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Received 7 January; accepted 11 February 2002.

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Acknowledgements

We thank M. Rosner and V. Chesneau for the gift of the pProExH6HA IDE expression vector, B. Zheng for ELISA analysis, S. Mansourian for assistance in the preparation of illustrations and W. T. Kimberly, W. P. Esler and D. M. Hartley for discussions. Supported by NIH grants (to D.J.S. and M.S.W.) and by Enterprise Ireland and the Health Research Board Ireland (M.R. and R.A.).

Competing interests statement

The authors declare that they have no competing financial interests.

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A global disorder of imprinting in the human female germ line

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Imprinted genes are expressed differently depending on whether they are carried by a chromosome of maternal or paternal origin. Correct imprinting is established by germline-specific modifications; failure of this process underlies several inherited human syndromes[1–5]. All these imprinting control defects are *cis*-acting, disrupting establishment or maintenance of allele-specific epigenetic modifications across one contiguous segment of the genome. In contrast, we report here an inherited global imprinting defect. This recessive maternal-effect mutation disrupts the specification of imprints at multiple, non-contiguous loci, with the result that genes normally carrying a maternal methylation imprint assume a paternal epigenetic pattern on the maternal allele. The resulting conception is phenotypically indistinguishable from an androgenetic complete hydatidiform mole[6], in which abnormal extra-embryonic tissue proliferates while development of the embryo is absent or nearly so. This disorder offers a genetic route to the identification of *trans*-acting oocyte factors that mediate maternal imprint establishment.

Although normally sporadic, complete hydatidiform mole (CHM) is occasionally familial, with affected women repeatedly having pregnancies of this type. These repetitive CHMs are not androgenetic but biparental (BiCHM)[7–9]. By analogy to disorders like Prader–Willi syndrome (which can result from sporadic uniparental disomy or from familial imprinting control mutations), we considered that BiCHM might arise from a global inherited failure of maternal imprinting.

We studied the sixth molar pregnancy of the index case in a BiCHM family with complex consanguinity, originating from the Mirpur region of Pakistan. We demonstrated biparental origin of the BiCHM DNA using markers on six autosomes.

Imprinted genes are associated with differentially methylated regions (DMRs), either 'primary' (established during gametogenesis) or 'secondary' (established later in embryogenesis). We used bisulphite sequencing[10] to compare methylation in the BiCHM and suitable controls, including uniparental DNAs and first-trimester chorionic villus samples, which like CHMs, are of trophoblastic origin.

The Beckwith–Wiedemann region of 11p15 contains two putative primary imprint control regions, at *H19* and *KCNQ1OT1*, ~500 kilobases (500 kb) apart. The DMR ~2-kb upstream of *H19* normally shows paternal-specific germline methylation[11], and is therefore an important control locus (Fig. 1a). Parthenogenetic (Pg) and androgenetic (Ag) control DNAs were respectively completely unmethylated and completely methylated at all CpG dinucleotides, as expected. The BiCHM DNA shows a differentially methylated pattern, like that of normal controls. Cloned polymerase chain reaction (PCR) products from BiCHM were either almost completely methylated or completely unmethylated, as expected for paternal or maternal alleles, respectively.) This maintenance of normal *H19* differential methylation in the BiCHM is as predicted, if only imprinting in the female germ line is affected.

At loci with a maternal methylation imprint (Fig. 1b–e), a very different pattern is seen. The *KCNQ1OT1* primary DMR[12,13] becomes methylated during oogenesis[14]. As expected, our normal control DNAs are uniformly haplo-methylated (C and T bands of similar intensity at each original CpG position), and the partheno-

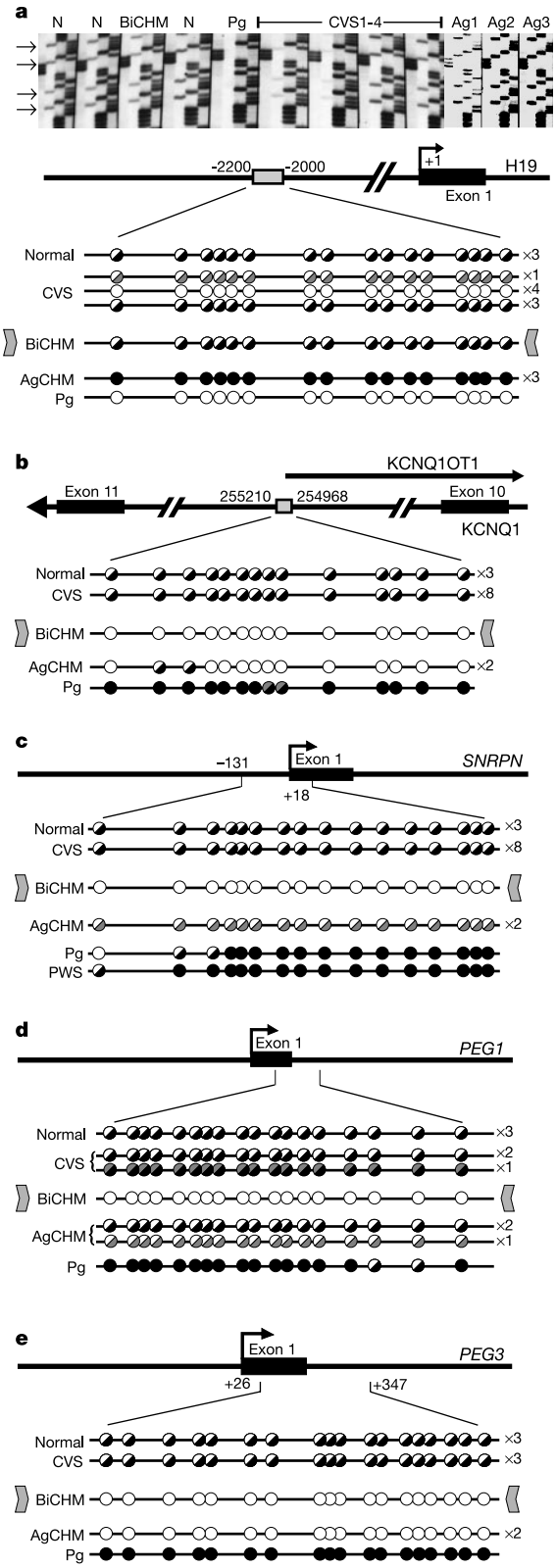

Figure 1 Bisulphite sequencing of DMRs in imprinted genes. Circles represent positions and methylation of individual CpGs, as follows: filled, methylated; open, unmethylated; half-filled black/white, haplo-methylated; black/grey, predominantly methylated; grey/white, predominantly unmethylated. Identical results from multiple controls are collated, numbers indicated to the right. **a**, *H19*. The first and last CpG are numbered relative to the transcriptional start site. Arrows indicate differentially methylated C residues on the gel. N, adult control DNA. Lanes: ACGT, left to right. **b**, *KCNQ1OT1*. Numbering refers to accession AJ006345. **c**, *SNRPN*. Numbering relates to the first nucleotide of exon 1. PWS, Prader–Willi syndrome. **d**, *PEG1*. **e**, *ZIM2/PEG3*. CVS, chorionic villus sample.

genetic sample fully methylated. In contrast, the BiCHM DNA is completely unmethylated, its maternal *KCNQ1OT1* allele thus having a paternal epigenotype.

The 5′ DMR of *SNRPN* (15q) behaved similarly. In the mouse, this is a primary imprint[15], but in humans may only become established in early post-zygotic development[16]. In the BiCHM, this DMR was completely unmethylated (paternal epigenotype), whereas the parthenogenetic and Prader–Willi controls had the opposite epigenotype (almost all CpGs completely methylated). Chorionic villus samples and other normal controls were uniformly haplo-methylated (Fig. 1c). AgCHM were hypomethylated compared to normal DNA, but unlike the BiCHM did show faint bands indicating some (presumably secondary) CpG methylation (see also Supplementary Information).

PEG1 (7q32) and *ZIM2/PEG3* (19q13.4)[17,18] both have maternally methylated DMRs. It is not known if these are primary imprints, although the demethylated paternal *PEG1* epigenotype is established during spermatogenesis[11]. In the BiCHM, these DMRs are both completely unmethylated (paternal epigenotype on both alleles). At *ZIM2/PEG3*, the controls appear as predicted, the normal DNAs being haplo-methylated, the PgDNA completely methylated, and the AgCHM, like the BiCHM, unmethylated (Fig. 1e). However, at *PEG1*, whilst the normal and parthenogenetic samples are respectively haplo-methylated and completely methylated (as expected) the AgCHM DNAs show a variable degree of incomplete methylation (Fig. 1d).

To test whether the BiCHM methylation abnormalities truly reflect a defect of maternal gametic imprinting, rather than being secondary to the molar phenotype, we examined a complex locus, *GNAS1*, that has multiple imprinted transcripts and at least three separate DMRs[19–22] (Fig. 2). In murine *Gnas*, the exon 1A DMR is a primary imprint, whereas the upstream DMRs only become established during the blastocyst stage[22]. Likewise, *GNAS1* imprinting mutations that cause type Ib pseudohypoparathyroidism (PHP-Ib) always alter exon 1A methylation, with the other DMRs only sometimes affected[5]. Therefore, a maternal germline imprinting defect should involve failure to methylate the maternal allele of exon 1A. The maternal NESP55, XLαs, and antisense promoter DMRs (all 35–50 kb upstream) should then secondarily assume a paternal epigenotype, becoming respectively methylated, unmethylated, and unmethylated. This prediction was almost completely fulfilled. At exon 1A, the parthenogenetic control, as expected, is completely methylated, whereas the BiCHM is completely unmethylated, indicating failure to establish the maternal primary imprint. There is some variability in methylation in control samples; two of ten chorionic villus sample DNAs are hypomethylated, and one of three AgCHM appears partially methylated, suggesting that some secondary methylation must have appeared at this locus. Nonetheless, the unmethylated status of the BiCHM is as predicted, and that this represents a true germline defect is supported by analysis of the other DMRs at this locus.

The NESP55 DMR becomes methylated on the paternal allele in the blastocyst stage, possibly secondary to antisense transcription[21,22]. BiCHM and AgCHM are both completely methylated at this DMR (paternal epigenotype on both alleles). All other controls show the expected methylation patterns. Thus, the postzygotic mechanism that sets up the secondary paternal NESP55 imprint remains operative in the BiCHM, but in the absence of a maternal gametic imprint at 1A this yields a paternal methylation pattern on both, rather than one, NESP55 alleles.

We also examined two regions ~3 kb apart, within a large (5-kb) CpG island spanning the antisense promoter and XLαs exon. At the antisense DMR, the BiCHM again shows a paternal epigenotype (this time unmethylated) on both alleles. This lack of methylation is distinctive, even though both AgCHMs show a minor degree of secondary methylation at this locus.

The maternal XLαs allele becomes methylated during the blas-

tocyst stage[22]. Here we initially saw no sign of abnormal methylation in the BiCHM, the DNA appearing haplo-methylated. Similar partial methylation at this DMR, independent of correct maternal methylation at exon 1A, has been seen with *cis*-acting *GNAS1* imprinting defects that cause PHP-Ib; several such families have an abnormal (paternal) methylation pattern at exon 1A and NESP55, whereas the XLαs DMR appears unaffected[5]. Cloning of the BiCHM bisulphite-PCR products, however, revealed a disordered pattern of partial methylation scattered irregularly across the clones, rather than the normal grouping into completely methylated and completely unmethylated clones (see Supplementary Information). A similar analysis has not been reported for the PHP-Ib mutations. Thus, despite the appearance of some methylation at the XLαs DMR, the overall evidence from the four DMRs argues compellingly for a *GNAS1* imprinting defect in BiCHM, very similar to that resulting from some maternally transmitted *cis*-acting imprinting mutations.

The contrasting behaviour of *H19* and *GNAS1*-NESP55 in the BiCHM is noteworthy. Although both DMRs are normally paternally methylated, for *H19* this is primary, and therefore unaffected by an oocyte defect. At NESP55, paternal methylation is secondary to lack of a maternal imprint at 1A, and hence occurs on both alleles in the BiCHM. This difference suggests that the BiCHM defect is not a generalized failure of methylation maintenance, but reflects specific events in the female germ line. Also consistent with this conclusion was the observation of a normal methylated status in the BiCHM at eight CpGs in an intragenic (non-CpG island) region of the non-imprinted *KHK* gene (not shown). Other evidence argues that the BiCHM methylation abnormalities reflect a specific imprinting defect, rather than changes peculiar to trophoblast derivatives. First, we see neither random nor generalized hypo- or hyper-methylation; instead, at each DMR, the direction of the BiCHM methylation abnormality is specifically as predicted for a maternal germline defect. Second, despite some minor inter-sample variation, chorionic villus sample DNAs (which, like the CHMs, are first trimester trophoblast derivatives) typically had normal differential methylation, and never had a 'paternal-only' epigenotype resembling that of the AgCHM and BiCHM.

Cis-acting mutations that disrupt imprinting at individual loci[1–5,12,13,23–24] show sex-dependent dominant (vertical) transmission. In contrast, BiCHM is a pure maternal-effect defect; affected women may have molar pregnancies with different partners[9], but are otherwise healthy. Its presumed autosomal recessive inheritance pattern implies a *trans*-acting molecular defect, consistent with the involvement of multiple dispersed imprinted loci. Of all imprinted loci examined, only *H19* (as predicted) showed a normal differentially methylated pattern in the BiCHM. Because most gametic imprints are imposed in the female rather than the male germ line[25], the great majority of all imprinted loci are probably affected by this genetic defect. A recessive *Dnmt3L* mutation, although also conferring male sterility, prevents specification of maternal imprints in the mouse germ line[26]. In the family studied here, lack of homozygosity for the corresponding human locus, as well as for a previously suggested 19q BiCHM locus[7](see

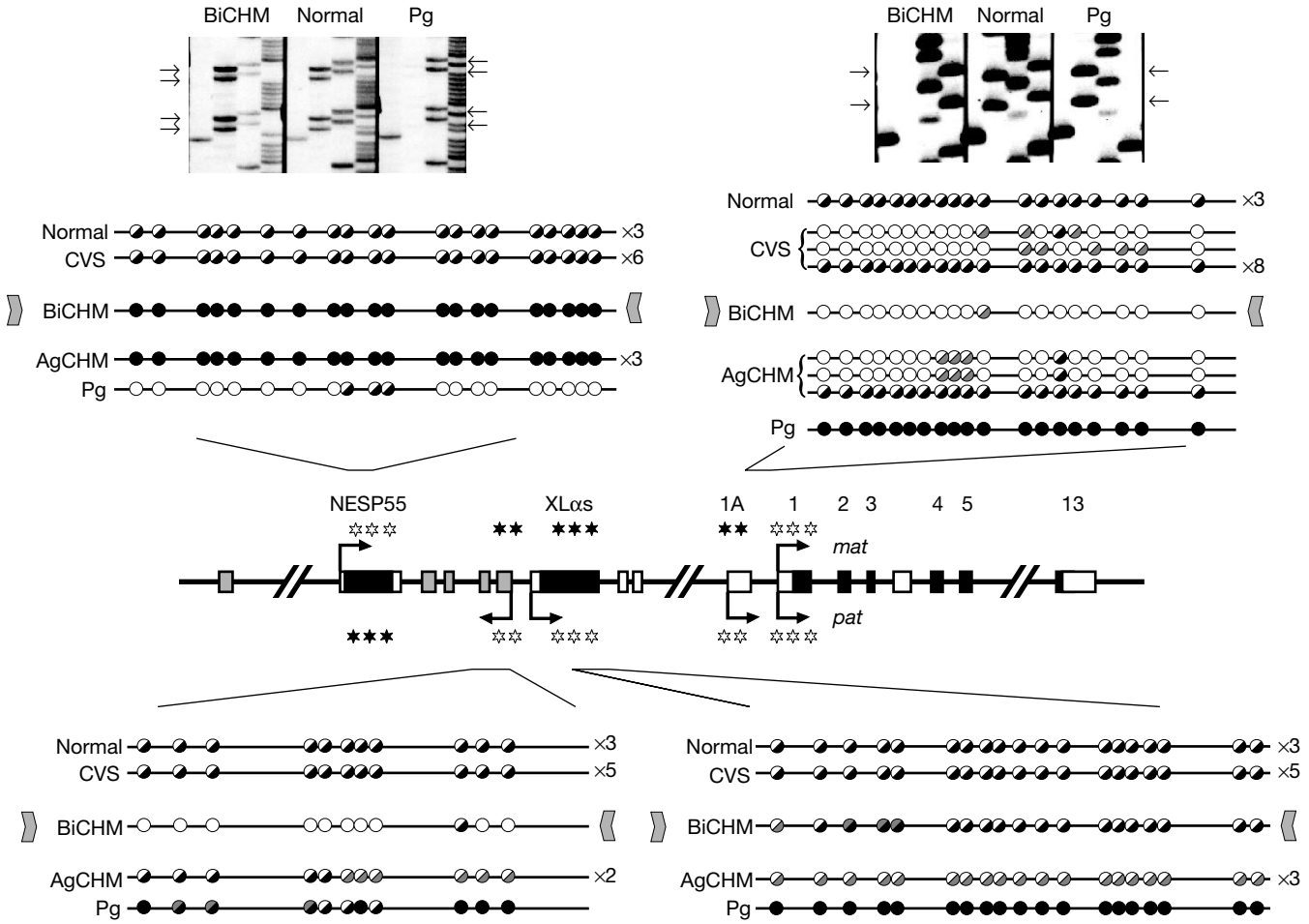

Figure 2 Bisulphite analysis of *GNAS1*. At the top, the contrasting methylation status at the 1A and *NESP55* DMRs is illustrated; arrows on the gels indicate differentially methylated residues. The remainder of the figure summarizes data for the whole locus (for symbols see Fig. 1). In the schematic map of *GNAS1*, coding regions are black, antisense exons grey. All alternative first exons on the sense strand splice onto exon 2 (refs 5, 21). Arrows above and below the line indicate maternal and paternal transcription, respectively. Normally methylated or unmethylated status of CpG islands is shown by filled or open stars, respectively.

Supplementary Information) makes involvement of either of these loci unlikely. However, the BiCHM defect should eventually be identifiable through autozygosity mapping. □

Methods

Detailed methods are available in Supplementary Information.

DNA samples

BiCHM DNA was extracted from a short-term culture of the evacuation products from the sixth pregnancy of the index case. Four of her first five conceptions had previously been histologically confirmed as CHM, and demonstrated to be biparental using archival pathological material. Parthenogenetic DNA was previously described[27]. Adult control blood DNAs were from the index case, her husband, and an unrelated individual. Fluorescent PCR analysis of markers D1S2691, D5S495, D10S189, D13S1293, D17S946, D19S210 and D19S413 was performed by standard methods on DNA from the cultured BiCHM and from the index case and her husband; all these markers were fully informative for demonstrating both maternal and paternal allelic contributions to the mole.

Bisulphite-PCR analysis of DNA methylation

The protocol was adapted from previously described methods[10,24]. Briefly, genomic DNA was denatured and bisulphite-treated to convert unmethylated cytosines to thymines. PCR products encompassing the DMRs of each imprinted locus were then generated. Only one strand was amplified at each locus. Products were analysed by direct sequencing, because at some loci the two modified alleles clone with different efficiencies. Cloning was therefore used only to assess the allelic separation of C and T at haplo-methylated loci (see text).

Received 18 September 2001; accepted 21 January 2002.

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Supplementary Information accompanies the paper on *Nature's* website (http://www.nature.com).

Acknowledgements

We thank R. Fisher for supplying androgenetic CHM DNAs, and G. Taylor for Prader–Willi and chorionic villus sample DNA samples. This work was supported by the Wellcome Trust.

Competing interests statement

The authors declare that they have no competing financial interests.

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Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion

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Recent studies have demonstrated that transplanted bone marrow cells can turn into unexpected lineages including myocytes, hepatocytes, neurons and many others[1]. A potential problem, however, is that reports discussing such 'transdifferentiation' *in vivo* tend to conclude donor origin of transdifferentiated cells on the basis of the existence of donor-specific genes such as Y-chromosome markers[1]. Here we demonstrate that mouse bone marrow cells can fuse spontaneously with embryonic stem cells in culture *in vitro* that contains interleukin-3. Moreover, spontaneously fused bone marrow cells can subsequently adopt the phenotype of the recipient cells, which, without detailed genetic analysis, might be interpreted as 'dedifferentiation' or transdifferentiation.

Recent progress in stem cell research indicates that certain mammalian cells, even from adults, maintain a high degree of plasticity for multilineage cell differentiation. The transferred nuclei from adult cells could be reprogrammed by a factor or factors in the cytoplasm of oocytes, showing the same potential for normal animal development as early embryonic nuclei[2]. More recently, neural stem cells were demonstrated to differentiate into virtually every cell type when they were injected into blastocysts *in vivo* or cultured *in vitro* with differentiating embryonic stem cells[3]. This indicated that the extracellular factor(s) of blastocysts or embryonic stem cells, or cell–cell interaction of neural stem cells with such embryonic cells, might be sufficient for reprogramming adult cells into a more pluripotent status. To this end, we attempted to establish a culture of pluripotent stem cells *in vitro* from adult cells (bone marrow cells) by nurturing them with embryonic stem cells. Bone marrow contains haematopoietic stem cells producing