Chromosome-wide identification of novel imprinted genes using microarrays and uniparental disomies

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ABSTRACT

Genomic imprinting refers to a specialized form of epigenetic gene regulation whereby the expression of a given allele is dictated by parental origin. Defining the extent and distribution of imprinting across genomes will be crucial for understanding the roles played by imprinting in normal mammalian growth and development. Using mice carrying uniparental disomies or duplications, microarray screening and stringent bioinformatics, we have developed the first large-scale tissue-specific screen for imprinted gene detection. We quantify the stringency of our methodology and relate it to previous non-tissue-specific large-scale studies. We report the identification in mouse of four brain-specific novel paternally expressed transcripts and an additional three genes that show maternal expression in the placenta. The regions of conserved linkage in the human genome are associated with the Prader–Willi Syndrome (PWS) and Beckwith–Wiedemann Syndrome (BWS) where imprinting is known to be a contributing factor. We conclude that large-scale systematic analyses of this genre are necessary for the full impact of genomic imprinting on mammalian gene expression and phenotype to be elucidated.

INTRODUCTION

Genomic imprinting refers to a specialized form of epigenetic gene regulation whereby the expression of a given allele is dictated by its maternal versus paternal origin. Imprinting plays a crucial role in mammalian reproduction and imposes an absolute requirement for maternal and paternal genomes for the generation of viable offspring (1,2). Approximately 80 imprinted genes have been identified in mouse (http://www.mgu.har.mrc.ac.uk/research/imprinted/), and around 40 in human (3). The extent of imprinting in mouse or human is not fully known, but has been estimated in mouse to range between 100 and 600 genes (4–6). Misregulation of the imprinted genes has been associated with growth and developmental abnormalities in mice (1,2,4), birth defects (7,8) and neoplasias in humans (9) as well as abnormalities in cloned mammals (10,11).

The rationale for the identification of new imprinted genes is based on further understanding imprinted gene regulation and the genetic components of developmental phenotypes in the mouse and birth defects in humans, such as Prader–Willi Syndrome (PWS), Beckwith–Wiedemann Syndrome (BWS) and cancer. Imprinted genes are frequently associated with asynchronous DNA replication (12) and epigenetic control mechanisms that can extend over large genomic regions. Studying aspects of epigenetic regulation requires examination of the genomic environment, especially in cases of placenta-specific imprinting where evidence suggests that not only DNA methylation but also histone modification is important for imprinted gene expression (13,14). Thus an inventory of monoallelic expression of genes and transcripts in a given region is advantageous.

Imprinted genes are characteristically differentially expressed in mice with a maternally derived uniparental duplication or disomy (matUpDp/UpD) compared to mice where the same UpDp/UpD is of paternal origin (patUpDp/UpD). Ideally, expression of an imprinted gene transcribed from the maternally inherited allele will increase 2-fold in a matUpDp sample compared to wild-type (wt) and will be undetectable in a patUpDp sample, and vice versa. In contrast, the expression of a non-imprinted gene is not expected to differ. Strains of mice carrying reciprocal and Robertsonian translocation chromosomes have been used to produce progeny where both copies of a particular chromosome or chromosomal region have been inherited from only the mother or the father. Here, the reciprocal translocation mouse strain T65H (15) was used to generate progeny with UpDps of Chromosomes (Chrs) 7 and 11 either proximal

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or distal of the translocation breakpoint as described previously (16). These mice provide the basis for both the computational and molecular studies presented here. They are viable until birth, so that differential gene expression profiles over a range of different tissues were investigated. Robertsonian translocation mouse strains that generated UpDs of Chr 18 (17) and Chr 12 (18) were used to gain additional statistical power for the evaluation of this method.

To a limited degree, the effectiveness of this method has already been demonstrated by the identification of a novel brain-specific imprinted gene, *Inpp5f_v2* (19). Here, we report on the results of systematically refining our approach and extending its coverage to the whole of Chr 7 and Chr 11. We describe this methodology, quantitatively evaluate its effectiveness and compare it to previous non-tissue-specific whole genome studies, reviewed in (20). This study has identified and validated four novel brain-specific paternally expressed transcripts and three placenta-specific maternally expressed genes.

MATERIALS AND METHODS

Tissue sources

Mouse: the tissue sources for the T65H translocation strain are described in (16). Essentially, newborn mice with maternal and paternal duplications for specific regions of Chrs 7 and 11 were generated using the T65H translocation (21,22). The tissues selected are shown in Table 1 and include 13.5 dpc embryo and placenta, newborn brain, carcass, heart and liver for matUpDp prox 7 versus patUpDp prox 7 (the same samples also generate data for patUpDp prox 11 versus matUpDp prox 11). MatUpDp distal 7 samples were compared to normal sibling samples at 13.5 dpc since the patUpDp distal 7 embryo is not viable at this stage of development. 8.5dpc embryos with maternal versus paternal UpD for Chr 18 were also compared using a Robertsonian translocation Chr Rb(2.8)2Lub(7.18)9Lub or RB92.82Lub (R) and C57BL/6JEi-Rb(7.18)9Lub (B) strains obtained

from the Cytogenetic Models Resource at the Jackson Laboratory (17). Placentae were dissected free from the decidua. However, due to the structure of the placenta with invading maternal vessels, some maternal material is likely to be included in these preparations. 15.5 dpc embryos and placentae from Chr 12 UpD (18) were obtained in collaboration with Anne Ferguson-Smith. Human: for *DHCR7* and *AMPD3*, anonymous placenta DNA and RNA samples were collected in collaboration with Dr B.S. Emanuel (CHOP) in accordance with ethical guidelines.

Microarray protocols

Affymetrix Genechips™ U74v2 and 430v2 microarrays were used (Table 1). The U74v2 triple array series represents ~36 000 genes and ESTs. The U74v2 series represents all sequences (~6 000) in the Mouse UniGene database (Build 74) that had been functionally characterized at the time plus EST clusters. The 430v2 dual array series probes ~39 000 genes and ESTs where the 430Av2 array predominantly represents well-characterized genes.

Caesium chloride isolated total RNA from samples in Table 1 were quantified on an Agilent Bioanalyser™ and 5–7 µg was used to prepare biotin-labelled cRNA target essentially as in the Affymetrix™ expression manual (23). Biotinylated cRNA targets were purified using cRNA Cleanup spin columns (Affymetrix™), fragmented in 5× fragmentation buffer (Affymetrix™), and quantified prior to hybridization on an Agilent Bioanalyser™. A total of 0.15 µg of labelled probe was hybridized per GeneChip expression array followed by washing and staining on the Affymetrix™ fluidics station 450. An Affymetrix Scanner 3000 was used to quantify the signal.

Gene chip operating software (GCOS) data analysis

Data from the scanner were analysed using the Affymetrix™ MASv5 or GCOS software. The software computes the signal for each pair of corresponding PM and MM probes in a probe set as $S_i = \log_2(PM_i) - \log_2(MM_i)$ where PM_i and MM_i are the measured fluorescence levels of the *i*th probes in the PM and MM sets. In cases where S_i would barely exceed the background noise level or be negative, GCOS makes adjustments to ensure a conservative and positive estimate of gene-specific hybridization. GCOS arrives at a single signal value (S) for each probe set by calculating the Tukey-biweight of the S_i, a weighted outlier-resistant average. GCOS reports 2^S as the estimate of the absolute expression level of the gene represented by the probe set. GCOS allows a comparative analysis of gene expression in two samples, each used to hybridize a separate microarray. GCOS computes a signal $\log_2$-ratio (SLR) for each probe set as a measure of the degree of differential expression between the two samples and a *P*-value as a measure of confidence in any measured difference in expression. In the most common case, the SLR equals the Tukey-biweight over *i* of $\log_2({}_APM_i) - \log_2({}_AMM_i) - [\log_2({}_BPM_i) - \log_2({}_BMM_i)]$; where ${}_APM_i$, e.g., is the measured fluorescence of the *i*th PM probe in a particular probe set on array A (hybridized with sample A). The *P*-value is based on a Wilcoxon signed rank test applied to the pairs of differences $[\log_2({}_APM_i) - \log_2({}_AMM_i), \log_2({}_BPM_i) - \log_2({}_BMM_i)]$ and $[\log_2({}_APM_i) - A, \log_2({}_BPM_i) - B]$, where

Table 1. Overview of the microarray data used in this study

UpDp/UpD	Microarray Series	Model	Tissue Pl	Em	Cc	Br	Lr	Ht
Prox Chrs 7 and 11	Affymetrix™ 430v2	A		x		x		
		B		x		x		
	Affymetrix™ U74v2	A	x		x		x	x
		B	x		x		x	x
		C	x		x		x	
	Incyte™ GEM 1		x				x	x
Dist Chrs 7 and 11	Affymetrix™ U74v2	A	x	x				
		B	x	x				
		C	x	x				
Chr 12	Affymetrix™ U74v2	A	x	x				
		B	x	x				
		C	x	x				
Chr 18	Affymetrix™ 430v2	A		x				
		B		x				

An 'x' indicates the availability of data for a specific combination of UpDp/UpD, microarray and tissue (Pl = placenta, Em = embryo, Cc = carcass, Br = brain, Lv = liver and Ht = heart). Note that for the experiment using a prox Chrs 7 and 11 sample of heart, there was only enough material to hybridize the U74v2 A and B arrays.

A and B are measures of the background noise of the respective array.

Expression analysis

Two assays were used; the first used cDNA prepared from maternal and paternal UpDp and wt RNA samples in RT–PCR assays. Briefly, 4 μg of total RNA was reverse transcribed using Superscript III (Invitrogen™) reverse transcriptase (manufacturers' instructions) and PCR-amplified using ABgene™ Reddymix and gene-specific primers (Supplementary Table S4). The second method used inter-subspecies hybrid RNA as described in (19). Essentially, single nucleotide polymorphisms (SNPs) between *Mus m. musculus* [C57Bl6J (B6)] and *Mus m. castaneus* (CAST) strains were identified and assayed by DNA sequencing (primers are listed in Supplementary Table S4). RT–PCR followed by sequencing identified the expressing allele(s) for each gene tested (Figures 2 and 3). Sequencing was performed on an ABI 3730 DNA analyser using ABI BigDye™ reagents (manufacturer's protocols). Fetal or parental human DNAs were sequenced for expressed SNPs, which were assayed as described above.

Gene prediction programs

Gene predictions tools used include Ensembl (24–26). SGP (27), Geneid (28), Genscan (29), Superfamily/SCOP (30), Twinscan (31) and Augustus (32).

RESULTS

The 'one chromosome at a time' approach excludes most false positives

Each of the microarrays used here measures the expression of thousands of distinct transcripts, all of which can potentially change in expression levels between the respective matUpDp/UpD and patUpDp/UpDs. However, most transcripts can be ruled out based on their location within the genome that places them outside the selected chromosomal duplication or disomy.

Four chromosome anomalies were investigated (Table 1). Filtering by genomic position reduced the number of potential false positives by between 85 and 94% (Table 2). For example, of the 45 037 distinct non-control probe sets, representing over 39 000 transcripts, on the Affymetrix™ 430v2 arrays, only 6595 (14.6%) generate a match to proximal Chrs 7 or 11 when their target sequences are aligned with the genome (March 2005 NCBI build 34) using BLAT (33). The remaining 38 442 probe sets do not pass the henceforth called 'map filter' for this UpDp and thus can be excluded from the search. On average, about a tenth of the transcripts represented on the microarrays mapped to the respective region of UpDp/UpD in our experiments and could legitimately change in expression between the matUpDp/UpD and pat UpDp/UpD samples as a direct result of being imprinted. This reduction of potential false positives by an order of magnitude compared to the analogous whole genome approach (parthenogenote versus androgenote), is compounded by the more limited downstream effects of UpDp/UpDs.

Gains in sensitivity by combining differential expression measures

Arrays hybridized with the matUpDp/UpD sample were compared to arrays hybridized with the patUpDp/UpD sample using the gene operating software (GCOS) that provides two measures of differential expression (34). The SLR of a probe set captures the fold change in expression of the represented transcript between the two samples, i.e., in simplified terms, $SLR = \log_2(\text{expression in matUpDp/UpD} / \text{expression in patUpDp/UpD})$. In addition, GCOS supplies a *P*-value that expresses the confidence in and direction of any measured expression difference, that is, $P = 0.5$ if there is no evidence for a change in expression, and *P* is the closer to 0 or 1 the more statistical evidence there is for an increase or decrease in expression, respectively.

SLR and *P*-value are closely correlated but non-redundant measures. Frequently, a probe set's absolute SLR (|SLR|) is large while $0.5 - |P - 0.5| \approx 0.5$, meaning that while a large fold change is measured, there is little statistical evidence for differential expression. The converse happens similarly frequently. Which of the two measures more truthfully represents the actual degree of differential expression is not immediately clear. Previously, SLR and *P*-value have been shown to give qualitatively different results (35). Here, we systematically evaluated the performance of SLR, *P*-value and combinations of both, namely, *P*-value-weighted SLRs (xSLR), in the context of imprinted gene detection. Specifically, $xSLR = 0$ if $0.5 - |P - 0.5| \geq x$, and otherwise, $xSLR = [1 - 1/x (0.5 - |P - 0.5|)] SLR$. Within the interval

Table 2. For each combination of microarray series, UpDp/UpD and tissue (Pl = placenta, Em = embryo, Cc = carcass, Br = brain, Lv = liver and Ht = heart) used in this study, the table shows the number of distinct non-control probe sets detecting differential expression that map to within (normal script) versus to only outside (*italic* script) the UpDp/UpD

UpDp/UpD: Array series	Prox Chrs 7 and 11 Number of distinct non-control probe sets								Dist Chrs 7 and 11			Chr 12			Chr 18	
		Mapping to within (normal) verus to only outside (*italic*) the UpDp/UpD														
	Total	Total	luSLR| ≥ 6						Total	luSLR| ≥ 6		Total	luSLR| ≥ 6		Total	luSLR| ≥ 6
			Pl	Em	Cc	Br	Lv	Ht		Pl	Em		Pl	Em		Em
430	45037	6595		222		212									3852	245
		38442		*1321*		*1084*									*41185*	*2246*
U74	36701	3679	161		305		325	125	3157	107	101	2240	58	267		
		33022	*1227*		*2451*		*2650*	*854*	*33544*	*1128*	*1321*	*34461*	*603*	*4369*		

The table also provides the total number of non-control probe sets per microarray series and, for each UpDp/UpD in turn, the total number of probe sets that map to within versus to only outside the UpDp/UpD.
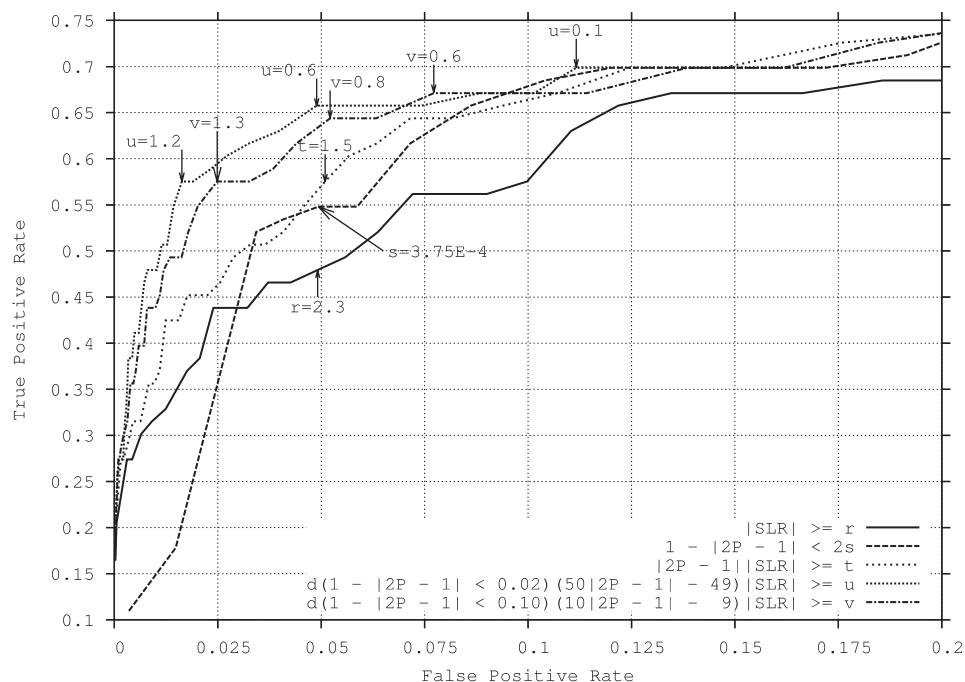
Figure 1. ROC curves for the SLR (solid), *P*-value (dashed), pSLR (finely dashed), uSLR (dotted) and vSLR (dash-dotted) differential expression measures. Each curve shows the TPR *y*-axis in relation to the FPR *x*-axis, estimated at various different decision thresholds for the respective measure and linearly interpolated in between the estimates. Labelled arrows point out thresholds of particular importance, specifically, the thresholds at which the estimated FPR was roughly 5% for all five measures, and/or, for uSLR and vSLR, thresholds that marked the end of steep increases in the TPR and therefore constituted particularly good trade-offs between TPR and FPR. The legend in the figure states for each measure the condition that a probe set needed to satisfy in order to be considered differentially expressed where r, s, t, u and v are the decision thresholds, and P and SLR are the *P*-value and SLR as computed by GCOS. d denotes Kronecker-δ, that equals 1 if the boolean expression in parenthesis (in our case unequality) is true, and that equals 0 otherwise. The uSLR consistently achieved the highest TPR for up to an FPR of 5%, closely followed by vSLR. Most significantly, *P*-value and SLR in isolation performed much worse as measures of differential expression than when combined in measures like uSLR.

$0 \leqslant 0.5 - |P - 0.5| \leqslant x$, xSLR linearly changes from SLR to zero where it remains at all lower levels of statistical confidence. We explored the whole range of possible values for x, i.e. $0 < x \leqslant 0.5$, and found x = 0.01 (uSLR) to perform best, closely followed by x = 0.05 (vSLR). The simple linear weighing of SLR over the whole range of *P*-values, (x = 0.5; pSLR) was not nearly as effective.

For each measure, we first estimated from our microarray data, the true positive rate (TPR) and false positive rate (FPR) for a range of differential expression thresholds. The TPR was estimated from a total of 43 known imprinted genes that map to within the investigated UpDp/UpDs and for which there are representative probe sets. For each tissue, we counted (a) the number of genes within the respective UpDp/UpD that the literature has reported to be imprinted in the tissue and for which tissue-matched array data were available and (b) the number of such genes for which in addition, at least one representative probe set detected an increase (for maternally expressed genes) or decrease (for paternally expressed genes) in expression that met or exceeded the differential expression threshold. The TPR was computed as the ratio between the two sums over all tissues of counts (b) and (a). The FPR is the fraction of probe sets mapping to the investigated UpDp/UpDs that do not represent known imprinted genes but nevertheless met or exceeded the differential expression threshold. Plotting the FPR versus the TPR over the range of decision thresholds yields a receiver operating characteristics (ROC) curve shown for SLR, *P*-value, uSLR, vSLR and pSLR (Figure 1).

A low FPR is paramount to achieve a good cost to discovery ratio in the molecular validation. Even with the map filter, an FPR of 5% still corresponds to, in the proximal Chrs 7 and 11 region, roughly 300 distinct probe sets on the 430 array that will be flagged as representing differentially expressed sequences, but are unlikely to represent imprinted transcripts. We therefore chose 5% as the upper limit on the FPR. Up to this limit, uSLR consistently performed best, i.e. gave the highest TPR compared to the other measures that we considered. Consequently, the ROC curve for uSLR dominates the other curves in Figure 1 for an FPR ≤ 5%. Specifically, the differential expression threshold of |uSLR| ≥ 0.6 delivered a TPR = 65.8% and an FPR = 4.9% so that henceforth, 'differentially expressed' will imply |uSLR| ≥ 0.6.

The map filter saturates candidates with imprinted genes

To prioritize the molecular validation experiments, we ranked the probe sets passing the map filter by degree of differential expression, i.e. in descending order of |uSLR|. On average, 19% (29%, 38%, 46% and 52%) of all genes known to be imprinted in the respective tissue and mapping to within the respective duplication were ranked among the top 10 (20, 50, 100 and 200). Compared to the analogous ranking of all probe sets, i.e. without the prior application of the

map filter, this corresponds to an average increase in the number of known imprinted genes in the top 10 (20, 50, 100 and 200) ranks by 85% (71%, 57%, 61% and 48%). Table 3 provides the absolute values. This illustrates how the map filter increases the saturation of the top ranks with known imprinted genes and, by extrapolation, with likely truely imprinted candidate genes.

Summary statistics of probe sets representing differentially expressed sequences

Table 2 gives a complete account, broken down by UpDp/UpD, tissue and microarray series, of how many probe sets whose target sequences map to within versus to exclusively outside the UpDp/UpD detected differential expression. On average, 4.9% (5.3%) of the probe sets mapping to within (outside) the UpDp/UpD detected differential expression. The differences underlying these averages are not statistically significant ($P = .083$; Wilcoxon signed rank test), which suggests that proportionately and in terms of differential expression, a UpDp/UpD affects the region of duplication approximately as much as the rest of the genome.

Differential gene expression profiles across tissues

For the UpDp of proximal Chrs 7 and 11, we conducted separate microarray experiments using placenta, embryo, carcass, brain, liver and heart samples (Table 1). Thus, we created tissue-specific differential expression profiles. Only limited or no tissue-specific data were available for the distal UpDp of Chrs 7 and 11 and the UpDs of Chrs 12 (embryo and placenta) and 18 (embryo).

Profiles of 43 known imprinted genes on Chrs 7, 11, 18 and 12 are compared with the literature-reported imprinting status

Table 3. For each combination of tissue (Pl = placenta, Em = embryo, Cc = carcass, Br = brain, Lv = liver and Ht = heart) and microarray series, the table first shows the union of the respectively studied UpDp/UpDs, then the total number of known imprinted genes located within the corresponding genomic regions, and finally, the number of known imprinted genes for which probe sets ranked in the top 10, 20, 50, 100 and 200, respectively, when the probe sets on all arrays of the series were arranged in descending order of |uSLR|, with (normal script) and without (*italic* script) prior application of the map filter.

Tissue	Array series	Union of UpDp/UpDs	Total					
				10	20	50	100	200
Br	430	Prox Chrs 7 and 11	20	4	6	10	11	13
				3	*6*	*7*	*9*	*11*
Cc	U74	Prox Chrs 7 and 11	12	4	5	6	6	7
				2	*3*	*4*	*5*	*6*
Em	430	Prox Chrs 7 and 11, Chr 18	21	3	7	8	12	12
				3	*6*	*7*	*7*	*9*
	U74	Dist Chrs 7 and 11, Chr 12	19	3	4	4	5	6
				1	*1*	*2*	*3*	*3*
Ht	U74	Prox Chrs 7 and 11	10	4	5	5	6	6
				2	*3*	*4*	*5*	*5*
Lv	U74	Prox Chrs 7 and 11	12	2	2	3	5	5
				1	*1*	*2*	*2*	*3*
Pl	U74	Chrs 7 and 11, Chr 12	31	4	7	11	13	16
				1	*1*	*4*	*5*	*7*

in Supplementary Table S1. Profiles of the 59 genes on Chrs 7 and 11 that were not known to be imprinted and for which we conducted imprinting validation experiments are listed in Supplementary Table S2, where the results of the validation assays appear side-by-side with the microarray data. The profiles of all transcripts on proximal Chrs 7 and 11 (935 transcripts represented by 1705 probe sets) that were differentially expressed in at least one of the tissues are shown in Supplementary Table S3.

Confirmation of seven differentially expressed candidates

Allele-specific assays using UpDp/UpD RNAs have been used extensively to determine parent of origin specific gene expression (36), but could be argued not to be independent of the microarray assay since the starting material is the same (37). Interspecies hybrids have been used to assay for parent of origin specific expression (38–40) but loss of imprinting of some genes has been detected in hybrid crosses (41). So, where possible we have used more than one method. Moreover, we have tested known imprinted genes and have in every case confirmed imprinting status.

A set of 59 candidate genes with an unknown imprinting status were selected and initially screened in an RT–PCR approach using matUpDp versus patUpDp RNA templates (where available). Candidates showing evidence of parental origin specific expression in this assay on the proximal regions of mouse Chrs 7 were subjected to further validation using RT–PCR combined with a SNP allele-specific assay in mouse inter-sub-specific hybrids.

On proximal Chr 11 in whole embryo or isolated brain, eight candidate imprinted transcripts were tested for monoallelic expression by RT–PCR in mat versus patUpDp material (Supplementary Table S2), along with two control known imprinted genes, *U2af1-rs1* and *Grb10*. The control genes showed imprinted expression and six out of eight non-control genes were biallelic. *Pnpt1* was expressed in paternally duplicated samples only but was not present in wt, *Gabra1* showed some maternal bias, but this difference was not considered robust enough to warrant further study. *Lsm11* is located within band B1.1 of Chr 11 containing the T65H translocation breakpoint thus may have been duplicated in the distal rather than the proximal experiment. However, the distal microarray data did not suggest differential expression of *Lsm11*.

On proximal Chr 7, 25 transcripts were tested for monoallelic expression in brain, 17 in whole embryo, 5 in placenta and 2 in heart (Supplementary Table S2). By UpDp assay, 10 transcripts showed allele bias in brain and were tested further by SNP analysis, which confirmed that four of these (BB077283, BM117114, AK080843 and AV328498) were paternally expressed (Figure 2). BB182944 and BB312372 were also validated as paternally expressed but were subsequently found to correspond to the imprinted *Pec2* and *Pec3* transcripts (42) and were excluded. Of the remainder, *Ampd3* was maternally expressed in placenta. The placenta UpDp assay showed a maternal bias with some paternal expression (data not shown) rather than the exclusive maternal expression seen with the interspecies SNP assay (Figure 3). BB264453 was robustly differentially expressed
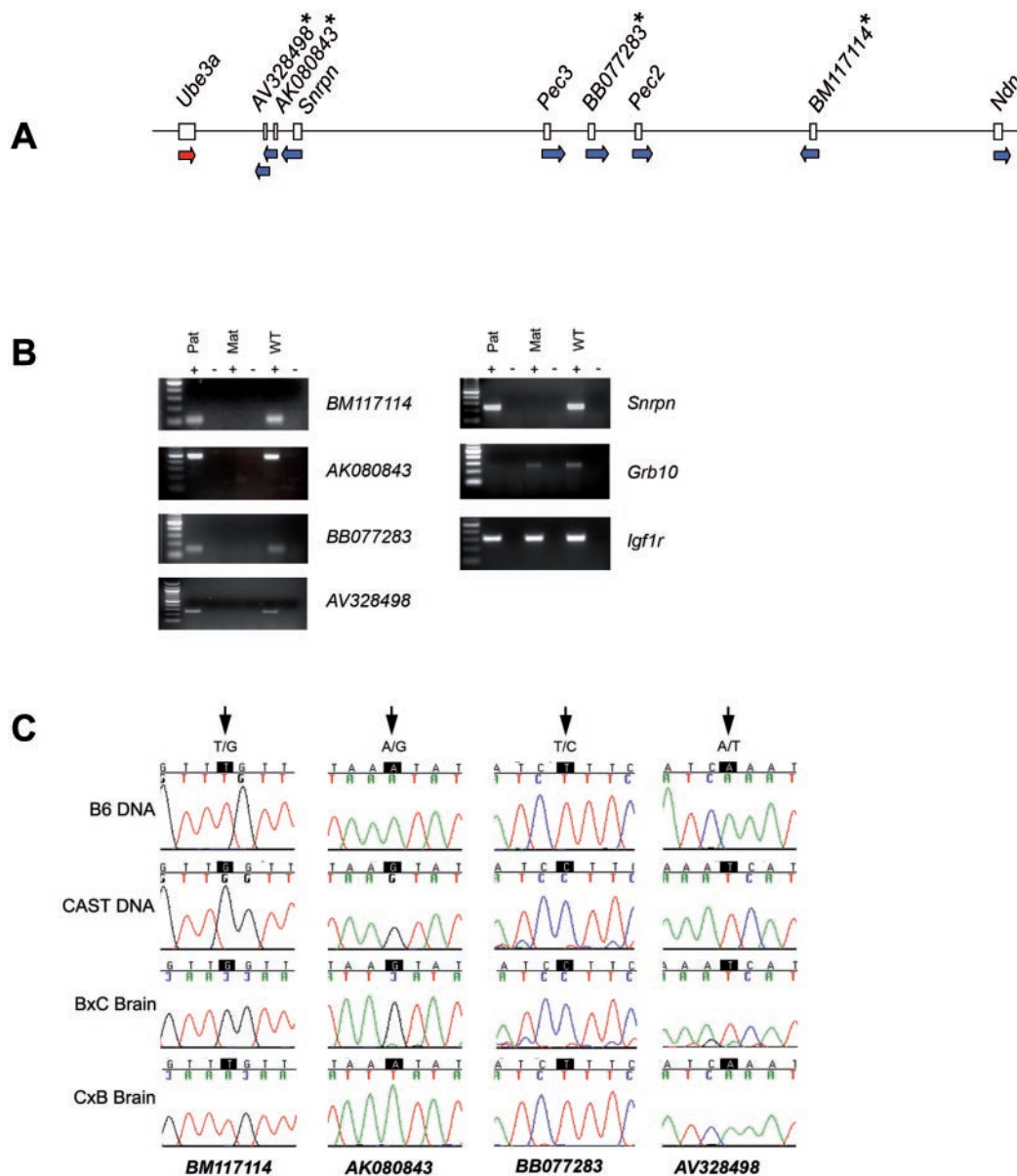
Figure 2. Identification of novel paternally expressed transcripts on mouse proximal Chr 7. (**A**) The 2 Mb PWS/AS orthologous region with positions of the novel paternally expressed transcripts indicated*. Genes are shown as open boxes with the relative transcriptional orientations defined by arrows. Blue, red or no colouring define paternal, maternal or biallelic expression respectively. (**B**) RT–PCR analysis of candidates identified on the proximal Chr 7 brain array in cDNA derived from patDp prox 7, matDp prox 7 and wild-type brain tissue. Controls for paternal (*Snrpn*), maternal (*Grb10*) and biallelic (*Igf1r*) expression are shown. The molecular weight marker is a 100 bp DNA ladder where the bright band corresponds to 500 bp. Samples treated with and without reverse transcriptase are indicated as + or −RT. ESTs *AK080843, BB077283, BM117114* and AV328498 were paternally expressed. (**C**) Allele-specific RT–PCR analysis of proximal Chr 7 candidates. Newborn brain tissues with expressed SNPs were obtained from reciprocal crosses between *M.m.musculus* (B6) and *M.m.castaneus* (CAST) animals. cDNA fragments containing the SNPs were recovered by RT–PCR and direct sequencing to determine allele-specific expression.

by RT–PCR in mat versus pat UpDp brain, but did not contain a SNP between B6 and cast and so was not considered further. The location of AI114950 within band F4 of Chr 7 makes it analogous to *Lsm11* and the distal microarray data did not suggest differential expression of AI114950. The remaining tested transcripts were biallelic by SNP assay. AK080843 and AV328498 are transcribed in the antisense orientation relative to *Ube3A* and could be part of the large imprinted antisense transcript LNCAT, (43,44) but

because the LNCAT cDNA is not clearly defined or publicly available, this is uncertain.

Ten transcripts were selected from distal mouse Chr 7 (Supplementary Table S2). Of these, *Th* and *Dhcr7* were maternally expressed in placenta (Figure 3) but were biallelic in embryo. These transcripts were not as robustly monoallelic as the novel brain transcripts in the proximal region, although the allele preferences exchanged with reversed parental transmission (Figure 3).
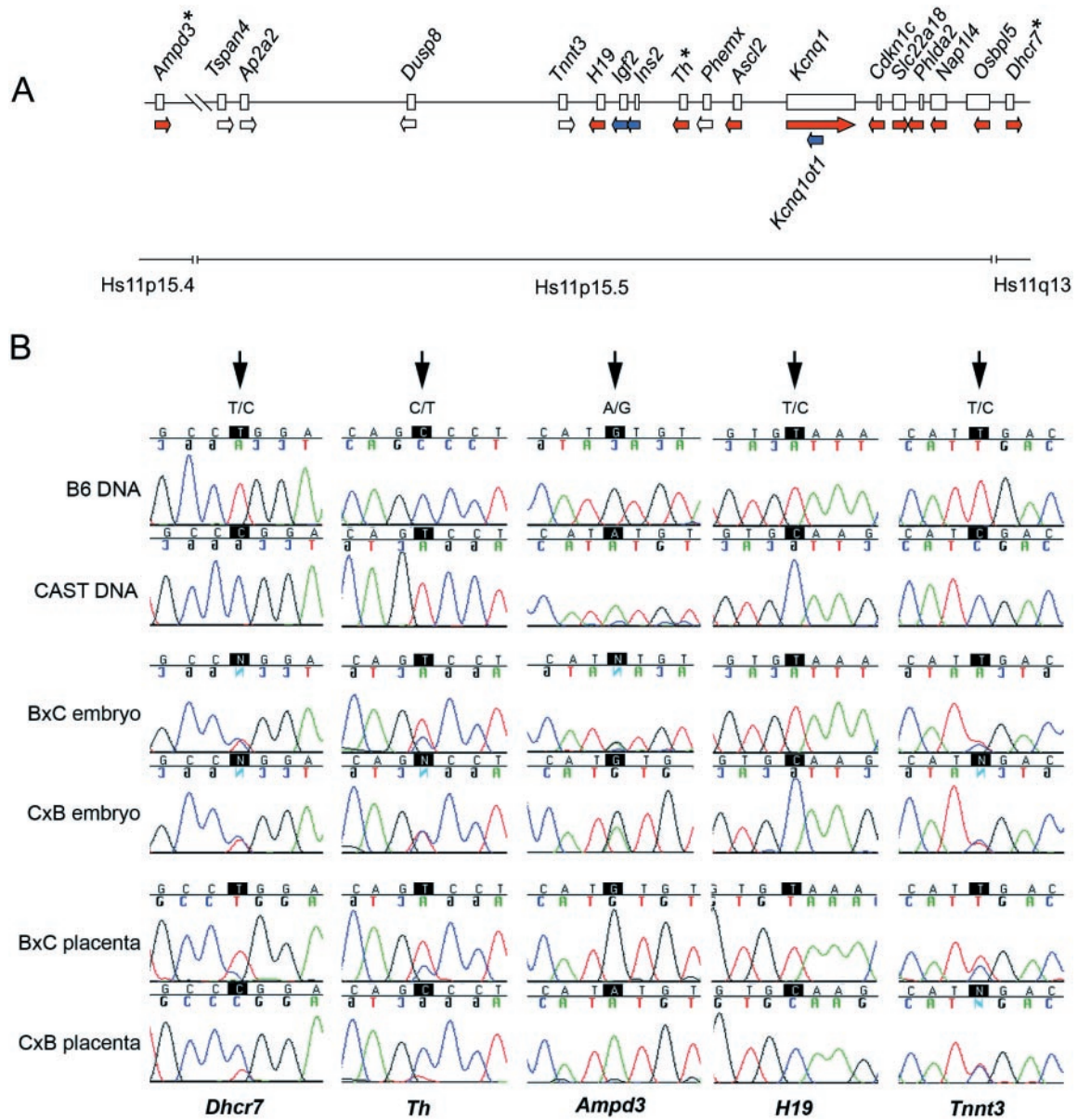
Figure 3. Identification of novel maternally expressed transcripts on mouse distal Chr 7. (**A**) The ~1 Mb BWS orthologous region shown with positions of the novel maternally expressed genes indicated*. Regions of conserved linkage on human chromosomes are indicated by horizontal bars. Genes are shown as open boxes with the relative transcriptional orientations defined by arrows. Blue, red or no colouring define paternal, maternal or biallelic expression respectively. (**B**) Allele-specific RT–PCR analysis of distal Chr 7 candidates. Embryo and placenta (E13.5) tissues with expressed SNPs were obtained by performing reciprocal crosses between *M.m.musculus* (B6) and *M.m.castaneus* (CAST) animals. cDNA fragments containing the SNPs were recovered by RT–PCR then direct sequenced to determine allele-specific expression. Distal Chr 7 candidates; *Dhcr7, Th* and *Ampd3* were maternally expressed in E13.5 placenta (lower panels) but biallelic in E13.5 embryo (middle panels). Ubiquitously imprinted (*H19*) and biallelic (*Tnnt3*) control genes are shown.

Verified imprinted ESTs: gene predictions and genomic context

Gene predictors were applied to the genomic regions containing the ESTs for which validation by RT–PCR and SNP analysis had confirmed imprinting (Figure 2). The genomic position of each EST was uniquely identifiable. In brain and embryo, the paternally expressed AK080843 and AV328498 map to a region between 6.7 and 13 kb centromeric of *Snrpn* (Figure 2A). Based on the genomic sequence of this region, no prediction program provided evidence for a separate gene or an extended transcript of *Snrpn*

whose coding region aligns with the ESTs. AV328498 is the 3′-read of the full insert sequence AK078094 that partially overlaps AK080843. Both AK078094 and AK080843 partially overlap BC070450, a transcript extending much further 3′. The distinct splicing patterns of these transcripts suggest that they might be alternative transcripts of the same transcriptional unit. Whether these transcripts are extensions of *Snrpn* is not completely clear but based on the ESTs in the region (including AK080843 and AV328498, AK078094), Ensembl predicts a gene (ENSMUSG00000016158) that is distinct from *Snrpn* at this location. To further ascertain whether transcripts AV328498 and AK080843 could splice
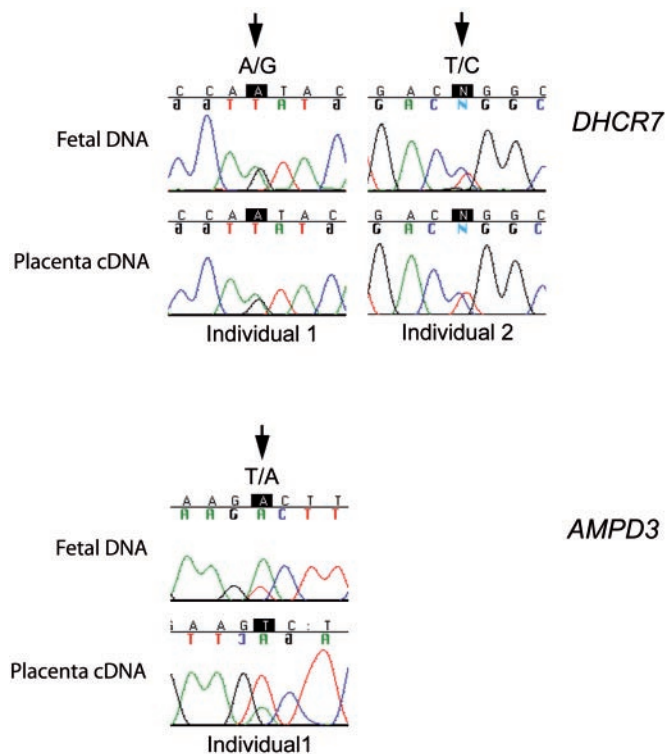
Figure 4. Imprinting analysis in human placenta. For *DHCR7* two distinct polymorphisms, a G/A SNP at nucleotide 364 and a known T/C SNP at nucleotide 382 (rs1790334) in the *DHCR7* cDNA were identified in two individuals by sequencing fetal DNA samples. For the *AMPD3* analysis a T/A SNP at nucleotide 3160 in the *AMPD3* cDNA was identified in one individual by sequencing fetal DNA samples. Allele-specific expression analysis in matched placenta cDNA showed the genes to be biallelic.

onto *Snrpn*, a *Snrpn* forward primer was combined with the AV328498 and AK080843 reverse primers (Supplementary Table S4, SNP assay primers) to amplify brain cDNA by RT–PCR. ABGene thermoprime plus *Taq* polymerase was used to amplify large products of up to 12 kb. No products were seen from any primer combination (data not shown). This provides empirical data to support these transcripts being independent of *Snrpn*, but they are limited in assaying the absence of a product. Similarly, no genes were predicted that coincide with BB077283 (corresponding 5′-read: BB625859) or BM117114 (no 5′-read available, but identical to BQ555876 with corresponding 5′-read BQ555877). These ESTs map telomeric of *Snrpn* and centromeric of *Ndn* (Figure 2A) and are located distant (1.8 Mb; BM117114 and 1.3 Mb; BB077283) from *Snrpn* and hence are not likely to be part of the *Snrpn* transcript.

Human orthologues of mouse distal Chromosome 7 genes are not maternally expressed in placenta

We examined the expression status of the human orthologues *DHCR7* and *AMPD3* in placenta using a combined RT–PCR and SNP assay essentially as performed in the mouse tissues. Both genes were biallelically expressed in human term placentae (Figure 4). The imprinting status of *TH* was not addressed, since informative polymorphisms could not be found in our sample sets. These genes may however be

imprinted in other non-tested human tissues since tissue-specific imprinting need not be the same between species (45).

Limited overlap with previous non-tissue-specific whole genome imprinting studies

A 2-fold change in expression was used as the cut-off for differential expression in RIKEN's FANTOM2 imprinting screen (46), which identified 2110 imprinting candidate transcripts. A large fraction of these transcripts were represented by probe sets on the Affymetrix™ arrays (430v2: 79%, U74v2: 65%), and between 17 (350) and 41% (870) of them were represented by probe sets mapping to one of the investigated UpDp/UpDs. Given that the above transcripts constitute imprinting candidates, we expected a large fraction of them to be represented by one or more differentially expressed probe sets in our experiments. However, at the most, this fraction was 45% (UpD of Chr 18), and across all UpDp/UpDs, tissues and microarray series', the average was 20.4%. The overlap between the RIKEN imprinting candidates and differentially expressed probe sets in our experiments increased when a less stringent differential expression threshold was used, permitting a higher FPR. For example, using $|pSLR| \geqslant 0.5$ with a TPR of 74% and an FPR of 21.1% (Figure 1), the overlap increases to 66.6% in the best case (UpD of Chr 18), and to 50.3% on average. This suggests that the $|uSLR| \geqslant 0.6$ is more stringent than the 2-fold change in expression threshold used in the RIKEN study.

Recent work that used bioinformatic methods predicted 600 out of 23 788 annotated (Ensembl) autosomal mouse genes to be imprinted based on their similarity to sequence features surrounding 44 known imprinted genes, and their dissimilarity to ~500 assumed non-imprinted genes (6). The TPR and FPR of the analysis, determined by cross-validation, was 100 and 7%, respectively. Using the same method, the authors also predicted allele preference, that cross-validation showed to be 97.7% accurate. We observed the largest overlap with our study on proximal Chrs 7 and 11 to which 27 of the 600 predicted imprinted genes mapped and were represented by probe sets on the Affymetrix arrays. For 10 of these genes there was at least one probe set that detected differential expression in at least one of the investigated tissues, and in three cases, the predicted allele preference consistently agreed with the direction of change detected by the probe sets (Supplementary Table S5).

DISCUSSION

Microarray measurements versus allele-specific assays

In principle, microarrays are an ideal tool for the large-scale detection of imprinted genes in UpDp/UpD material because imprinted genes are expected to exhibit an extreme expression differential between the matUpDp/UpD and patUpDp/UpD samples. Empirically however, the array measurements will lead to both false negatives and false positives due to, among other factors, the sharing of probe sets between multiple alternative transcripts, cross-hybridization and downstream effects of the chromosome anomaly. Most downstream targets can be excluded by map position.

Table 4. Contingency table summarizing Supplementary Table S2 by contrasting the microarray-derived mode of expression (either M = maternal: uSLR $\geqslant$ .6, P = paternal: uSLR $\leqslant$ −.6 or B = biallelic) with the mode determined by the UpDp/UpD PCR and/or SNP sequencing assays

Array-based expression mode: Tissue	Total	Maternal M	P	B	Paternal M	P	B	Biallelic M	P	B
Pl	13	2 **Ampd3** Tspan4						2 **Dhcr7** **Th**		9
Em	28			9 A330103N21Rik Ccndl Ceacam11-14 Cebpa Pank3 Teadl		2 **AK080843** BB264453	4 Ebf3 Gabra5 Hbxap Rtn4			13
Br	30	1 Gabral*		5 Rkhd3 Rgs10 MGI:2446326 Arrdc4 0710005119Rik		8 Inpp5f_v2 **AK080843** **BM117114** BB312372* BB264453* BB182944 **BB077283** **AV328498**	11 Ebf2 Txnl2 Mlstd2 Kcnq5 Dlgh2 AI114950 AA123443 4933439C20Rik 4632427E13Rik 4632419K20Rik 2310047115Rik			5
Ht	2			1 Sh3gl3						1

For each combination of microarray-derived and assay-derived expression mode, the number of distinct genes that exhibited this combination is provided. Empty entries correspond to zero occurrences. The gene names are given except for genes that showed a biallelic mode of expression in both the microarray and assay experiments. New imprinted genes/transcripts are in bold. *indicates no SNP between Bb and Cast.

However some false positives are retained, and unless a gene has been verified by allele-specific assay, its imprinting status remains uncertain. Thus, the fine-tuning of the true and especially the FPR in microarray data analysis is of paramount importance in a screen, such as this and has been a focus of this study.

Probe sets representing differentially expressed sequences (|uSLR| $\geqslant$ .6) were present for 40 of the 59 genes that were tested for imprinting using the UpDp and/or the SNP assay. Nineteen transcripts were assayed based on past analyses using outdated Affymetrix™ software and annotation (MAS4). For individual tissues, Table 4 shows the total number of transcripts for individual tissues that were tested using an allele-specific assay and compares the results with the microarray measurements. The control gene, *H19,* showed robust maternal expression in the SNP assay (Figure 3). However, contrary to expectation, on examining the differential expression on the embryo and placenta distal matUpDp versus wt microarrays, *H19* had similar expression levels in both samples (Supplementary Table S1). In contrast, *Igf2,* was expressed in wt and vastly reduced in matUpDp RNA as would be predicted (Supplementary Table S1). The probe sequences for *H19* were examined but there was no evidence for cross-hybridization in the Affymetrix™ annotation. Since *H19* is a very highly expressed transcript in the cell, the fluorescent signal may have saturated, but this was not the case because GCOS excludes saturated readings from the comparative analysis, and for *H19*, all probes were included. Hence this false negative result may be an artifactual problem, but could also be due to a biological modulation of *H19* RNA levels in the matUpDp samples by an unknown mechanism.

More than 66% of the 40 genes with microarray evidence for differential expression were found to be biallelic in the UpDp and/or SNP assays (Table 4). This is in stark contrast to the FPR of 5% that we estimated for our threshold for differential expression (|uSLR| $\geqslant$ .6; Figure 1). However, this estimate is with respect to differential expression detected by a single probe set. Each of the tested 59 genes is on average represented by four probe sets that were each used for measurements in at least two different tissues. Assuming independence, the probability of one of these probe sets detecting differential expression in one of the tissues by chance is roughly one-third. However, only 9 of the 31 genes detected as differentially expressed by their probe sets subsequently exhibited an allele preference in the UpDp assay. For a per-gene and cross-tissue FPR of 1/3 the expected number is 20. The UpDp assay is not strand-specific, so an antisense transcript could mask an allele bias in the UpDp assay, but this is unlikely to explain the ~50% shortfall in genes with an allele bias. The probe sets that apparently mistakenly indicate differential expression do not obviously share any characteristics that distinguish them from 'correct' probe sets. So, a large fraction of the inconsistencies between the microarray and the UpDp assay results remain unexplained.

Limited overlap with previous whole genome imprinting studies

Previous studies report that the genome-wide identification of imprinted genes via differential expression between parthenogenotes and androgenotes likely suffers from a high FPR

due to the extreme and different downstream effects of partheno- and androgenesis (39,47). This is likely to explain our limited overlap with the results of the RIKEN FANTOM2 imprinting study (46), especially since relaxing our threshold for differential expression increased the overlap significantly.

The small overlap between our results and the genes predicted to be imprinted in (6) has several possible explanations. The predictor may have misclassified imprinted genes for which the training set was not representative, e.g. imprinted genes with a distinct regulatory mechanism that would have made the sequence features appear atypical for an imprinted gene. For a given gene prediction, it is still unknown in which tissue(s) and developmental stages the imprinting occurs. Our microarray measurements may not have covered the relevant tissues or developmental stages. A general difficulty in establishing a closer correspondence between the classifier and our microarray-based approach is the classifiers' limited applicability to characterized genes, while the microarrays contain a large number of EST-complementary probe sets.

Maternally expressed genes in placenta

Two of the three genes found to be maternally expressed in the placenta, *Dhcr7* and *Th* are located within or in close association with the BWS orthologous region on mouse distal Chr 7. The third, *Ampd3*, is not associated with the cluster and maps ∼33 Mb centromeric of *H19* in apparent isolation from any other imprinted gene. The majority of genes contained within the respective human and mouse BWS regions are highly conserved, both in structural organization and, with few exceptions, in imprinting status (48,49). We could not address the imprinting status of *TH* in humans due to the absence of informative polymorphisms in the available tissues. *DHCR7* and *AMPD3* were biallelic in placenta, a finding consistent with their respective locations on 11q13 and 11p15.4, neither region being associated with imprinting (Figure 4).

The boundaries of the cluster have been defined by the maternally expressed *H19* and *Osbpl5* genes (48–50). The finding of preferential maternal expression at *Dhcr7*, which is located outside these arbitrary boundaries could extend this region of imprinting. Current evidence supports regional imprinting control in the BWS cluster by two imprinting centres (IC's), the *H19* differentially methylated domain (DMD) (51,52), and the *Kcnq1* (Kv) DMR1 (40,53). Paternal inheritance of a KvDMR1 deletion leads to loss of imprinting of several maternally expressed imprinted genes with disruption of the repressive paternally expressed *Kcnq1ot1* antisense transcript thought to be responsible for this effect. Though not formally addressed here, it is conceivable that the maternal expression of *Th* and *Dhcr7*, both of which are located adjacent to KvDMR1 could be regulated by this region, or by the *Kcnq1ot1* transcript in *cis*. Analysis of both genes within the context of a KvDMR1 deletion allele could address this expectation.

One of the limitations of examining monoallelic expression in placenta compared to other organs is the presence of invading maternal blood vessels. The allele-specific assay in interspecies hybrids were performed on placenta with the deciduum removed but the precise fetal:maternal

contribution is not known. Some maternal expression could be derived from maternal contamination. The maternal and paternal UPD placentae would not be susceptible to this issue since it applies equally to these tissues harvested for the arrays. Thus the arrays provide independent evidence for differential expression. Maternal allele preference observed in these genes is largely consistent with the directionality of imprinting of other known placental imprinted genes, an exception provided by the *Igf2 P0* transcript, expressed exclusively from the paternal allele in labyrinthine trophoblast (54,55).

While *Ampd3* shows almost exclusive expression from the maternal allele, for *Dhcr7* and *Th,* transcription was also evident (albeit at a much lower level) from the paternal allele, which indicates that maternal expression of these genes may not be absolute. On the other hand we note the caveat of alternative (non-imprinted) transcripts or imprinting in a cell lineage-dependent manner that potentially complicates this interpretation. However it has not escaped our notice that similarly 'incomplete' imprinting has been observed for other placenta-specific genes, *Nap1l4, Phlda2* and *Osbpl5*, located immediately centromeric to *Dhcr7* (48,49). Incomplete silencing of the paternal allele in these examples, it has been argued, reflects their relatively distant separation from the KvDMR1 compared with other genes, *Kcnq1, Kcnq1ot1* and *Cdkn1c,* that are more closely associated with this element and robustly imprinted (49), providing a possible mechanistic explanation for these observations.

Imprinted EST transcripts identified in the PWS/Angelman Syndrome region

PWS is thought to arise as a consequence of the loss in expression of several paternally expressed imprinted genes on human 15q11–13. Studies of transgenic models have not revealed an obvious candidate, though the *Ndn* gene, when disrupted in mice, causes failure to thrive, a frequent observation in PWS (56). The characterization of additional imprinted transcripts within this region could therefore contribute to the further genetic dissection of PWS. Four novel imprinted transcripts *AK080843, BM117114, BB077283* and *AV328498* were identified in the PWS/AS orthologous region on proximal Chr 7. Bioinformatic analysis did not reveal evidence that these transcripts were contained within larger transcription units or possessed a capacity for protein coding. Significantly, human orthologues could not be found for two transcripts (*BM117114* and *BB077283*), suggesting that they do not have a role in PWS. For *AK080843,* a 100 bp sequence shares 96% identity in the human genome although extensive RT–PCR analysis failed to detect transcripts from this region (T. R. Menheniott, unpublished data).

Unifying hypotheses to explain imprinting disorders will require a comprehensive mapping of genes in the pertinent critical regions. Methods permitting the global detection of imprinted genes across multiple developmental lineages are likely to shed light upon the role of imprinting processes in such disorders. Indeed a prominent conclusion of this study is that the total number of imprinted genes is likely to exceed the number of currently known imprinted genes and the incidence of tissue-specific imprinting will be significant.

ACKNOWLEDGEMENTS

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