

What does genetics tell us about imprinting and the placenta connection?

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Abstract Genomic imprinting is an epigenetic gene silencing phenomenon that is specific to eutherians in the vertebrate lineage. The acquisition of both placentation and genomic imprinting has spurred interest in the possible evolutionary link for many years. In this review we examine the genetic evidence and find that while many imprinted domains are anchored by genes required for proper placenta development in a parent of origin fashion, an equal number of imprinted genes have no apparent function that depends on imprinting. Examination of recent data from studies of molecular and genetic mechanisms points to a maternal control of the selection and maintenance of imprint marks, reinforcing the importance of the oocyte in the healthy development of the placenta and fetus.

Keywords Genomic imprinting · Placentation · Maternal effect · Differential methylation · Targeted mutation · Conflict hypothesis · Trophoblast · Oocyte

Introduction

Genomic imprinting was discovered in mammals 30 years ago with the publication of two seminal papers describing the same experiment: pronuclear exchange between fertilized zygotes generated two kinds of abnormal embryos—

androgenotes (two paternal genomes) and gynogenotes (two maternal genomes) [1, 2]. Development of both kinds of embryo proceeded relatively normally to the blastocyst stage; however after implantation, embryogenesis in both groups began to go awry, but in different ways. Androgenotes that survived to mid-gestation possessed hyperplastic extraembryonic tissues of trophoblast origin to the exclusion of embryonic structures, while gynogenetic survivors at mid-gestation were characterized by poor to non-existent extraembryonic tissues of trophoblast origin and small, although normally developed embryos. The vast majority of androgenotes and gynogenotes die at or shortly after implantation [3], with occasional survival up to mid-gestation [4].

The results of these experiments suggested that one set of genes is silenced on the maternal allele, while another is silenced on the paternal allele [5, 6]. In the intervening 30 years, this prediction has been proven correct with the discovery of approximately 100 imprinted genes in mice and 50 in humans (the two most extensively studied species). Importantly, the discovery of genomic imprinting provided an explanation for several human genetic diseases whose inheritance patterns had stumped investigators for many years. The ensuing ramping up of research in this area provided the stimulus, both intellectual and financial, for studies in other fields of epigenetics, with the result that great progress in our understanding has been achieved, in part because technological strides have made it possible to address very complex questions in very specific ways.

The restriction of genomic imprinting in vertebrates to mammals is highly suggestive of an evolutionary link to placentation. Indeed, the phenotypes of androgenetic and gynogenetic embryos, with major defects in trophoblast, clearly indicate that at least early acting imprinted genes are involved in placenta development. These observations

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prompted proposals in the 1990s that genomic imprinting arose as an evolutionary protective mechanism to counter the potentially lethal effects of excessive placentation [7], or of ectopic trophoblast in females [8]. Other ideas about the evolution of imprinting have focused on functions outside of the extraembryonic tissues, including the brain [9, 10]. Widely cited is the popular conflict hypothesis [11] that has dominated thinking in the field for several decades. In this review we will examine the evidence that has accumulated in the past 30 years, with an emphasis on the roles played by imprinted genes in placenta development and function revealed by genetic manipulation.

Genomic imprinting has been studied in greatest depth in mice; while most of the studies in humans have been largely correlative, more recent high-throughput analyses in pathological placentas [12] or rare genome wide uniparental disomy (UPD) samples [13] revealed several novel imprinted genes, indicating that significant species differences probably exist. Studies in other species have been much more limited, although important observations of genomic imprinting in marsupials have provided insight into the evolutionary mechanisms [14]. This review will focus on the genetic evidence gathered in murine studies, with mention of other species where appropriate. Readers are also referred to a review by Tunster et al. [15].

Organization of imprinted genes and regulation of expression

Many imprinted genes reside in clusters. However, this is not carved in stone. Of 31 imprinted gene loci in mice, 12 are represented by a single gene; imprinting of 10 is not conserved with humans, at least in the tissues assayed. This, and recent high-throughput data from human studies [12, 13], supports the idea that genomic imprinting is evolutionarily dynamic (see review by Renfree et al. [14]). Most, although not all, imprinted domains are regulated by a germline differentially methylated region (gDMR). Of the 23 confirmed gDMRs, 20 are methylated on the maternal allele, while 3 are paternally methylated (Fig. 1; Table 1). Multi-gene imprinted domains can contain genes that are maternally expressed, paternally expressed and biallelic, indicating that the gDMRs regulate chromatin structure over large distances rather than merely shutting off expression of a single target gene.

Maternal gDMRs are all associated with CpG islands near the transcriptional start sites (TSS) of protein-coding genes, while paternal gDMRs are intergenic. In general, methylation of the TSS CpG island is associated with repression of the target gene, whereas paternal intergenic gDMRs are associated with activation of at least one gene in the cluster, in two cases (*H19* and

Rasgrf1) through action of a CTCF boundary element [16, 17]. Interestingly, oocyte-specific knockdown of CTCF leads to methylation of the maternal *H19* DMR, suggesting that methylation of this element may be the default state [18]. Indeed, germline-specific methylation as a default may be a general rule unrelated to imprinting, not surprising given the major differences between sperm and egg [19–21]. This point will be returned to later in the review.

Not all imprinted domains possess an identifiable gDMR. For example, the CpG island spanning the TSS of the paternally expressed murine *Sfmbt2* gene is unmethylated [22]. This gene, imprinted only in old world rodents, is distinguished from orthologs in other species by the presence of a large block of miRNAs in intron 10. A similar situation exists in the older primate-specific *C19M* locus, although in this case a gDMR has been identified [23]. The correlation between the presence of the miRNAs and imprinting of the locus is suggestive of an RNA-based mechanism driving imprinting at this gene. Interestingly, imprinted genes are sixfold more likely to be closely associated with an miRNA than other biallelic genes. Two other imprinted domains are characterized by the presence of large blocks of either miRNAs or SnoRNAs, or both: the *Dkl1* locus and the *Snrpn* locus. Interestingly, all but three—mir-296-5p, mir-483 and mir-494 (GEO accession GSE17966)—of the miRNAs associated with imprinted genes, regardless of their allelic expression bias later in development, are silent in oocytes. In addition, several loci are regulated by ncRNAs (*Kcnq1ot1*, *Airn*, *Xist*, *H19*); additional loci may be found to be similarly regulated as high-throughput datasets reveal unknown non-coding transcripts (e.g., Fig. 2).

Imprinted genes in placenta

The notion that imprinting and placentation are evolutionarily linked is reinforced by the observation that a number of genes display extraembryonic tissue-only imprinting/expression; this includes both placenta and yolk sac. The most compelling evidence comes from a series of targeted mutations of imprinted genes (Table 1). In particular, genes closest to the gDMR, which may be the primary target of imprinting at a multi-gene domain (referred to below as the anchoring gene), often show a placenta phenotype. For example, *Peg10*, whose DMR regulates a domain on proximal Chr. 6, is required for the development of the spongiotrophoblast layer of the placenta; loss of function (LOF) is embryonic lethal because of failed placentation. A similar scenario can be described for several other genes that anchor imprinted domains (see below).

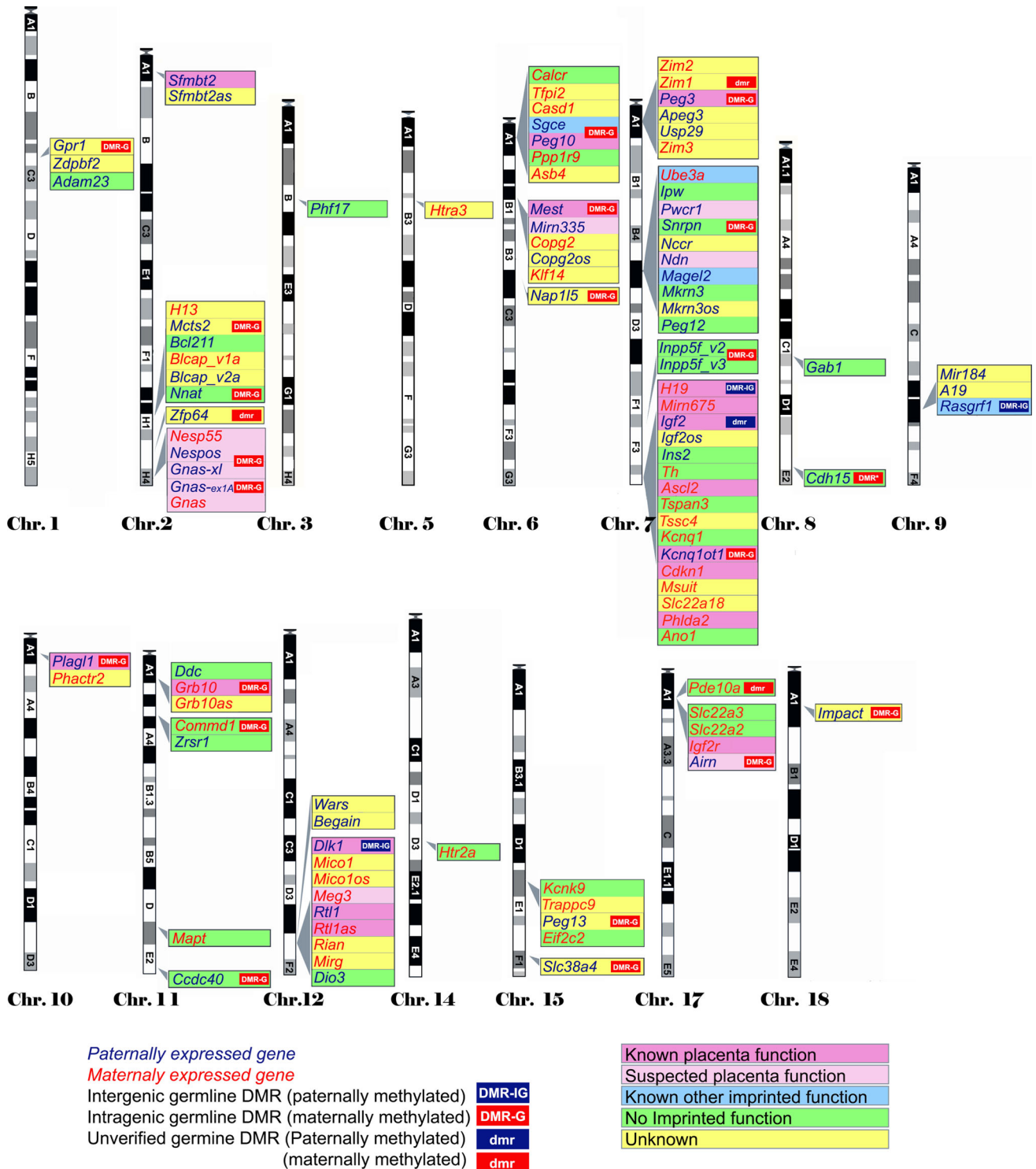


Fig. 1 Map of imprinted genes. Known imprinted genes are displayed on a map of the mouse genome. Color coding highlights features discussed in the text, including known placental function (dark pink), suspected placental function (pale pink), non-placental imprinted function (blue), no imprinted function (green) and

unknown (yellow). The location of verified gDMRs is indicated by capital letters and for unverified gDMRs by lowercase letters, with maternal DMRs represented by red boxes and paternal DMRs by blue boxes. Several domains do not possess gDMRs

A recent analysis of imprinted genes restricted to extraembryonic tissues (EXEL) suggested that imprinting in the placenta and yolk sac may be governed by a different

epigenetic mechanism [24, 25] than the traditional gDMR-regulated silencing, in line with an earlier study of genes at the *Kcnq1* locus [26]. A similar interpretation was made

Table 1 Imprinted gene function

Gene	Chr/loc	Expr.	DMR	sRNA	Notes	Human gene imprinted	Ref
<i>Gpr1</i>	1/63 mB	P	M - g	Intronic miRNA	No KO. In UCSC, long transcript overlaps partially with <i>Zdbf2</i> on os; expressed mainly in placenta where biallelic; DMR becomes methylated after blastocyst stage; no KO; not expressed in TS cells	yes	[74]
<i>Zdbf2</i>	1/63 mB	P		5' UTR miRNA; intronic snRNA	No KO. Expressed in brain, ES cells, placenta; biallelic in placenta; imprinted in humans; <i>Zdbf2</i> paternal DMRs are erased in early embryos and re-established after the blastocyst stage; noKO; not expressed in TS cells	yes	[74]
<i>Adam23</i>	1/63 mB	P			Secretory and transmembrane protein gene trap. Recessive postnatal lethal, brain defects; not expressed in TS cells	no	[104]
<i>Sfmbt2</i>	2/10 mB	P		110 miRNA in mir-467 family, intronic	Null gene trap. No DMR; paternal in TS cells; KO results in developmental arrest of placenta at e8.5; paternal in TS cells	no	[29]
<i>Sfmbt2 as</i>					No KO. NoRNA; paternal in TS cells	no	[34]
<i>H13</i>	2/15 mB	M			No KO. Weak placenta expression; biallelic in TS cells	no	[105]
<i>Mcts2</i>	2/15 mB	P	M - g		No KO. In intron of <i>H13</i> ; <i>Mcts2</i> promoter methylated DMR; retrotransposon; weak expression in placenta; paternal expression in TS cells	No human orthologue	[106]
<i>Bcl2l1</i>	2/15 mB	P			Targeted deletion of first coding exon. Recessive lethal at e13; biallelic in TS cells	no	[107]
<i>Blcap</i>	2/15.7 mB	M/P		Intronic miRNA	No KO. Wide expression; one transcript is maternal and the other is paternal, both encode protein; bisected by <i>Nnat</i> in intron. Weak biallelic expression in TS cells	Yes	[108]
<i>Nnat</i>	2/15.7 mB	P	M - g		Targeted deletion coding exons. Encoded on os of <i>Blcap</i> in intron; mainly brain; KO normal phenotype; not expressed in TS cells	Yes, expressed only in brain	[108, 109]
<i>Zfp64</i>	2/16.9 mB	P	m - g		No KO. Weak expression everywhere; biallelic in TS cells	No human orthologue	[110]

Table 1 continued

<i>Nesp55</i>	2/17.4 mB	M	P - s		Targeted deletion exons 1 and 2, includes Nesp DMR. IUGR with edema, similar to hydrops fetalis in humans that is sometimes linked to placenta dysfunction; placenta not examined; not expressed in TS cells	yes	[67, 111, 112]
<i>GnasXL</i>	2/17.4 mB	P			Targeted deletion <i>GnasXL</i> exon1. <i>GnasXL</i> paternal KO slightly reduced at birth but then fail to thrive; not expressed in TS cells	yes	[66]
<i>Gnas</i>	2/17.4 mB	P/M			Targeted insertion of neo cassette into common exon 2 or Oed/Sml point mutation in exon 6. Maternal transmission of KO allele results in edema, wide bodies, ataxia, postnatal death; paternal transmission causes smaller birth weight, narrow bodies, postnatal death; phenotypes mirror Oed-Sml point mutation in <i>Gnas</i> ; placentas not examined; not expressed in TS cells; human IUGR placentae show reduced expression of <i>Gnas</i>	yes	[113 – 115]
<i>Nespos</i>	2/17.4 mB	P	M - g	Mir-296, mir-298 in 3' flank	Targeted deletion of DMR and several <i>Nespos</i> exons results in biallelic <i>Nesp</i> expression and downregulation of <i>Gnas</i> transcripts; paternally lethal with phenotype similar to <i>GnasXL</i> ; maternal Oed and paternal DMR deletion rescue each other; paternally expressed in TS cells, reads extend down to miRNAs	yes	[116]
<i>Phf17</i>	3/41.5 mB	P			Aka Jade1. Gene trap null insertion upstream of first coding exon. Homozygotes are viable and fertile, although there may be some partial lethality; not clear whether from maternal or paternal side; biallelic in TS cells.	no	[117]
<i>Htra3</i>	5/35.6 mB	M			No KO. Not expressed in TS cells	no	[110]
<i>Calcr</i>	6/3.68 mB	M		Mir-498; mir-653, intronic	Targeted deletion exons 5-7. Recessive embryonic lethal; not expressed in TS cells	yes	[118]
<i>Tfpi2</i>	6/3.96 mB	M			No KO. Not expressed in TS cells. Imprinting suspect due to contaminating maternal tissue (Okae et al)	Yes? Placenta specific, so may be contaminating maternal tissue	[119, 120]
<i>Casdl</i>	6/4.6 mB	M			No KO. Biallelic in TS cells	no	[120, 121]

Table 1 continued

<i>Sgce</i>	6/4.6 mB	P			Floxed exons 4-5 crossed with ubiquitous cre transgenic. Imprinted myoclonic dystrophy; paternally expressed in TS cells	yes	[122]
<i>Peg10</i>	6/4.7 mB	P	M - g		Targeted deletion coding exons. Placenta defects; paternally expressed in TS cells	yes	[39]
<i>Ppp1r9a</i>	6/4.9 mB	M			Targeted deletion of first coding exon. Recessive mild neurological defects; biallelic in TS cells. Imprinting in placenta suspect due to potential contaminating maternal tissue (Okae et al)	yes	[123]
<i>Asb4</i>	6/5.3 mB	M			No KO. Not expressed in TS cells	no	[110, 119]
<i>Mest</i>	6/30.7 mB	P	M - g	Mir-335 in intron	Gene trap reporter insertion in intron 2 creates null allele. Placentas and embryos both growth restricted at mid-gestation; not expressed in all TS cell lines; mir-335 retained in mutant allele, but processing unknown	yes	[41]
<i>Mirn-335</i>	6/30.7 mB				Within Mest transcription unit	Yes?	[124]
<i>Copg2</i>	6/30.7 mB	M			No KO. Biallelic in TS cells	No?	[125]
<i>Copg2os</i>	6/30.7 mB	P			No KO. Not expressed in TS cells	yes	[125]
<i>Klf14</i>	6/30.9 mB	M			No KO. Not expressed in TS cells	yes	[110]
<i>Nap115</i>	6/58.9 mB	P	M - g		No KO. Retrotransposon in intron of Herc3; may drive alternative pA use on Herc3 which truncates COOH terminus, but doesn't delete any annotated domains; not expressed in TS cells	yes	[106, 126]
<i>Zim2</i>	7/6.6 mB	M			No KO. Poorly annotated; not in Ensembl and called imprinting zinc finger 2 in UCSC; no RNAseq reads at map location	Yes, but paternally expressed	[127]
<i>Zim1</i>	7/6.6 mB	M	m - g		No KO. Not expressed in TS cells	No human orthologue	[110, 120, 127]
<i>Peg3</i>	7/6.7 mB	P	M - g		Targeted deletion exons 5-7. Placenta growth retarded, see footnote in KO paper; paternally expressed in TS cells	yes	[43]

Table 1 continued

<i>Apeg3</i>	7/6.7 mB	P		mir-3099 within the range of the unannotated AS transcript which may be orthologue of MIMT1	No KO. Not expressed in TS cells. There is another paternally expressed AS transcript in the RNA-seq database which is probably the orthologue of human MIMT1, an imprinted ncRNA	Yes	[128]
<i>Usp29</i>	7/6.9 mB	P			No KO. In UCSC <i>Usp29</i> transcripts coincide with location of MIMT1; in Ensembl transcripts annotated further downstream. Not expressed in TS cells	no	[129, 130]
<i>Zim3</i>	7/6.9 mB	M			No KO. Antisense to <i>Usp29</i> ; not expressed in TS cells	no	[131]
<i>Ube3a</i>	7/59.2 mB	M			Targeted deletion exon 2. Postnatal neurological defects; biallelic in TS cells	yes	[132]
<i>Ipw</i>	7/59.6 mB	P		SNORD115	Radiation induced deletion. Probably part of nc transcript encoding SNORD115; other blocks of SNORD downstream; large deletion including <i>Ube3a</i> and this block of SNORD has no obvious phenotype; not expressed in TS cells	yes	[133]
<i>Pwcr1</i>	7/59.7 mB	P		SNORD116	Targeted deletion of MBII-85 SNO RNA cluster. Postnatal growth restriction, although authors note that mutants are identifiable at birth; measured weight of placentas only which is inadequate (<i>Dlk1</i> KO placentas are normal weight but have abnormal histology); not expressed in TS cells	yes	[65]
<i>Snrpn/Snurfn</i>	7/59.7 mB	P			Targeted deletion of 0.9 kb exon 1 (hypomorph) or exons 5-7 (null). <i>Snrpn</i> itself is dispensable; deletion of the ICR causes IUGR; paternally expressed in TS cells	yes	[63, 134]
<i>Snrpn ICR</i>	7/59.7 mB	P	M - g		Targeted deletion of 4.8 kb promoter and exon 1. Paternal inheritance of ICR deletion results in PWS like symptoms including IUGR		[63]
<i>Nccr</i>	7/61.3 mB	P		12 mir-344	No KO. Multiple ncRNAs between <i>Snrpn</i> and <i>Ndn</i> , including <i>Pec2</i> , <i>Pec3</i> , <i>U80893</i> , <i>DOKist</i> ; ref to <i>Nccr</i> in Otago web site; no reads in TS RNAseq database	No human orthologue of mir-344 (rodent specific)	
<i>Ndn</i>	7/62.3 mB	P	M - s		Targeted deletion of <i>Ndn</i> orf. Phenotype evident at birth; strongly dependent on genetic background; not expressed in TS cells	yes	[64]
<i>Magel2</i>	7/62.3 mB	P	M - s		LacZ knock-in allele. Some perinatal lethality dependent on C57BL6 genetic background; postnatal	yes	[135, 136]

Table 1 continued

					circadian rhythm defects; placentas normal; not expressed in TS cells		
<i>Mkx3</i>	7/62.4 mB	P			No KO. Weak biallelic expression in TS cells	yes	[137]
<i>Mkx3os</i>	7/62.4 mB	P			No KO. Not expressed in TS cells	unknown	[137]
<i>Peg12</i>	7/62.4 mB	P			LacZ knock-in. KO normal; weak biallelic expression in TS cells	no	[138]
<i>Inpp5f_v2</i>	7/128.6 mB	P	M - g		Targeted deletion exons 7-13. Only one transcript imprinted; the coding part is biallelic; recessive mild heart defect; biallelic expression in TS cells	yes	[139]
<i>H19</i>	7/142.5 mB	M	P - g P - s	Mir-675	Targeted deletion of transcription unit. Increased neonate weight; placentomegaly; biallelic in TS cells	yes	[44, 45, 140, 141]
<i>Mir-675</i>	7/142.5 mB	M		Mir-675	Part of H19 transcript; LOF results in placentomegaly; biallelic in TS cells	yes	[44]
<i>Igf2</i>	7/142.6 mB	P	p - g P - s	Mir-483	Targeted deletion of placenta specific exon. Growth retardation effect mediated by placenta specific expression of Igf2; biallelic in TS cells	yes	[36, 142]
<i>Igf2os</i>	7/142.6 mB	P			No KO. Biallelic in TS cells	yes	[143]
<i>Ins2</i>	7/142.6 mB	P			KO normal phenotype; not expressed in TS cells	yes	[144]
<i>Th</i>	7/142.6 mB	M			Targeted disruption exons 7-8. Recessive embryonic lethal; not expressed in TS cells. Imprinting in placenta needs additional confirmation due to possible maternal contamination (Okae et al).	no	[145]
<i>Ascl2</i>	7/142.9 mB	M			Targeted deletion of coding exon. Impaired placenta development; reduced spongiotrophoblast, increased trophoblast giant cell layer; weak biallelic expression in TS cells	no	[27]
<i>Tspan32</i>	7/143 mB	M			Gene trap null allele. KO normal; not expressed in TS cells. Imprinting in placenta suspect due to maternal contamination (Okae et al)	no	[146]
<i>Tssc4</i>	7/143 mB	M			No KO. Biallelic in TS cells. Imprinting in placenta may be suspect due to maternal contamination (Okae et al)	no	[147, 148]
<i>Kcnq1</i>	7/143.1 mB	M			Targeted deletion exon 1. Recessive deafness; not expressed in TS cells	yes	[50]

Table 1 continued

<i>Kcnq1ot1</i>	7/143.2 mB	P	M - g		Targeted deletion of <i>Kcnq1ot1</i> CpG islands. KO phenotype is IUGR, placental growth restriction; probably due to loss of imprinting of other genes in locus; paternally expressed in TS cells	yes	[47, 48]
<i>Cdkn1c</i>	7/143.4 mB	M	P - s		Targeted deletion of coding exons. KO results in placentomegaly; maternally expressed in TS cells	yes	[52, 149, 150]
<i>Msu1</i>	7/143.4 mB	M			No KO. ncRNA upstream and AS to <i>Cdkn1c</i> ; EST BX529363; no reads in region in TS cells		[151]
<i>Slc22a18</i>	7/143.4 mB	M			No KO. Weak maternal expression in TS cells	yes	[110, 152]
<i>Phlda2</i>	7/143.5 mB	M			Targeted deletion coding exons. Placentomegaly; maternally expressed in TS cells	yes	[54]
<i>Ano1</i>	7/144.5 mB	M			Targeted deletion of exon 12 that encodes one of transmembrane domains critical for function. KO recessive postnatal lethal; not expressed in all TS cells	yes	[153]
<i>Gab1</i>	8/80.7 mB	P			In-frame reporter insertion null allele. Recessive embryonic lethal with placental and muscular defects; biallelically expressed in some TS cells; unannotated antisense paternal transcript in TS RNAseq dataset (Fig. 2)	no	[154]
<i>Cdh15</i>	8/122.8 mB	P	m - g		Targeted lacZ knock-in null allele. KO normal; not expressed in TS cells	no	[155]
<i>mir184</i>	9/89.8 mB	P			No KO. On (-) strand; not expressed in TS cells	no	[156]
<i>4930524 OORik/A19</i>	9/89.8 mB	P			No KO. ncRNA, includes A19; + strand; not expressed in TS cells	no	[157]
<i>Rasgrfl</i>	9/89.9 mB	P	P - g		Targeted deletion of catalytic domain exon. Postnatal growth restriction; not expressed in TS cells	no	[158]
<i>Plagl1</i>	10/13.1 mB	P	M - g		Targeted deletion exons 1-2. Placenta growth restriction, reduced labyrinth; paternally expressed in TS cells	yes	[57]
<i>Hymai</i>	10/13.1 mB	P			No KO; ncRNA.		[55]
<i>Phactr2</i>	10/13.2 mB	M			No KO. Biallelic in TS cells	yes	[110]

Table 1 continued

<i>Ddc</i>	11/11.8 mB	P			Knock-in splicing error found in human patients. Recessive postnatal growth restriction and brain/behaviour defects; weak biallelic expression in TS cells	no	[159]
<i>Grb10</i>	11/11.9 mB	M	M - g		Targeted deletion exons 2-4. Placental and fetal overgrowth; maternal bias in TS cells	Yes, although not sure about placenta?	[58]
<i>Grb10as</i>	11/11.9 mB	M			No KO. Maternal bias in TS cells	no	
<i>Commd1</i>	11/22.9 mB	M	M - g		Targeted deletion exon 2. Recessive embryonic lethal, with reduced vascularization of placenta; biallelic in TS cells	no	[160]
<i>Zrsr1</i>	11/22.9 mB	P			Targeted deletion of coding sequence and CpG island. KO normal; paternally expressed in TS cells	No orthologue	[161]
<i>Mapt</i>	11/104.2 mB	M			Targeted deletion exon 1. Recessive mild neurological defects; weakly biallelic in TS cells	no	[162]
<i>Ccdc40</i>	11/119.2 mB	P	M - g		Natural nonsense SNP mutation. Recessive laterality defects; embryonic lethal; not expressed in TS cells	no	[163]
<i>Wars</i>	12/108.8 mB	P			No KO. tRNA synthetase; biallelic in TS cells	no	[164]
<i>Begain</i>	12/109 mB	P			No KO. Not expressed in TS cells	no	[165]
<i>Dlk1</i>	12/109.4 mB	P	P - g P - s		Targeted deletion exons 5-6. Neonatal lethal, reduced growth, abnormal placenta; not expressed in TS cells	yes	[59, 166, 167]
<i>Micol</i>	12/109.5 mB	M			No KO. ncRNA; no reads in TS RNAseq	No orthologue	[168]
<i>Mico1os</i>	12/109.5 mB	M			No KO. No reads in TS RNAseq	No orthologue	[168]
<i>Meg3/Gil2</i>	12/109.5 mB	M	P - s	Multiple annotated miRNAs	Targeted deletion exons 1-5. Meg3 ncRNA transcripts extend down to Mirg, which also contains numerous miRNAs (Fig. 2A); both maternal and paternal transmission have phenotypes that involve LOI in both embryo and placenta similar to Rtl1 deletion, only milder; strongly maternally expressed in TS cells	yes	[169]

Table 1 continued

<i>Rtl1</i>	12/109.5 mB	P		AS to some of miRNAs	Simultaneous targeted deletion of most of <i>Rtl1</i> and <i>Rtl1as</i> transcription units. Retrotransposon; paternal KO yields IUGR, maternal KO causes placentomegaly because of upregulation of paternal <i>Rtl1</i> ; not expressed in TS cells	yes	[60]
<i>Rtl1as</i>	12/109.5 mB	M		Six miRNAs	Placentomegaly due to loss of miRNA repression of <i>Rtl1</i>		[60]
<i>Rian</i>	12/109.6 mB	M		miRNAs and SnoRNAs	No KO. ncRNA; maternally expressed in TS cells	unknown	[170]
<i>Mirg</i>	12/109.7 mB	M		Many annotated miRNAs	No KO. Maternally expressed in TS cells	unknown	[171]
<i>Dio3</i>	12/110.2 mB	P			Triple point mutation knock-in. Recessive hypothyroidism; not expressed in TS cells	no	[172]
<i>Htr2a</i>	14/74.6 mB	M			Targeted deletion of promoter; null allele. Recessive neural defects; not expressed in TS cells	no	[173]
<i>Kcnk9</i>	15/72.5 mB	M			Targeted deletion exon 2. Recessive mild neural defects; not expressed in TS cells	Yes	[174]
<i>Trappc9/1810044 A24Rik</i>	15/72.9 mB	M			No KO. Biallelic in TS cells	no	[175]
<i>Peg13</i>	15/72.8 mB	P	M - g		No KO. In <i>Trappc9</i> intron, same strand	no	[126]
<i>Eif2c2</i>	15/73.1 mB	M			Transgene insertional null allele. Recessive embryonic lethal (includes placenta and ys); biallelic in TS cells	no	[176]
<i>Slc38a4</i>	15/96.9 mB	P	M - g		No KO. Paternally expressed in TS cells	no	[126]
<i>Pde10a</i>	17/8.8 mB	M	m - g		Targeted deletion exons 14-16. Recessive behavioural defects; weak maternal expression in TS cells	no	[177]
<i>Slc22a3</i>	17/12.4 mB	M			Targeted deletion exon 1. Recessive mild neurological defects; not expressed in TS cells	yes	[178]
<i>Slc22a2</i>	17/12.5 mB	M			Targeted deletion exon 1. KO normal; not expressed in TS cells	yes	[179]
<i>Igf2r</i>	17/12.6 mB	M	P - s		Targeted deletion exons 13-18. Placentomegaly; maternally expressed in TS cells	no	[62, 180, 181]
<i>Airn</i>	17/12.7 mB	P	M - g		Targeted deletion <i>Airn</i> CpG island. IUGR; no Placenta histology; paternally expressed in TS cells	unknown	[182]
<i>Impact</i>	18/12.9 mB	P	M - g		No KO. Biallelic in TS cells	no	[183]

Imprinted genes listed here were obtained from the Otago Catalogue of Parent of Origin Effects website (<http://igc.otago.ac.nz/home.html>). Where imprinting of a gene is listed as “provisional” or “other”, it has been left out of the table. TS cell expression data were obtained from the dataset published by Calabrese et al. [34]. DMRs with lowercase letters are derived from Kobayashi et al. [74] that have not been confirmed experimentally; “g” denotes germline; “s” denotes somatic. Annotated miRNAs are named unless there are too many. Domains are separated by grey bars

following the discovery of several novel placenta-specific imprinted genes in humans [13]. However, some caution should be used in the interpretation of the data, especially allelic expression in placenta, which is “contaminated” with maternal tissue. Examination of the data in mice, for example, reveals that of 16 so-called EXEL genes, strong evidence exists for imprinting of only five (*Ascl2* [27]; *Ins2* [28]; *Sfmbt2* [29]; *Slc22a2*; and *Slc22a3* [30]); for most of the rest, imprinting can be discounted due to contamination by maternal tissue, or very weak maternal bias [31]. In the human studies, which focused on differential methylation [12, 13], the number of DMRs far outnumbered the number of genes showing consistent monoallelic expression in placenta, and between the two studies there was minimal overlap, suggesting that methodology may also introduce artifacts. As an example of the latter, the initial report of the murine *Sfmbt2* gene described differential methylation of the maternal allele in placenta [32], based on PCR–RFLP analysis of genomic DNA that had been digested with methylation-sensitive restriction enzymes. However, in a later report [22], bisulfite sequence analysis clearly demonstrated very low methylation levels of both alleles in placenta, not surprising given the resistance of extraembryonic lineages to loss of DNA methyltransferases [33]. The most robust test of imprinting is derived from genetic analysis of targeted mutations, either reporter constructs in which expression is visualized following transmission from only one parent, or transcriptional null alleles in which expression of the endogenous gene can be assessed following transmission from either the mother or father.

Many imprinted genes (29) appear to be “innocent bystanders” [8]. Their loss is either without any effect (e.g., *Nnat*, *Peg12*) or, while they are clearly required for survival, this function is not dependent on imprinted expression, since it can only be observed when both alleles are mutated (e.g., *Commd1*, *Dcn*, *Eif2c2*), suggesting that the key expression is in a tissue or at a stage of development when both alleles are expressed and the imprints have been reprogrammed during development. There remains the possibility that a subtle imprinted effect has been overlooked in the phenotypic analysis of these mutations.

Targeted mutations in several anchoring imprinted genes have known phenotypes affecting the placenta. In some additional cases, placenta function can only be inferred from the reported phenotype, such as intrauterine growth restriction (IUGR), because the authors did not perform any placental analysis, or limited examination to measurement of weight. Many genes also have functions in later stages of development or in postnatal animals, in particular in the brain. This review will discuss only the placenta or early development functions of imprinted gene mutations. Given that almost the entire X chromosome is

imprinted in trophoblast [34], we will only discuss autosomal genes.

Imprinted genes with known placenta function

Of the 31 imprinted domains, 10 are anchored by genes with known function in the placenta and 2 by genes with suspected placental function. The remaining 20 domains harbor genes with no known function or with no imprinted function (see Fig. 1). The relevance of these domains will be discussed below. Some large domains contain more than one gene required for proper placenta development and can be expressed either maternally or paternally. The following are brief descriptions of what we know about their roles in placentation. Analysis of placenta development and function is variable as there are no standard protocols, although several laboratories have adopted some sophisticated methodologies that allow examination of more subtle phenotypes [35], including physiological adaptation to reduced placental mass [36], demonstrating the capacity of the placenta to work overtime when pressed, and altered nutrient management [37]. This kind of thorough examination is warranted especially in cases where the phenotype does not “jump up off the bench and hit you on the head”.

Sfmbt2 is part of a murine-specific domain on proximal Chr. 2 consisting of the protein-coding gene and an anti-sense non-coding transcript (ncRNA); both are expressed from the paternal allele in extraembryonic tissues. *Sfmbt2* encodes a PcG protein required for maintaining the trophoblast stem cell (TS) pool; its loss results in severe reduction of all trophoblast layers of the placenta and is accompanied by embryonic developmental arrest and death at mid-gestation [29]. Murine *Sfmbt2* lacks a DMR [22], but possesses a large block of miRNAs in intron 10.

Peg10 is a retrotransposon-derived gene on proximal Chr. 6 that is paternally expressed and regulated by a maternal gDMR. Its loss results in severe reduction of the labyrinthine layer and almost complete loss of the spongiotrophoblast layer of the placenta by early mid-gestation. PEG10 protein may be involved in protection against apoptosis [38]. Placenta dysfunction is accompanied by embryonic developmental arrest and death at mid-gestation [39]. *Peg10* is one of the earliest imprinted genes to arise during mammalian evolution [40] and is an example of retrotransposon-driven imprinting. It anchors a domain that contains six additional imprinted genes.

Mest is a paternally expressed epoxide hydrolase gene on Chr. 6 that is regulated by a maternal gDMR and anchors a domain containing four additional genes, one of which is an ncRNA. *Mest* is expressed in embryonic and extraembryonic mesoderm (e.g., allantois); its loss results in reduced growth of both the fetus and the placenta and in behavioral defects in females inheriting a paternal null

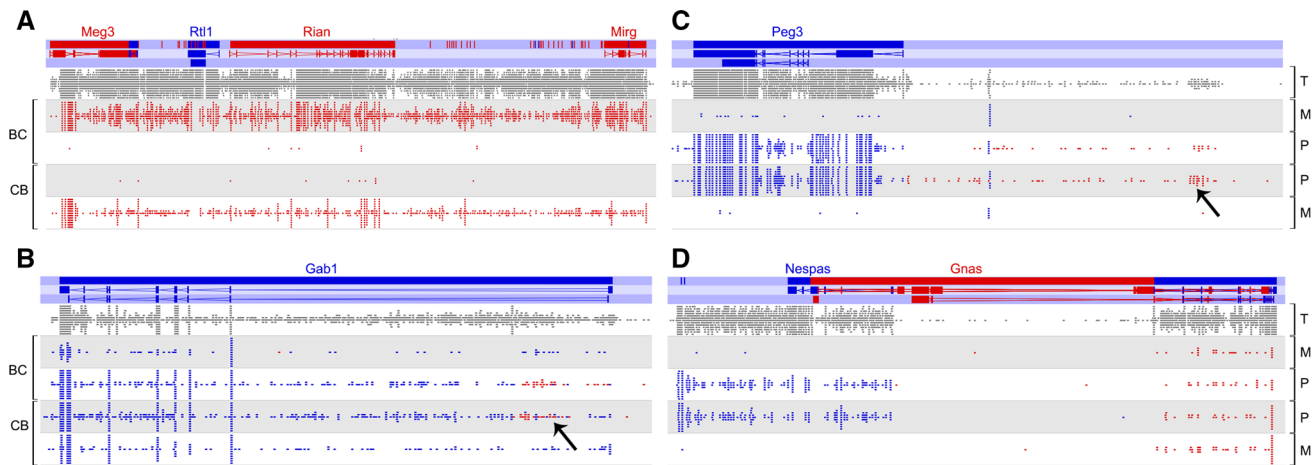


Fig. 2 TS cell RNA-seq allele-specific expression. RNA-seq SNP specific reads from Calabrese et al. [34] are displayed using SeqMonk Mapped Sequence Data Analyser (<http://www.bioinformatics.babraham.ac.uk/projects>), version 0.24.1, onto the NCBIM37 genome. It should be noted that there is variability among TS cell lines in expression of genes, including imprinted genes [29]. Forward strand is red; reverse strand is blue. Arrows highlight unannotated antisense transcripts. The top two tracks of each panel are from C57BL/6 X Cast

TS cells (BC); the bottom two tracks are from Cast X C57BL/6 TS cells (CB); T total, M maternal, P paternal. **a** *Meg3* to *Mirg* interval, including all of the miRNAs at the locus. Note the lack of expression of *Rtl1*. **b** *Gab1* is biallelic except for an unannotated antisense transcript that overlaps the region assayed by Okae et al. [31]. **c** *Peg3*. **d** *Gnas* locus. Images are screenshots of SeqMonk output and are not to scale

allele [41]. *Mest* harbors an intronic mir-335, which has been linked to mesendoderm development through targeting of several transcription factors [42]. While the *Mest* mutant allele, a reporter construct inserted downstream of mir-335, has the potential to express mir-335, it is unknown whether the miRNA is processed from the mutant transcript. Unpublished data from the Lefebvre laboratory indicates that mir-335 is processed from the mutant allele, but not at the same levels as the wild-type allele (L. Lefebvre, personal communication).

Peg3 is a paternally expressed Zn finger gene regulated by a maternal gDMR and anchoring a domain containing five additional genes, one of which is a paternally expressed ncRNA anti-sense to *Peg3*; a second antisense transcript can be seen in the 3' flank of *Peg3* in the RNA-seq data from TS cells that is likely orthologous with the imprinted human ncRNA MIMT1 [34], (Fig. 2c). While the authors of the knockout paper focused on behavioral defects in females inheriting a paternal null allele, they note in the footnotes that placentae are 30 % smaller, suggesting that *Peg3* is involved in placenta growth/function [43]. It is expressed robustly in TS cells.

H19 is a maternally expressed ncRNA that harbors a miRNA now known to regulate placenta growth and development [44]; loss of *H19*/mir-675 results in placentomegaly. *H19* is regulated by a paternal intergenic gDMR that is thought to act as a boundary element through the activity of CTCF binding. Knockdown of CTCF in oocytes results in methylation of maternal DMR CpGs, suggesting that methylation is the default state for this

element [18]. The *H19* DMR anchors an imprinted domain consisting of four additional imprinted genes, including another ncRNA. An earlier study of *H19* reported increased weight of neonates, but did not examine the placenta [45]; re-examination of this mutant line revealed placentomegaly [44], illustrating the utility of thorough analysis.

Igf2 is a paternally expressed growth factor that is reciprocally regulated by the *H19* intergenic DMR on Chr. 7 [46]. Loss of a placenta-specific alternative transcript (P0) results in both placental and fetal growth restriction; this mirrors the phenotype described for the original knockout, suggesting that the placental function of *Igf2* is the critical factor in how the gene regulates fetal growth [36].

Ascl2 (*Mash2*) is a maternally expressed transcription factor gene on distal Chr. 7 that is required for the development of the spongiotrophoblast layer of the placenta. Embryos inheriting a mutant allele from their mothers cease development and die at mid-gestation [27]. *Ascl2* is regulated by the *Kcnqlot1* maternal gDMR which controls imprinting of a cluster of nine genes.

Kcnqlot1 is a paternally expressed ncRNA in the intron of the *Kcnql* gene. Its promoter comprises the maternal gDMR that regulates the domain [47]. Fetuses inheriting a deletion of the *Kcnqlot1* DMR from their fathers are growth restricted [47]; this is reflected by growth restriction of the placenta [48], thought to reflect loss of imprinting/overexpression of maternally expressed genes in the cluster, such as *Phlda2* and *Cdkn1c*. A transcription termination mutation at *Kcnqlot1* that results in partial loss

of imprinting (LOI) of *Cdkn1c* in placenta but not somatic tissues produces a milder growth retardation phenotype [49]. However, no histological analysis of mutant placentas was undertaken in either the complete or partial *Kcnq1ot1* mutants [47, 49]. Interestingly, the phenotype of the *Kcnq1* mutant—recessive deafness—suggests that the function of this gene is unaffected by imprinting [50].

Cdkn1c is a maternally expressed cell cycle protein gene that is regulated by the *Kcnq1ot1* gDMR. Maternal loss of function is generally lethal, although on a mixed genetic background growth-restricted mutant pups can survive [51]. Mutant placentas exhibit placentomegaly [52], and females pregnant with *Cdkn1c* mutant embryos show symptoms of pre-eclampsia [53].

Phlda2 encodes a pleckstrin homology domain protein that is maternally expressed; it is regulated by the *Kcnq1ot1* gDMR and is one of the genes responsible for the *Kcnq1ot1* deletion phenotype. Loss of *Phlda2* results in placentomegaly, with no effect on fetal growth or survival [54].

Plagl1 encodes a paternally expressed Zn finger protein and at least two ncRNAs of unknown function [55]. It is regulated by a maternal gDMR that spans its transcriptional start site (TSS) [56] and anchors a domain with one other gene. Loss of *Plagl1* results in IUGR up to 25 % and, while the authors claim there is minimal effect on the placenta, the data in the supplementary files indicate reduced placental weights and significantly reduced labyrinthine layer development [57]. A more thorough analysis of placenta in *Plagl1* KO mice is warranted.

Grb10 encodes a maternally expressed pleckstrin homology domain protein that is regulated by a maternal gDMR near one of its TSS. Loss of maternal *Grb10* results in placenta and fetal overgrowth [58]. *Grb10* anchors a domain containing two other genes, one of which is a *Grb10* antisense ncRNA.

Dlk1 is part of the complex *Dlk1* imprinted domain that is regulated by the paternal intergenic gDMR. *Dlk1* encodes a paternally expressed Delta-like protein, containing EGF domains. Its loss results in IUGR with reduced and abnormal labyrinth layer, although with no change in placenta weight. Conditional knockouts in pancreas, pituitary and endothelial cells were normal [59].

Rtl1 is another retrotransposon-derived gene that is expressed paternally. It is part of the complex *Dlk1* locus on Chr. 12 that contains several protein-coding genes as well as numerous ncRNAs and large blocks of miRNA and SnoRNA genes, all of which are expressed in TS cells (Fig. 2a). One of the ncRNAs—*Rtl1AS*—antisense to *Rtl1*, is maternally expressed and encodes several miRNAs that directly target *Rtl1* for RNAi-mediated repression. Deletion of part of the *Rtl1* coding sequence simultaneously deletes both *Rtl1* sequence and some of the miRNAs encoded by

Rtl1AS. Paternal transmission of this deletion, which generates *Rtl1* null embryos, results in IUGR and placental infarcts in the labyrinth due to excessive phagocytosis of the fetal endothelial cells that normally express *Rtl1*; maternal transmission, which generates *Rtl1* overexpressing embryos, results in placentomegaly but normal fetal growth [60]. While the function of RTL1 protein remains to be established, the phenotype is suggestive of a role in protection against cell death, possibly similar to PEG10.

Igf2r is a maternally expressed *Igf2* receptor that is thought to act as a sink for *Igf2*. It is regulated by the maternal gDMR that overlaps the promoter of the antisense ncRNA *Airn*, whose transcription through the *Igf2r* TSS represses *Igf2r* transcription [61]. Maternal transmission of a null allele results in placentomegaly and fetal overgrowth [62].

Potential placenta function

Several imprinted genes result in phenotypes that are likely to involve the placenta, but that were never analyzed thoroughly for placental function. For example, the *Snrpn* locus is of great interest because of the link with Prader Willi syndrome/Angelman syndrome (PWS/AS) in humans. PWS is characterized by IUGR, neonatal respiratory distress and later developmental abnormalities, including neurological impairment and eating disorders, which likely have a root cause in defects of neural development during gestation. The *Snrpn* locus comprises several protein-coding genes (*Ube3a*, *Snrpn*, *Ndn*, *Mkrn3*, *Magel2* and *Peg12*) and a large block of SnoRNAs encoded by several long ncRNAs. The mouse locus also harbors a block of rodent-specific miRNAs. Imprinting of the locus is regulated by a maternal gDMR near the *Snrpn* TSS. Intragenic knockout of *Snrpn* is without phenotypic consequence [63]; however, deletion of the gDMR results in IUGR and neonatal lethality, and is associated with loss of expression of several genes in the cluster. This phenotype is mimicked in part by targeted deletion of *Ndn* [64]. Deletion of one of the SnoRNA clusters (SNORD116/MBII-85) results in neonatal growth restriction that persisted into postnatal life; the authors reported no effect on placenta weight, but did not examine the histology [65]. It should be noted that the *Dlk1* knockout results in abnormal placentas with normal weight.

Another locus that warrants closer examination of placenta function is the complex *GNAS* suite of genes. The *Gnas* locus generates multiple transcripts that encode different proteins or are non-coding; some (*Gnas-XL*, *Gnas-exon1A*, *NespAS*) are expressed from the paternal allele, some (*Nesp55*) are expressed from the maternal allele and others (*Gnas*) are biallelic in some tissues and monoallelic in others. The *NespAS* ncRNA may encode two miRNAs;

NespAS reads in the TS cell RNA-seq dataset extend down to the region of the two miRNAs [34] (Fig. 2d). Inheritance of a paternal null allele of *Gnas-XL* results in pups with morphological body-type defects that fail to thrive and die in early postnatal stages [66]; these defects clearly arose during development as they were present at birth. Inheritance of a maternal deletion of *Nesp55* leads to IUGR with edema [67], a phenotype that is similar to hydrops fetalis in humans, which can be associated with placental dysfunction [68–70]. Paternal transmission of a deletion of the gDMR associated with *NespAS* results in partial LOI at the locus and generates a phenotype similar to *GnasXL* mutants. Mutations in *Gnas* result in phenotypes that differ depending on the direction of transmission: maternal inheritance results in edema, wide bodies and failure to thrive, while paternal inheritance results in low birth weight, narrow bodies and failure to thrive. The edema and the lower birth weights are symptoms associated with dysfunctional placentas, although Okae et al. [31] reported biallelic expression of *Gnas* in whole transcriptome analysis of dissected placentas.

Finally, *Airn* deletion results in biallelic expression of *Igf2r* [71]. Fetal weights were reduced, but placental weights were unaffected, leading the authors to conclude there was no link between placenta function and fetal weight. However, the *Airn* deletion rescues maternal deletion of *Igf2r*, which is known to cause placentomegaly. It would be interesting to know whether placentomegaly is also rescued in this genetic system.

Evolutionary significance of imprinting

While many imprinted genes are either directly or potentially involved in placenta function, only five have imprinted functions that do not, and of these only one (*Rasgrf1*) represents a separate domain; the other four are located in clusters that are regulated by gDMRs anchored by genes that have demonstrated or potential placenta phenotypes. The genomic evidence suggests that imprinting starts out with a placenta-specific gene and then spreads to other genes in the locale [40], lending weight to the notion that imprinting arose to regulate placenta development [7, 8]. However, the dominant hypothesis in the field of genomic imprinting—the conflict hypothesis—still guides thinking among researchers [11]. How does the evidence stack up?

The conflict hypothesis in its simplest form posits that paternal genomes strive to increase fitness of their offspring by increasing their growth/size (“bigger is better”), while maternal genomes strive to spread the resources among all offspring by dampening growth. This has led investigators to analyze the effects of

imprinted genes on growth. The fact that paternally expressed genes are often found to be required for growth, as assessed by the effects of loss of function (LOF) mutations, is seen as strong evidence in support of the hypothesis. However, there is an internal flaw in this argument that is often overlooked, i.e., that mammalian development is accompanied by growth; LOF mutations in genes essential for development will, a priori, result in either arrest or delay in development and consequently in growth. The effect of paternal genes as regulators of development cannot be distinguished from any role in conflict-driven growth enhancement. This means that the flip side of the argument, the maternal growth-dampening effect, is the only means of assessing the validity of the conflict hypothesis. Of 27 maternally expressed genes for which LOF mutations are available (Table 1), only three fit the hypothesis: *H19*, *Grb10* and *Igf2r* (which, along with *Igf2*, inspired the conflict hypothesis more than 20 years ago). All three have compromised placentae associated with fetal overgrowth, so these examples also support hypotheses directed at maternal control of placentation. Placental defects do not always translate into fetal overgrowth; *Phlda2* and *RtlIAS* mutations both result in placentomegaly with no alteration in fetal growth; *Cdkn1c* results in fetal growth retardation with pathological effects on placenta, including evidence of pre-eclampsia; *Nesp55* KO results in edema, thought to be a result of placental dysfunction. The evidence is inconclusive regarding the validity of the conflict hypothesis. However, the support for hypotheses directed at maternal control of placentation driving genomic imprinting, while strong, is not overwhelming. A glance at the map of imprinted domains illustrates that more than half do not seem to be doing much of anything (Fig. 1; Table 1). Why, therefore, are they imprinted? Part of the answer to this question may come from analyses of the mechanisms of imprinting.

Mechanism matters

Genomic imprinting is a gene-silencing phenomenon. Research over the past 30 years has revealed the great depth and breadth of epigenetic regulation in nature. In mammals, gene silencing is accomplished by a range of mechanisms that include DNA methylation, histone modifications, chromatin protein associations and RNA-based transcriptional and post-transcriptional gene silencing. All of these mechanisms have been co-opted to mediate genomic imprinting [69, 70].

The differentiation between the two parental genomes must take place while they are apart. The obvious place is in the germline. Significant effort has been expended on

assessing the molecular differences between oocyte and sperm genomes, in spite of technical challenges. Until new tools became available, older technology limited analysis to DNA methylation. Consequently, there is a wealth of data on differential methylation between the maternal and paternal genomes. Exhaustive searches near imprinted genes have revealed that DMRs can be either germline or somatic, with the latter arising after fertilization and probably reflecting chromatin remodeling during development. Germline DMRs become methylated during germ cell development [72, 73]; spermatogonia acquire DNA methylation imprints during embryogenesis and before the onset of meiosis, while oocytes acquire their methylated DMRs during postnatal oocyte maturation. The number of imprinted genes is very small, so the results from a recent methylome analysis revealing the presence of several thousand germline DMRs that survived to the blastocyst stage was quite surprising [74]. They were found on every chromosome, including those shown to not harbor imprinted genes with a developmental function, suggesting that many may be ephemeral. It is tempting to speculate that roughly 30 genes that display no imprinted function retain their imprints, because there is no need to remodel them, except perhaps in select tissues. Also, striking from the data in this study were two other observations: sperm DNA is very heavily methylated everywhere, and both oocyte and sperm DNA have some mechanism that protects CpG islands from methylation. The rare exceptions are germline DMRs.

The heavy methylation of the paternal genome is likely necessary to aid the tight packaging required to fit the DNA into a tiny sperm head. We now know that this methylation is largely erased shortly after fertilization by maternal TET3 [75, 76]. The burning question is how the gDMRs escape the remodeling that occurs during the first cell cycle [77]. One likely mechanism is protection by other factors, such as ZFP57, which has been shown to bind to methylated gDMRs containing the consensus hexanucleotide sequence (TGCCGC) [78]. Maternal loss of *Zfp57* results in embryonic failure due to LOI [79]. Another maternal protection factor is DPPA3/PGC7, which has been shown to bind DMRs where it inhibits TET3-mediated demethylation [76, 80]. Maternal DPPA3/PGC7 binds H3K9Me2 in the maternal pronucleus; depletion of H3K9Me2 leads to loss of both DPPA3/PGC7 and DNA methylation from the maternal pronucleus [76]. Interestingly, maternal LOF of DPPA3/PGC7 results in loss of methylation at both maternal and paternal gDMRs in zygotes [81].

Other maternal factors have been shown to play significant roles in both establishment and maintenance of gDMRs. Maternal effect mutations in several genes disrupt imprinting: *Dnmt1o* [82], *Dnmt3l* [83], *Dnmt3A* [84], *Pgc7* [81] and to a lesser extent *Zfp57* [79]. In other species,

maternal effect mutations in human *NLRP7* and *KHDC3L* result in biparental hydatidiform molar pregnancies as a result of catastrophic loss of imprinting in zygotes [85, 86]; interspecific hybrids involving *Peromyscus* (deer mice) result in placental defects related to imprinting dysregulation that can be traced to maternal effect genes [87].

Not all imprinted genes possess a gDMR (*Sfmbt2*, [22]), or are sensitive to loss of maintenance methylation (*Ascl2*, [88]). This suggests that other epigenetic marks regulate imprinted expression. Reports of chromatin marks such as H3K4Me2, H3K9Me2 and H3K27Me3 at imprinted loci at the *Kcnq1* locus in placenta are difficult to interpret, given that half of the genes tested are likely not imprinted [26, 89]; the same technical problem with ChIP in placenta as was demonstrated for expression [31]—contamination with maternal tissues—confounds interpretation of results. De novo methylation may also play a minimal role in placenta-specific imprinting. Maternal loss of *Dnmt3l* [90], while lethal, has only subtle effects on placenta development. It does not preclude establishment/maintenance of TS cells, in spite of widespread loss of methylation at gDMRs [83]; *Sfmbt2*, required for TS cell maintenance [29], retains imprinted expression in *Dnmt3l* and *Dnmt3a* mutants [31].

Finally, the role played by ncRNAs in imprinting at some loci (e.g., *Airn/Igf2r* [61]; *Kcnq1ot1/Kcnq1* [49]) and by oocyte transcription across gDMRs [91] suggests that some aspect of RNA biochemistry may be involved in silencing genes/domains in the maternal germline. The emerging picture points to initial marks on both maternal and paternal genomes being selectively maintained by the zygote after fertilization. The second burning question is: “Why those particular sites?”

When things go wrong

The consequences of LOI can be devastating. BWS, PWS/AS and Russell Silver syndrome (RSS) diseases are, in many cases, caused by LOI. In some BWS and RSS patients, global LOI is seen at multiple imprinted loci, suggesting that an early breakdown in the imprinting machinery has occurred [92–97], sometimes associated with conception via assisted reproductive technology (ART). A similar breakdown is thought to underly familial hydatidiform moles, caused by maternal effect mutations in two genes—*NLRP7* and *KHDC3L*—and resulting in recurrent molar pregnancies [85, 86, 98]. Expression of both genes during oocyte development coincides with the timing of maternal gDMR methylation [73, 86, 99]. The connection between ART-induced LOI and maternal effect mutation-induced LOI strongly links oocyte health and proper imprinting. The ART studies also implicate the environment as a major factor in epigenetic health. Most

studies focusing on diet, especially folate metabolism, are limited to gestation and lactation (e.g., [100]), although there have been some recent studies of periconceptual maternal diet [101, 102], possibly in response to a study of the Dutch Famine cohort [103] which demonstrated a more dramatic effect of diet during an unspecified periconceptual period compared with late gestation. Given that gDMR methylation occurs during oocyte development, starting around the secondary follicle stage, the need to ensure good nutrition in girls and women starting in childhood to ensure a healthy next generation warrants closer investigation.

The third burning question is: “Why does LOI have such devastating effects?” The stock answer is that there is a need to regulate gene dosage (to fulfill the requirements of the conflict hypothesis). However, organisms have evolved much better mechanisms to regulate the levels of gene products over the past 4 or 5 billion years, and mammals make use of all options. For example, expression from one allele of *Sfmbt2* in extraembryonic tissues is about 50-fold higher than that from two alleles in somatic tissues [32]. Gene dosage is not a very satisfactory explanation. An alternative reason for the severe developmental abnormalities accruing from LOI is ectopic expression, either plus or minus, and spatial or temporal, or both, brought about by altered chromatin structure. Expression (or lack) of a key gene during development in cells where it is normally silent/active can wreak havoc. Thorough unbiased investigation of all tissues during development in imprinted gene mutations may yield some unexpected treasures.

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