 placentas by *in situ* hybridization. The lower panels show enlarged views of the boxed areas. Scale bars indicate 400 μm. 'Dec', decidua; 'Sp', spongiotrophoblast layer; 'Lab', labyrinth layer.

the 18 genes for which there were SNPs were authentically expressed from the maternal allele (*Ppp1r9a*, *Ascl2*, *Th*, *Tssc4*, *Slc22a3* and *Slc22a2*) (Table 1 and Supplementary Material, Fig. S2), while 11 genes were potentially falsely identified as imprinted due to their relatively high expression in maternal material (*Wt1*, *Tfpi2*, *Pon3*, *Pon2*, *Ampd3*, *Osbpl5*, *Dhcr7*, *Mst1r*, *Dcn* and *Scin* are shown in Supplementary Material, Fig. S2; *Tspan32* is shown in Fig. 2C). *Nap1l4* was biallelically expressed in the [B6xJF1]F1 and [JF1xB6]F1 placentas obtained by normal mating (Supplementary Material, Fig. S2).

For the 12 genes which did not show maternal allele-specific expression in the [JF1xB6]F1 placentas obtained by the embryo transfer, it was still possible that these genes showed maternal allele-specific expression but that this expression was obscured by the contaminating material. TS cells can differentiate into all cell types of the placenta and do not contain contaminating maternal cells (23). First, we confirmed the faithful expression of 10 well-known imprinted genes (*Igf2r*, *H19*, *Meg3*, *Grb10*, *Phlda2*, *Cdkn1c*, *Peg10*, *Sgce*, *Snrpn*, *Mest*) in [B6xJF1]F1 and [JF1xB6]F1 TS cells (Supplementary Material, Fig. S3A). Using undifferentiated and differentiated TS cells, we were able to confirm imprinting for only 2 of the 12 genes (*Tspan32* and *Tfpi2*) (Fig. 2C and Supplementary Material, Fig. S3B). Similar results were obtained in at least two [B6xJF1]F1 and [JF1xB6]F1 TS cell lines. Furthermore, we analyzed [JF1xB6]F1 and [C3HxB6]F1 placentas at E9.0 and confirmed that *Wt1*, *Gatm*, *Pon3*, *Pon2*, *Ampd3*, *Osbpl5*, *Dhcr7*, *Mst1r*, *Dcn*, *Scin* and *Qpct* did not show maternal allele-specific expression (Supplementary Material, Figs S1B and S2B).

Whole transcriptome sequencing analysis of placental imprinting

To determine how significant this issue of maternal contamination might be to the identification of novel imprinted genes, we applied whole transcriptome sequencing to the dissected F1 material obtained by normal mating. About 300 million reads were sequenced and SNPs with biased allelic expression were identified (see Materials and Methods for details). Preferential expression from paternal and maternal alleles were observed at 323 and 1930 SNP sites, respectively, equivalent to 133 and 955 candidate imprinted genes with paternal allele- and maternal allele-specific expression (Supplementary Material, Tables S1 and S2). Expression of 49 genes

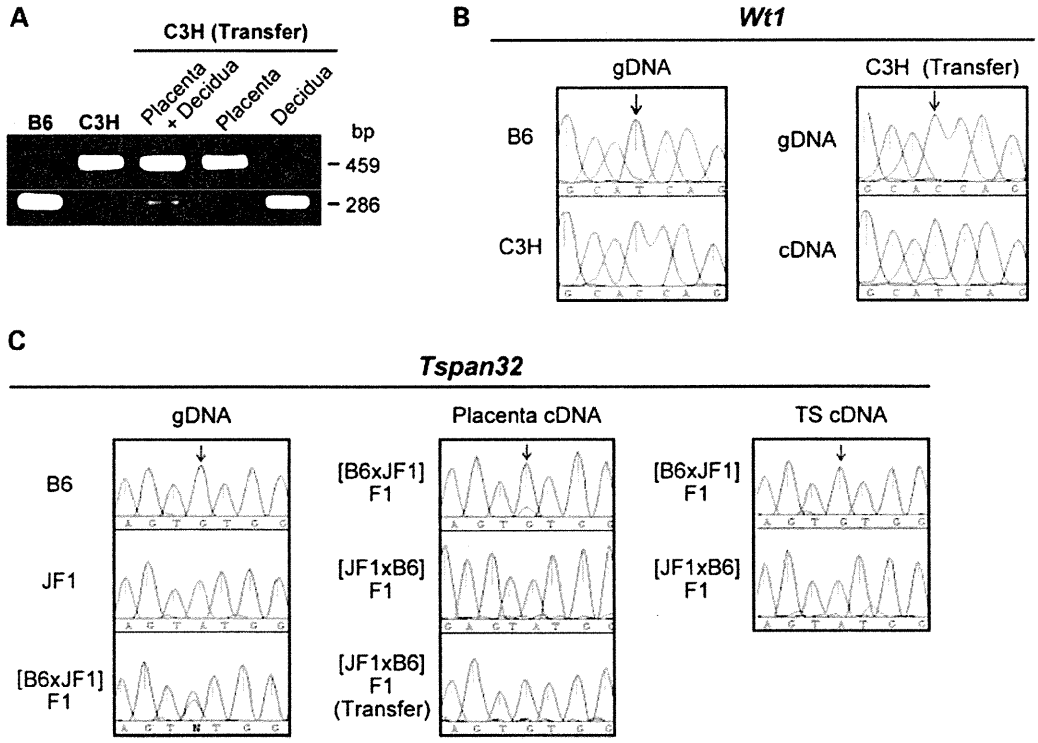

Figure 2. Analyses of the placenta-specific imprinting using embryo transfer and TS cells. (**A**) The proportion of maternal cells in the placenta. E13.5 placentas were obtained from C3H embryos transferred to recipient B6 mice [C3H (Transfer)]. Genomic DNA was PCR amplified with *Gapdh*-specific primers and digested with AflII. (**B**) Predominant expression of *Wt1* from maternal cells. cDNA and gDNA were PCR amplified with *Wt1*-specific primers and sequenced. (**C**) The allelic expression of *Tspan32* in [JF1xB6]F1 placentas transferred to recipient B6 mice ([JF1xB6]F1 (Transfer)) and TS cells. The SNP site between B6 and JF1 is indicated by arrows.

previously reported to be imprinted were at sufficient level to assess allelic expression. Of these, 36 showed imprinted expression (Supplementary Material, Table S3). The success rate of imprinted gene identification was comparable with the previous work (24–27).

Identification of novel genes with placenta-specific imprinted expression

Among the novel genes identified, 6 and 269 candidate genes with paternal allele- and maternal allele-specific expression, which contain more than two SNP sites with biased allelic expression, were chosen for further analysis. The allelic expression of the six candidate genes with paternal allele expression was analyzed by Sanger sequencing. *Gab1* was confirmed to be imprinted (Fig. 3A). *Gab1* also showed paternal allele-specific expression in TS cells, but biallelically expressed in the embryo and yolk sac at E13.5 and in adult tissues (Fig. 3A). The other five genes were biallelically expressed in the placenta (Supplementary Material, Fig. S4A).

The allelic expression of 269 candidate genes with maternal allele-specific expression was analyzed in the [JF1xB6]F1 placentas obtained by the embryo transfer strategy (Supplementary Material, Table S5). All these candidate genes showed a higher or equal level of expression from B6 allele, implying that the maternal allele-specific expression identified in the natural mating strategy could be attributed to maternal cell contamination. But again, it was possible that the maternal allele-specific expression was obscured by the contaminating material. To address this possibility, we examined the allelic expression of 269 genes in the TS cells. Only *Ano1* was found to be expressed from the maternal allele (Fig. 3B). *Ano1* was biallelically expressed in the embryo, yolk sac and adult tissues (Fig. 3B).

Epigenetic modification of *Gab1*, *Ano1* and *Sfmbt2*

As imprinted genes tend to be clustered, the allelic expression of neighboring genes for *Gab*1 and *Ano1* was analyzed in the [B6xJF1]F1 and [JF1xB6]F1 TS cells. None was found to be imprinted in the TS cells (Supplementary Material, Fig. S4B). We also analyzed the *Sfmbt2* locus. *Sfmbt2* shows placenta-specific paternal allele expression and does not map to a known imprinted gene clusters (28). The neighboring genes of *Sfmbt2* were also biallelically expressed in the TS cells (Supplementary Material, Fig. S4B).

The DNA methylation patterns of *Gab1*, *Ano1* and *Sfmbt2* were analyzed in E13.5 placenta by bisulphite sequencing, but no differentially methylation was found (Fig. 4A–C). Furthermore, the paternal allele-specific expression of *Gab1* and *Sfmbt2* was not affected in E9.5 placenta obtained from *Dnmt3l*-deficient or oocyte-specific *Dnmt3a/3b*-deficient female mice (Fig. 4D). This indicates that the establishment of imprinting of *Gab1* and *Sfmbt2* does not require maternal germline methylation. Because *Ano1* was mainly expressed

Table 1. Summary of genes with placenta-specific imprinted expression

Chr.	Imprinting status in the mouse			Imprinting status in the human placenta
	Gene	Placenta	TS cells	
2	***Sfmbt2***	**Imprinted (P)**	**Imprinted (P)**	Non-imprinted
2	*Wt1*	Non-imprinted	Non-imprinted	Imprinted (polymorphic) (59)
2	*Gatm*	Non-imprinted	NA	Non-imprinted (48)
6	***Tfpi2***	**Non-imprinted**	**Imprinted (M)**	Imprinted (polymorphic) (30)
6	***Ppp1r9a***	**Imprinted (M)**	**Imprinted (M)**	Imprinted (polymorphic) (60)
6	*Pon3*	Non-imprinted	Non-imprinted	Non-imprinted (30)
6	*Pon2*	Non-imprinted	Non-imprinted	Non-imprinted (30)
6	*Cntn3*	ND	ND	NA
6	*Klrb1f*	ND	ND	NA
7	*Art5*	ND	ND	NA
7	*Ampd3*	Non-imprinted	Non-imprinted	Non-imprinted (49)
7	***Th***	**Imprinted (M)**	**ND**	NA
7	***Ascl2***	**Imprinted (M)**	**Imprinted (M)**	Non-imprinted (48)
7	***Tspan32***	**Non-imprinted**	**Imprinted (M)**	Non-imprinted (48)
7	*Cd81*	NA	NA	Non-imprinted (48)
7	***Tssc4***	**Imprinted (M)**	**Imprinted (M)**	Non-imprinted (48)
7	*Nap1l4*	Non-imprinted	ND	Non-imprinted (48)
7	*Osbpl5*	Non-imprinted	Non-imprinted	Imprinted (61)
7	*Dhcr7*	Non-imprinted	Non-imprinted	Non-imprinted (49)
7	***Ano1***	**Non-imprinted**	**Imprinted (M)**	Imprinted (polymorphic)
8	***Gab1***	**Imprinted (P)**	**Imprinted (P)**	Non-imprinted
9	*Mst1r*	Non-imprinted	Non-imprinted	NA
10	*Dcn*	Non-imprinted	ND	Non-imprinted (48)
12	*Scin*	Non-imprinted	ND	NA
13	*Cmah*	ND	ND	NA
13	*Drd1a*	ND	ND	NA
16	*Fbxo40*	ND	ND	NA
17	***Slc22a3***	**Imprinted (M)**	**Imprinted (M)**	Imprinted (polymorphic) (48)
17	***Slc22a2***	**Imprinted (M)**	**ND**	Imprinted (polymorphic) (48)
17	*Qpct*	Non-imprinted	NA	NA

Genes where paternal and maternal allele expression was confirmed in the placenta and/or TS cells are shown in bold. 'M', maternal allele-specific expression; 'P', paternal allele-specific expression; ND, not detected; NA, not analyzed.

from contaminated maternal cells, the allelic expression was not analyzed in those mutant mice.

Allele-specific expression of some genes with placenta-specific imprinted expression is reported to be regulated by the histone methylation (18,29,30). We analyzed the allelic histone modifications in E13.5 [B6xJF1]F1 placenta or [B6xJF1]F1 TS cells by chromatin immunoprecipitation (ChIP) analyses (Fig. 4). Following antibodies were used: dimethylated H3-Lys4 (H3K4me2), trimethylated H3-Lys4 (H3K4me3), H3K9me2, H3K27me3, H3K4me2 and H3K4me3 are markers of active genes and H3K9me2 and H3K27me3 are repressive markers. At the *Ano1* transcription start site, H3K4me2 and H3K4me3 were enriched on the maternal allele in the placenta and TS cells. Allelic enrichment of H3K9me2 or H3K27me3 was not observed (Fig. 4B). At *Sfmbt2*, maternal enrichment of H3K9me2 and H3K27me3 and paternal enrichment of H3K4me2 and H3K4me3 were observed (Fig. 4C). The histone modification around the *Gab1* transcription start site could not be analyzed as no SNPs were found between B6 and JF1. Maternal enrichment of H3K9me2 and paternal enrichment of H3K4me3 were observed at the intron 1 of *Gab1* (Fig. 4A). The levels of H3K9me2 and H3K27me3 at *Sfmbt2* and *Gab1* were comparable with those at *Tssc4* and *Slc22a3*, which are reported to be regulated by H3K9me2 and/or H3K27me3 (31,32) (Supplementary Material, Fig. S5).

Preferential expression of *ANO1* from the maternal allele in the human placenta

We next examined the imprinting status of *ANO1*, *GAB1* and *SFMBT2* in the human placenta. Preferential expression of *ANO1* from the maternal allele was observed in one of the two term placenta samples where we had informative parental genotyping (Fig. 5A and Supplementary Material, Fig. S6). The maternal allele expression of *ANO1* was also confirmed by restriction fragment length polymorphism (RFLP) analysis (Fig. 5B). Importantly, we were also able to show monoallelic expression in three of five term placenta samples where both the mothers and the fetuses were heterozygous for the SNP, which formally excludes maternal contamination (Supplementary Material, Fig. S6). *GAB1* and *SFMBT2* were biallelically expressed in four human term placenta samples (Fig. 5A and Supplementary Material, Fig. S6). *ANO1*, *GAB1* and *SFMBT2* were biallelically expressed in one or two first trimester placenta samples (Supplementary Material, Fig. S6). Biallelic expression of *SFMBT2* in the human placenta was quite recently reported (33).

DISCUSSION

The key finding from this study is that maternal contamination is a confounding factor when analyzing imprinted gene

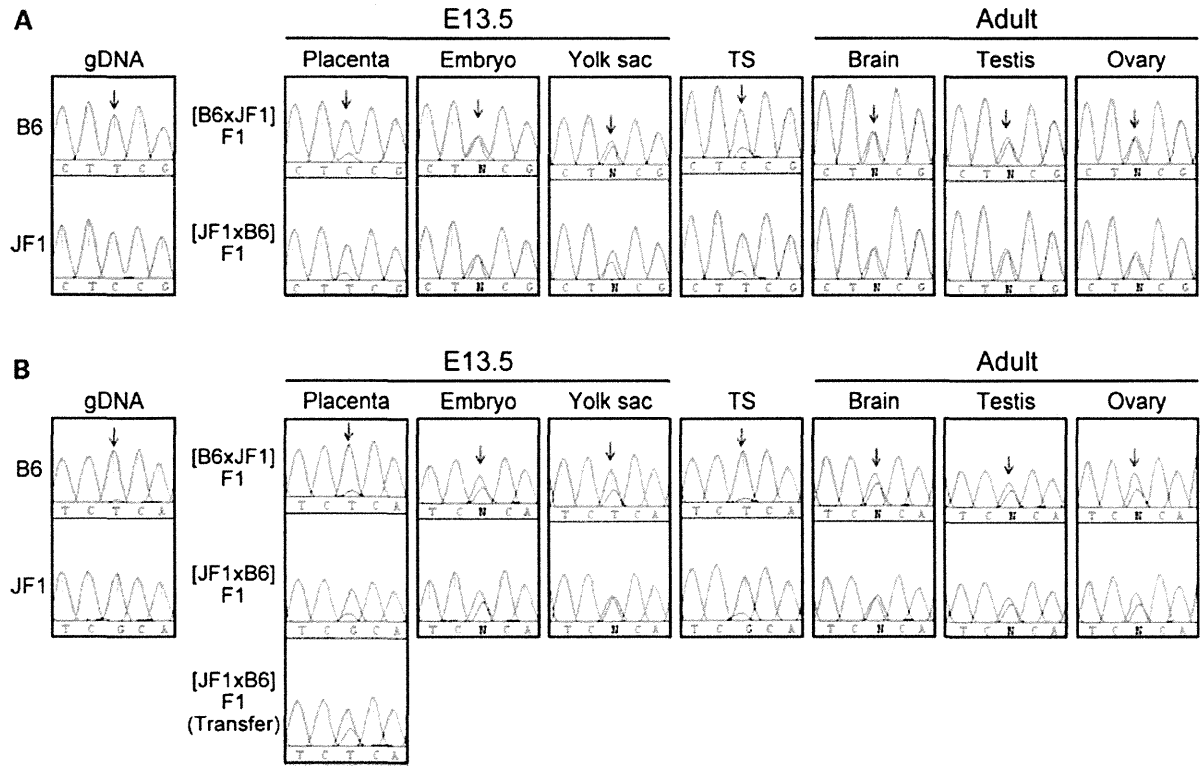

Figure 3. Allelic expression of novel imprinted genes identified by transcriptome sequencing. (**A**) Allelic expression of *Gab1*. *Gab1* showed paternal allele-specific expression in the placenta and TS cells but not in the embryo and adult tissues. (**B**) Allelic expression of *Ano1*. In the placenta, *Ano1* was predominantly expressed by contaminated maternal cells. *Ano1* showed maternal allele-specific expression in TS cells but not in the embryo, yolk sac and adult tissues.

expression in the mouse placenta. As a result of our analysis, we can provide a new map of genes with placenta-specific imprinted expression, shown in Figure 6. We have also identified two novel genes with placenta-specific imprinted expression, *Ano1* and *Gab1*. Our data provide a more accurate picture of imprinting in the placenta, which will help in understanding of the function of imprinting in the mammalian placenta and evolution of genes with placenta-specific imprinted expression.

Re-examination of genes with placenta-specific imprinted expression

We have shown that genes highly expressed in the maternal decidua can be falsely identified as showing placenta-specific imprinted expression. For *Dcn* and *Osbpl5*, the predominant expression from the decidua has already been reported (34,35) and we provide a list of additional genes that fall into this category. The expression level in the decidua may be a good indicator of the risk that the allelic expression is affected by maternal cell contamination. We have shown that, for genes highly expressed in the decidua, maternal allele-specific expression in the placenta can be explained by maternal cell contamination.

Although maternal cell contamination can be misleading, it does not exclude the possibility that some genes, in fact, show maternal allele-specific expression. For example, *Tfpi2* and *Tspan32* were found to be expressed from contaminated maternal cells in the placenta but were also found to show maternal allele expression in TS cells. Maternal contamination can therefore result both in the false identification of imprinted expression and also obscure genuine imprinted expression. TS cells, which do not contain any maternal cells, are useful model systems for studying genomic imprinting in the placenta. However, this approach may not be full proof because it is possible that some genes are biallelically expressed in TS cells despite definitive maternal allele-specific expression in the placenta. In addition, it is already known that *in vitro* fertilization and embryo culture could disturb the imprinted expression of some genes (36,37), and this might also affect our analysis because *in vitro* fertilized embryos were used for the embryo transfer experiments. For *Gatm*, *Pon3*, *Pon2* and *Osbpl5*, which are not confirmed to be imprinted in this study, preferential expression from the maternal allele was reported in the yolk sac, but the bias is very weak and should be carefully interpreted (35,38,39). One way to resolve these problems would be to utilize a knock-in reporter system where allele-specific expression can be finely assigned to specific tissues.

Identification of novel genes with placenta-specific imprinted expression

By the whole transcriptome analysis of the placenta, >1000 genes were identified as showing supposedly allele-specific differences. We identified 19 genes with paternal allele-specific expression, which contain more than two SNP sites with biased allelic expression. Among them, 13 were known

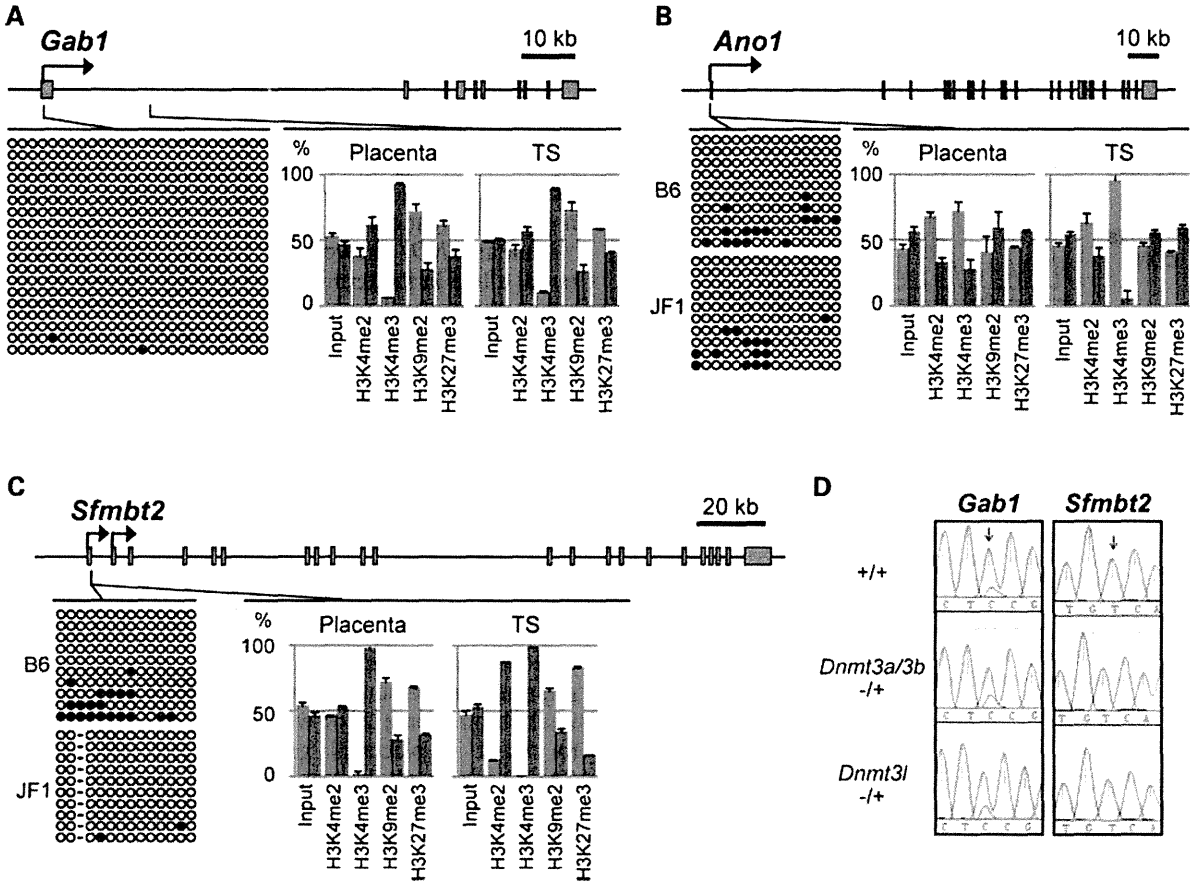

Figure 4. Epigenetic modification of *Gab1*, *Ano1* and *Sfmbt2*. (**A**–**C**) DNA methylation in [B6xJF1]F1 placenta was analyzed by bisulphite sequencing. Black and white circles indicate methylated and unmethylated residues. Histone modifications in [B6xJF1]F1 placenta and TS cells were analyzed using ChIP and SNuPE. The allele-specific histone modification was expressed as a percentage of maternal (red bars) or paternal (blue bars) alleles in the total immunoprecipitate. The error bars indicate the means + SD from two replicates. The genomic structure of *Gab1*, *Ano1* and *Sfmbt2* is shown and arrows indicate transcription start sites. (**D**) The allelic expression of *Gab1* and *Sfmbt2* in the E9.5 placentas obtained from *Dnmt3l*-deficient and oocyte-specific *Dnmt3a/3b*-deficient females. *Dnmt3a/3b* and *Dnmt3l* knockout female mice were crossed with WT JF1 male mice.

imprinted genes and the other 6 candidate genes were analyzed in detail. *Gab1* was confirmed to show imprinted expression, but the other five genes were biallelically expressed in the placenta. It is possible that for the five genes, only some splicing variants show imprinted expression and others are biallelically expressed. Alternatively, it is also known that candidate genes identified by RNA sequencing involve some false positive genes (24). Of the 269 candidate genes with maternal allele expression, which we analyzed in [JF1xB6]F1 placenta obtained by embryo transfer, only *Ano1* was confirmed to show maternal allele-specific expression, by the TS cell strategy. These data indicate that there are many genes highly expressed from contaminating maternal cells in the placenta.

Recently, Wang *et al.* (40) identified two paternally expressed and three maternally expressed novel imprinted genes using E17.5 mouse placenta samples from reciprocal cross F1 progeny of AKR/J and PWD/PhJ. The two genes with paternal allele-specific expression are not included in our candidate imprinted genes and this may reflect the differences in developmental stages and mouse strains. For the three genes with maternal allele-specific expression, the possibility of maternal cell contamination was not considered. Importantly, Brideau *et al.* (17) reported 10 novel imprinted genes using very similar samples to those used by Wang *et al.* (40), but we failed to confirm the imprinting of the 10 genes. Among them, four genes (*Wt1*, *Mst1r*, *Scin*, *Qpct*) were predominantly expressed by the contaminating maternal cells and the other six (*Cntn3*, *Klrb1f*, *Art5*, *Cmah*, *Drd1a*, *Fbxo40*) were not detected in E13.5 placenta. It is possible that the maternal cell contamination is greater at E17.5 and the six genes are detectable at E17.5 but not at E13.5. These data suggest that E17.5 placenta obtained by natural mating is not suitable for the identification of imprinted genes because of significant maternal cell contamination.

In this study, *Gab1* was found to show paternal allele-specific expression. Recently, *Gab1* was reported to show lower expression in parthenogenetic blastocysts than in fertilized embryos (41), consistent with our data. *Ano1* and *Gab1* appear to be genes with placenta-specific imprinted expression because we have shown that they are not imprinted in the embryo, yolk sac or adult tissues. Including these two genes, there are now 11 confirmed genes with placenta-specific imprinted expression (Fig. 6). While most imprinted genes

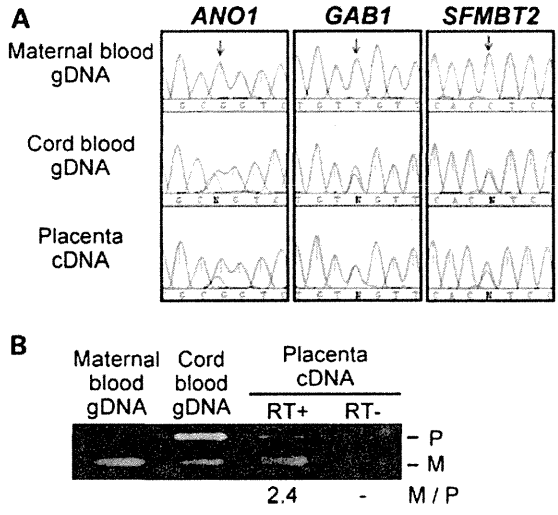

Figure 5. Allelic expression of *ANO1*, *GAB1* and *SFMBT2* in the human term placenta. (**A**) The A/G polymorphic site (SNP ID: rs2276067) in *ANO1*, T/G polymorphic site (SNP ID: rs1397529) in *GAB1* and C/G polymorphic site (SNP ID: rs2692756) in *SFMBT2* are indicated by the arrows. (**B**) Preferential expression of *ANO1* from the maternal allele was confirmed by the RFLP analysis. cDNA and gDNA were PCR amplified and digested with *Bst*UI. The ratio between maternal and paternal band intensity is represented.

with placenta-specific maternal allele expression are included in known imprinted gene clusters, *Ano1*, which was imprinted in the mouse and human, is unlikely to be included in the *Kcnq1* imprinting cluster because the distance between *ANO1* and *KCNQ1* is over 67 Mb. *Gab1* and *Sfmbt2* are not included in any known imprinted gene clusters. For the maintenance of allele-specific expression of most imprinted genes with placenta-specific maternal allele expression, H3K9me2 and/or H3K27me3 are known to play important roles while maintenance of DNA methylation seems to be less important (18,29,30). Differentially methylated regions (DMRs) were not identified at the promoter region of *Ano1*, *Gab1* and *Sfmbt2*, but allelic enrichment of histone modifications was detected. Recently, we reported a genome-wide screening of DMRs using mouse TS cells (42) and no DMR was found near *Ano1*, *Gab1* or *Sfmbt2*. This suggests that perhaps DNA methylation is not required for inducing allelic expression of *Ano1*, *Gab1* or *Sfmbt2*. We showed that for the establishment of imprinting of genes with placenta-specific paternal allele expression, *Gab1* and *Sfmbt2*, maternal germline methylation was not required. It remains possible that methylation in the paternal germline is required for their imprinted expression at a DMR not identified in our genome-wide screen, but it is also possible that the establishment of imprinting of these genes does not need DNA methylation at all.

Among two novel imprinted genes we identified, *Gab1* is already known to be important for placental development (43,44). Deficiency of *Gab1* results in the reduction in the number of trophoblast cells in the labyrinth region. Gab1 functions as a signaling mediator of various receptor tyrosine kinases and regulates multiple signaling effectors, such as phosphatidylinositol 3-kinase and Shp2 (45). While the placentas of heterozygous *Gab1*$^{+/-}$ have not been analyzed, we predict that placental abnormalities will be present as the inactive allele is inherited from their father. Ano1 is a subunit of calcium-activated chloride channels and *Ano1* knockout mice are reported to exhibit severe defects in tracheal development and death within 1 month of birth, while no overt phenotype has been observed for heterozygous *Ano1*$^{+/-}$ mice (46,47). The role of *Ano1* in placental development has not been determined, but our data suggest this will merit investigation.

Human *ANO1* showed preferential expression from the maternal allele in the term placenta. We find that, for most of the genes authentically expressed from the maternal allele in the mouse placenta, there is evidence of allele-specific expression in the human placenta, albeit polymorphic in most cases (Table 1). Polymorphic imprinting might imply that imprinting in the human placenta is stage or cell type specific. Nonetheless, our data indicate that, contrary to previous suggestions (48,49), imprinting in the placenta is well conserved between the mouse and human. The only exception to this is the proximal region of *Kcnq1* imprinting cluster where *Th*, *Ascl2*, *Tspan32* and *Tssc4* all show imprinted expression in either the mouse placenta or TS cells but not in the human placenta.

Mouse *Gab1* and *Sfmbt2* are two imprinted genes with placenta-specific paternal expression, which were not imprinted in the human placenta. *Sfmbt2* is located in the proximal chromosome 2, of which maternal duplication is known to result in placental growth retardation (50). Currently, *Sfmbt2* is the only known imprinted gene with paternal allele-specific expression in the proximal chromosome 2 and this suggests that Sfmbt2 might be important for placental development. *Gab1* is already known to be required for placental development (43,44). Both *Gab1* and *Sfmbt2* may therefore positively regulate placental growth. We cannot say whether the mouse *Gab1* and *Sfmbt2* genes have gained imprinted expression in mice or whether the human homologues have lost their imprints, but it is interesting to speculate that these genes are not imprinted to increase the capacity of the human placenta to support fetal growth.

MATERIALS AND METHODS

Whole transcriptome sequencing and data analysis

B6 females were mated with JF1 (51) males to generate [B6xJF1]F1 mice and reciprocally crossed to generate [JF1xB6]F1 mice. Total RNA was extracted from four [B6xJF1]F1 and four [JF1xB6]F1 placentas at E13.5, respectively, and pooled. Twelve micrograms of total RNA was used for rRNA depletion (RiboMinus Eukaryote Kit for RNA-seq, Invitrogen, CA, USA) and RNA-seq library construction (SOLiD Whole Transcriptome Analysis Kit, Life Technologies, CA, USA) according to the manufacturer's protocol. Libraries were clonally amplified on SOLiD P1 DNA Beads by emulsion PCR and sequenced using SOLiD3Plus System (Life Technologies). All SOLiD3+ reads were aligned with AB WT Analysis Pipeline (Applied Biosystems, CA, USA) against mouse NCBI genome build 37 (mm9) and against RefSeq Genes to capture alignment to splice sites. Reads aligned to rRNA, tRNA or sequence-adapter were filtered. Among total 319 701 254 reads obtained ([B6xJF1]F1: 154 500 642 and [JF1xB6]F1: 165 200 612), 144 406 747 (45.2%) were

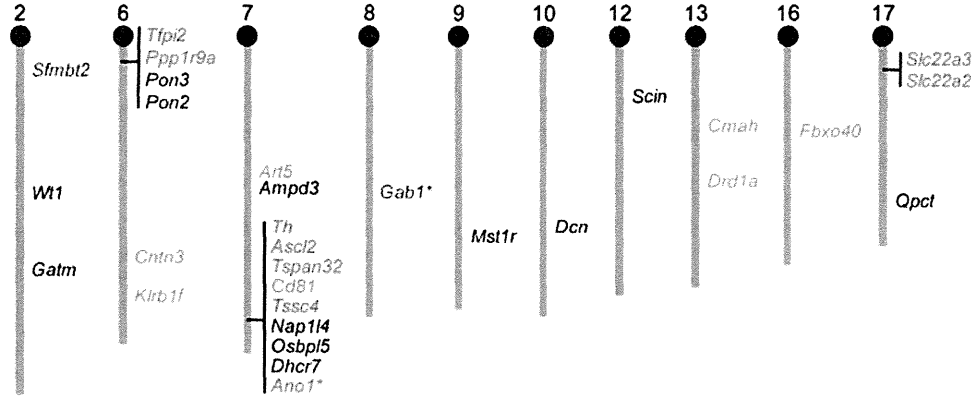

Figure 6. Chromosome map of genes with placenta-specific imprinted expression. Genes, of which paternal allele- and maternal allele-specific expression were confirmed in this study, are shown in blue and red, respectively. For genes shown in black, imprinted expression was not confirmed. Genes which do not contain SNPs (*Cd81*) or are poorly expressed in the placenta (*Cntn3*, *Klrb1f*, *Art5*, *Cmah*, *Drd1a* and *Fbxo40*) are shown in gray. Novel genes are marked by an asterisk (*).

aligned and passed filter. AB WT Analysis Pipeline aligns reads with up to five colorspace mismatches and provides mapping quality for each read. In order to collect highly reliable SNP candidates in each [B6xJF1]F1 and [JF1xB6]F1 on forward and reverse strand, we applied SAMtools 'pileup' software (52) with 15 over coverage and Phred-scaled likelihood SNP quality over 20 (as accuracy of SNP call 99%) to divided reads. Finally, we detected 128 837 candidate SNPs in transcriptome. Allele counts were tallied independently by transcript coordinates. To exclude minor alignment bias against sequences, we set the coverage threshold as 15 for each SNP. SNPs with biased allelic expression was determined using the following criteria: the ratio of maternal or paternal reads to total reads was >65% both in [B6xJF1]F1 and [JF1xB6]F1 samples. Unless otherwise indicated, 'transcripts' in this study comprise mouse UCSC known genes, RefSeq genes and Ensemble genes. Transcripts mapping to the sex chromosomes and mitochondrial chromosome were not considered.

Preparation of DNA and RNA

Production of mice with the conditional alleles, referred to as *Dnmt3a*^{2lox} and *Dnmt3b*^{2lox}, was described previously (53,54). To disrupt the conditional alleles in growing oocytes, the mice were crossed with those carrying a *Zp3*-Cre gene (55). The precise timing of conditional deletion of *Dnmt3a* and *Dnmt3b* by *Zp3*-Cre is described elsewhere (56). By crossing (*Dnmt3a*$^{2lox/2lox}$, *Dnmt3b*$^{2lox/2lox}$, *Zp3*-Cre) females with wild-type JF1 male mice, we obtained (*Dnmt3a*$^{-/+}$, *Dnmt3b*$^{-/+}$) E9.5 embryos. *Dnmt3l* knockout female mice (57) were also crossed with wild-type JF1 male mice to obtain *Dnmt3l*$^{-/+}$ E9.5 embryos.

For embryo transfer experiments, *in vitro* fertilized embryos were transferred to pseudopregnant recipients. The mating between B6 and JF1 does not efficiently occur even if superovulation is used, and we used *in vitro* fertilization to obtain enough embryos for the embryo transfer. [B6xJF1]F1 TS cells and [JF1xB6]F1 TS cells were derived and cultured in the absence of mouse embryonic fibroblasts (MEF) as previously described (23). For differentiation, TS cells were cultured in the absence of MEF conditioned medium and FGF4 for 5 days. Total RNA was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) and further purified using RNeasy mini Kit and RNase-free DNase (Qiagen, CA, USA). For human polymorphic analyses, human samples were obtained following informed consent at Yoshida Lady's Clinic, Sendai, Japan. DNA was prepared from umbilical cord blood after delivery and from the mothers' peripheral blood using standard protocols.

In situ hybridization analysis

cDNA probes for *Gatm*, *Tfpi2* and *Ampd3* were generated by PCR and used to prepare sense and antisense riboprobes by *in vitro* transcription using the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). The primer sets are shown in Supplementary Material, Table S4. Sagittal sections of paraffin embedding mouse placentas at E13.5 were used for *in situ* hybridization as described previously (42).

Real-time RT–PCR

First-strand cDNA was synthesized from total RNA using PrimeScript II (Takara Bio, Shiga, Japan). Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). The amount of target mRNA was determined from the appropriate standard curve and normalized to the amount of β-actin mRNA. The primer sets are shown in Supplementary Material, Table S4.

Analysis of allelic expression

PCR amplification was performed using KOD FX (TOYOBO, Osaka, Japan). PCR products were Sanger-sequenced and the sequence chromatograms were analyzed with Sequencing Analysis Software v5.4 (Applied Biosystems). Multiple sequence alignments were done using GENETYX ver. 10.0.3 (GENETYX, Tokyo, Japan). For RFLP analysis, the PCR

products were digested and electrophoresed, and the band intensity was measured with ImageJ (National Institutes of Health, Bethesda, MD, USA). In all experiments, similar results were obtained in at least two independently collected tissues and cell lines. The primer sets are shown in Supplementary Material, Tables S4 and S5.

Bisulphite sequencing

DNA sample was treated with sodium bisulphite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) and PCR amplified using *Ex taq* Hot Start DNA Polymerase (Takara Bio). The PCR products were cloned into the pGEM-T Easy vector (Promega, Wisconsin, USA) and individual clones were sequenced. Primers used are listed in Supplementary Material, Table S4.

ChIP and single nucleotide primer extension (SNuPE)

ChIP analysis was performed using Magna ChIP G Chromatin Immunoprecipitation Kit (Millipore, Temecula, CA, USA) according to the manufacturer's protocol. We used the following antibodies: dimethylated H3-Lys4, trimethylated H3-Lys4, dimethylated H3-Lys9 and trimethylated H3-Lys27 (Millipore). The precipitated DNA was PCR amplified and the allelic histone modifications were analyzed using single nucleotide primer extension (SNuPE). SNuPE analysis was performed using SNaPshot Multiplex kit (Applied Biosystems) according to the manufacturer's protocol. The peak height was determined by GeneMapper v4.1 (Applied Biosystems) as described previously (58). Primers used are listed in Supplementary Material, Table S4.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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High-throughput detection of aberrant imprint methylation in the ovarian cancer by the bisulphite PCR-Luminex method

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Abstract

Background: Aberrant DNA methylation leads to loss of heterozygosity (LOH) or loss of imprinting (LOI) as the first hit during human carcinogenesis. Recently we developed a new high-throughput, high-resolution DNA methylation analysis method, bisulphite PCR-Luminex (BPL), using sperm DNA and demonstrated the effectiveness of this novel approach in rapidly identifying methylation errors.

Results: In the current study, we applied the BPL method to the analysis of DNA methylation for identification of prognostic panels of DNA methylation cancer biomarkers of imprinted genes. We found that the BPL method precisely quantified the methylation status of specific DNA regions in somatic cells. We found a higher frequency of LOI than LOH. LOI at *IGF2*, *PEG1* and *H19* were frequent alterations, with a tendency to show a more hypermethylated state. We detected changes in DNA methylation as an early event in ovarian cancer. The degree of LOI (LOH) was associated with altered DNA methylation at *IGF2/H19* and *PEG1*.

Conclusions: The relative ease of BPL method provides a practical method for use within a clinical setting. We suggest that DNA methylation of *H19* and *PEG1* differentially methylated regions (DMRs) may provide novel biomarkers useful for screening, diagnosis and, potentially, for improving the clinical management of women with human ovarian cancer.

Keywords: Genomic imprinting, Ovarian cancer, DNA methylation, Bisulphite PCR-Luminex(BPL)method, LOI (loss of imprinting)

Background

Human ovarian cancer (HOC) is the leading cause of death from gynecological malignancies, primarily due to the lateness of detection when the cancer is already at an advanced stage. Effective screening protocols for early stages are not currently available. HOC is characterized by complex genetic and epigenetic alterations, including loss of heterozygosity (LOH) and loss of imprinting (LOI) [1,2]. Such alterations are presumed to represent the second hit, according to Knudson's two-hit hypothesis (OMIM #167000) [3]. However, alterations in DNA methylation can also occur as the first hit during human carcinogenesis [4].

For childhood cancers such as retinoblastoma (OMIM #180200), Wilms' tumor (OMIM #194070) and osteosarcoma (OMIM #259500), changes primarily occur on the paternal allele first, followed by a second hit on the maternal allele [5,6]. Complete hydatidiform moles, which are of androgenetic or paternal origin, are characterized by malignant transformation whereas ovarian teratomas, which are of parthenogenetic or maternal origin, are benign [7,8]. These observations suggest a role for altered genomic imprinting in the malignant transformation process.

Alterations in the expression of imprinted genes represent one of the most common changes seen in cancer [9,10]. Some imprinted genes, including *H19*

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[11], *GTL2* [12], *PEG1, PEG3* [13], *LIT1* (*KCNQ1OT1*) [14] and *ZAC* [15], are known to act, or strongly implicated to act, as tumor suppressor genes (TSGs). The monoallelic expression of imprinted genes is reliant on epigenetic mechanisms, most notably DNA methylation, which initiates the imprinting process in the male and female germlines at discrete locations termed differentially methylated regions (DMRs) [16]. Imprinted domains generally contain several genes displaying allele-specific expression and these DMRs, which can be located over the promoter of a protein coding gene or the promoter of a functional non-coding RNA or within intergenic regions, are known to control imprinted gene expression within the domain, acting as imprinting centers or imprint control regions [17]. We recently developed a new high-throughput, high-resolution DNA methylation analysis method called bisulphite PCR-Luminex (BPL) for the rapid analysis of DNA methylation [18]. In this study, we applied this method to 21 HOC cell lines and 74 HOC tissues to efficiently and accurately determine the methylation status of DMRs at eight imprinted loci, six of which contained TSGs. To determine whether abnormal methylation of these DMRs acts as an indicator for potential LOH and/or LOI, we also examined the association between abnormal hypermethylation and LOI or LOH. We found a higher frequency of LOI than LOH. LOI at *IGF2*, *PEG1* and *H19* was a frequent alteration, with a tendency to show a more hypermethylated status. The degrees of LOI and altered DNA methylation were similar among histology, progression and tumor grades. This suggests that DNA methylation of the *H19* and *PEG1* DMRs may provide novel biomarkers useful for screening, diagnosis and, potentially, for improving the clinical management of women with HOC.

Results

Frequencies of the 8 imprinted gene profiles in HOC

We first determined whether the ovarian malignancies showed LOH by comparing the restriction fragment length polymorphism (RFLP) patterns of normal lymphocyte DNA and 74 matching primary HOC DNA samples. Samples where RFLPs were present in the lymphocyte DNA sample but absent or with an altered ratio in the tumor sample were considered to exhibit LOH in the regions of 8 imprinted genes (*H19, IGF2, KCNQ1, LIT1, GTL2, PEG1, PEG3* and *NDN*). The average percentage of heterozygosity was 48.0% (16.2-58.5%). We found only 14 cases of LOH in the 8 imprinted genes in the 74 HOC samples we analysed (Table 1). The most frequent gene with LOH was *IGF2* (9.0%, 3/33), followed by *PEG1* (8.1%, 3/37) and *GTL2* (7.1%, 3/42). LOH of *NDN* and *LOT1* was not detected (0/31 and 0/12). The samples with LOH were not from the same cases (Additional file 1: Table 1).

We next performed RT-PCR and RFLP analysis to identify the samples of LOI without LOH. The frequency of LOI was higher than that of LOH for all 8 imprinted genes and we found a total of 46 cases of LOI (Table 1, Additional file 1: Table S1). The most frequent sites of LOI were *PEG1* (45.9%, 17/37), *IGF2* (45.4%, 15/33) and *H19* (29.2%, 12/41). *NDN* had the lowest frequency. In 19 of the 46 cases, the abnormal gene expression pattern was apparent at two or more imprinted loci. A normal imprinting pattern, maintenance of imprint (MOI), was most frequent in *NDN* (93.5%, 29/31). ND (not determined) means no amplification of RT-PCR at 3 times in several samples, perhaps indicating low expression of the genes. In 9 of the 14 LOH cases, LOI was also found in at least one gene. In HOC cell lines, LOI was found in 2 of 3 informative cases for *IGF2*, and 3 of 9 cases for *PEG1*. We did not find any LOH or LOI in 7 normal ovarian surface tissues and 4 normal cell lines. We compared patients' ages, progression, histology and tumor grades with imprinted gene expression pattern profiles. Patients with LOI had a tendency to be younger than patients with LOH (mean ages for LOH and LOI: 55.0 ± 7.4 and 47.7 ± 6.9, respectively), but the difference was not statistically significant by ANOVA, and no other correlations were apparent.

Analysis of the methylation status of DMRs in ovarian cancers by the BPL method

The proof-of principle experiment of the BPL method has been described in detail [18]. Briefly, bisulphite-DNA can be used to distinguish between methylation and non-methylation status in the genome, e.g. cytosine and uracil. The BPL method can determine one base substitution by specific hybridization and detect the ratio of methylation to non-methylation. We examined the quality of the BPL method in spermatic DNA, which should show 100% methylation of the paternally methylated DMRs: *ZDBF2, H19* and *GTL2*, whereas the maternally methylated DMRs: *PEG1, ZAC, SNRPN, PEG3* and *LIT1* are non-methylated. We applied the classic methylation assay COBRA technique and our recently devised BPL method to the DNA of 7 normal ovarian surface epithelium tissues, 4 primary cultures of normal human ovarian surface epithelium (OSE1-4) and 21 HOC cell lines, and performed statistical analysis with Spearman's and Pearson's rank correlations. For all 8 DMRs a good correlation was found between these two methods (Figure 1, Table 2, Additional file 2: Figure S1).

We next determined the methylation status of the 8 DMRs from the 74 samples of primary ovarian cancer tissue by the BPL method. Overall, we compared the average DNA methylation status of cancer and normal samples for each DMR and found that *PEG1* from ovarian cancers was