Differential regulation of imprinting in the murine embryo and placenta by the Dlk1-Dio3 imprinting control region

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Genomic imprinting is an epigenetic mechanism controlling parental-origin-specific gene expression. Perturbing the parental origin of the distal portion of mouse chromosome 12 causes alterations in the dosage of imprinted genes resulting in embryonic lethality and developmental abnormalities of both embryo and placenta. A 1 Mb imprinted domain identified on distal chromosome 12 contains three paternally expressed protein-coding genes and multiple non-coding RNA genes, including snoRNAs and microRNAs, expressed from the maternally inherited chromosome. An intergenic, parental-origin-specific differentially methylated region, the IG-DMR, which is unmethylated on the maternally inherited chromosome, is necessary for the repression of the paternally expressed protein-coding genes and for activation of the maternally expressed non-coding RNAs: its absence causes the maternal chromosome to behave like the paternally inherited one. Here, we characterise the developmental consequences of this epigenotype switch and compare these with phenotypes associated with paternal uniparental disomy of mouse chromosome 12. The results show that the embryonic defects described for uniparental disomy embryos can be attributed to this one cluster of imprinted genes on distal chromosome 12 and that these defects alone, and not the mutant placenta, can cause prenatal lethality. In the placenta, the absence of the IG-DMR has no phenotypic consequence. Loss of repression of the protein-coding genes occurs but the non-coding RNAs are not repressed on the maternally inherited chromosome. This indicates that the mechanism of action of the IG-DMR is different in the embryo and the placenta and suggests that the epigenetic control of imprinting differs in these two lineages.

KEY WORDS: Genomic imprinting, Dlk1-Dio3 domain, Mouse development

INTRODUCTION:
Genomic imprinting in mammals is an epigenetic marking process that causes a subset of genes to be regulated depending on their parental origin (Reik and Walter, 2001; Da Rocha and Ferguson-Smith, 2004). This results in the functional non-equivalence of at least thirteen subchromosomal homologues in the mouse and the requirement for both a maternal and paternally inherited copy of these domains for normal development to occur (http://www.mgu.har.mrc.ac.uk/research/imprinting/largemap.html). Embryological studies involving the generation of parthenogenetic and androgenetic conceptuses and chimaeras containing such cells in the presence of normal biparental cells, showed that imprinted genes are required for the formation of the placenta and for the development of particular lineages including mesodermal and ectodermal derivatives (Barton et al., 1984; McGrath and Solter, 1984; Barton et al., 1991; Fundele et al., 1991). The function and expression of the subsequently identified imprinted genes is consistent with this and in particular emphasises a role for imprinted genes in the regulation of pre and postnatal resources (Constancia et al., 2004). For example, all imprinted genes tested to date are expressed in the developing extraembryonic lineages where most function to control lineage specification, morphogenesis and physiological processes within the placenta (Coan et al., 2005). Indeed, for many imprinted genes, the imprinting is placenta-specific with other tissues exhibiting biallelic expression. In addition to a placental function, imprinted genes also function to control tissue development and growth within the embryo with alterations in imprinted gene dosage causing defects in a range of tissues including muscle, skeleton, adipose tissue and liver (Georgiades et al., 2000; Barton et al., 1991; McLoughlin et al., 1997; Moon et al., 2002; Charalambous et al., 2003). Finally, defects involving energy homeostasis and behaviour indicate a role for imprinted genes in postnatal processes (Plagge et al., 2004; Plagge et al., 2005; Curley et al., 2005; Charalambous et al., 2007) (S.T.R., S.-P.L. and A.F.S., unpublished).

The distal portion of mouse chromosome 12 is subject to genomic imprinting and harbours a 1 Mb imprinted domain containing developmentally regulated protein-coding genes and non-coding RNA genes (Takada et al., 2000; Schmidt et al., 2000; Cavaille et al., 2002; Tsai et al., 2002; Yevtodiyenko et al., 2002; Seitz et al., 2003; Seitz et al., 2004; Youngson et al., 2005). The 5’ end of the domain harbours the Dlk1 gene and the 3’ end, the Dio3 gene. The need for correct dosage of imprinted genes at this domain is demonstrated by the lethality and developmental abnormalities observed in conceptuses with maternal and paternal uniparental disomy for chromosome 12 [MatD12(12) and PatD12(12), respectively]. Embryos with PatD12(12) have two copies of chromosome 12 inherited from their father with none from their mother. They die late in gestation and have prenatal muscle hypertrophy and defects in muscle maturation, costal cartilage defects and hypo-ossification of mesoderm-derived bones (Georgiades et al., 2000; Sutton et al., 2003). Placentomegaly is also evident, accompanied by cellular defects in the junctional and labyrinthine trophoblast zones and in
the decidua basalis (Georgiades et al., 2001). Conceptuses with the reciprocal MatDi(12) defect, with two copies of maternally inherited chromosome 12, die perinatally and have proportional growth-retardation of both the embryo and the placenta. They have a reciprocal skeletal muscle phenotype as compared with PatDi(12), skin defects and defective neural crest-derived middle ear ossicles (Georgiades et al., 2000; Tevendale et al., 2006). Thus, during prenatal stages, imprinted genes on chromosome 12 are essential for fetal viability, the regulation of prenatal growth, the normal development of extraembryonic tissues and of some mesodermal and neural crest-derived lineages.

Some human individuals with uniparental disomy for chromosome 14 (mUPD14 and pUPD14) have been reported. Part of chromosome 14 shares syntenic homology with distal mouse chromosome 12. Maternal and paternal disomy patients have distinct growth, developmental and neural defects. Clinical features of mUPD14 include intrauterine growth retardation (IUOG), followed by postnatal hypotonia. Patients have short stature, precocious puberty, small hands and feet, obesity, joint laxity, scoliosis, recurrent otitis media, hydrocephalus and mild mental retardation (Temple et al., 1991; Mitter et al., 2006; Georgiades et al., 1998; Sanlaville et al., 2000; Sutton and Shaffer, 2000; Martin et al., 1999). By contrast, patients with pUPD14 have polyhydramnios, a small thorax with rib deformities and associated respiratory insufficiency, facial anomalies, short limbs, ventral wall hernia and moderate to severe mental retardation (Georgiades et al., 1998; Kurosawa et al., 2002; Sanlaville et al., 2000; Sutton and Shaffer, 2000). The similar phenotypes observed in human UPD14 patients and mouse uniparental disomy 12 mutants suggests some conservation of growth and neuronal functions related to imprinted genes that reside in this chromosomal region.

The extent to which the Dlk1-Dio3 domain might be responsible for most of the defects observed in the MatDi(12) and PatDi(12) mutant mice and UPD14 patients is not known. The region contains three protein-coding genes expressed from the paternally inherited chromosome: delta-like 1 (Dlk1), retrotransposon-like 1 (Rtl1) and Dio3 (Takada et al., 2000; Schmidt et al., 2000; Tsai et al., 2002; Seitz et al., 2003; Youngson et al., 2005). On the maternally inherited chromosome these genes are repressed and several non-coding RNA transcripts are expressed. These include gene trap locus 2 (Gtl2), microRNAs overlapping with and expressed in an antisense orientation to Rtl1, C/D small nucleolar RNAs (snRNAs) and the microRNA-containing gene (Mirg) located in the vicinity of a cluster containing approximately 40 microRNAs (Seitz et al., 2004). The parentale-specific expression of these imprinted genes is regulated by an intergenic imprinting control element, the IG-DMR located 13 kb upstream of Gtl2. When an IG-DMR deletion is transmitted from the mother, the entire 1 Mb Dlk1-Dio3 imprinted cluster changes from a maternal epigenotype into a paternal epigenotype. This results in activation of the normally maternally repressed imprinted genes (Dlk1, Rtl1 and Dio3), whereas the transcripts normally expressed from the maternally inherited chromosome (Gtl2, microRNAs and C/D snoRNAs) become repressed. Furthermore, the Gtl2 promoter, normally only methylated after fertilisation on the normally methylated chromosome, becomes hypermethylated at the maternal promoter (Lin et al., 2003). As further evidence of the epigenotype switch, a region further downstream that is usually methylated on the maternally inherited chromosome, loses its methylation (N. Youngson, M. Ito, S.P.L. and A.F.S., unpublished). By contrast, no significant imprinting defect was observed when the IG-DMR deletion was transmitted from the father (Lin et al., 2003).

Because of the maternal to paternal epigenotype switch at the Dlk1-Dio3 domain in this IG-DMR knockout model (ΔIG-DMR/+), these mutant mice demonstrate the consequence of imprinted gene dosage alteration in this domain. The main difference between the ΔIG-DMR/+ mutants and the PatDi(12) is the fact that, in PatDi(12), both copies of chromosome 12 are paternally-derived hence both complete chromosomes harbour the paternal epigenotype, whereas in ΔIG-DMR/+ embryos only the 1 Mb Dlk1-Dio3 imprinted domain has switched into a paternal epigenotype on the mutant maternal chromosome 12. The remainder of the maternal chromosome 12 in the ΔIG-DMR/+ mutant maintains its maternal epigenotype. Therefore, comparing the phenotypes of ΔIG-DMR/+ conceptuses with those of PatDi(12), enables us to clarify the extent to which imprinted genes in the Dlk1-Dio3 locus contribute to the phenotypes observed in the murine PatDi(12) mutants, and provides insight into whether other imprinted genes might be located elsewhere on chromosome 12. Here, we have correlated phenotype to epigenotype in the fetus. In addition, our results have revealed a difference in the mechanisms regulating imprinting at this domain between the embryo and the placenta.

MATERIALS AND METHODS

Mice

Animals harbouring deletion of the IG-DMR were maintained on a C57BL/6J background by crossing males heterozygous for the deletion with wild-type females. Females carrying the deletion were mated to wild-type C57BL/6J or DBA/2 males to generate conceptuses with the maternally inherited deletion. In all experiments, controls were wild-type littermates. Genotyping was as described previously (Lin et al., 2005). For the methylation analysis, DNA was isolated from conceptuses derived from heterozygous intercrosses between animals with the balanced translocation T(4;12)47H on C3H and 129Sv/101 genetic backgrounds. Conceptuses with uniparental duplication/deficiency of the distal portion of mouse chromosome 12 were distinguished from wild-type littersmates as described previously (Tevendale et al., 2006).

Skeletal analysis

The freshly dissected embryos were kept in tap water at room temperature or 4°C overnight and then incubated at 65°C for 3–5 minutes before skinning and eviscerating. The carcasses were stained with Alcian Blue and Alizarin Red using standard procedures (McLeod, 1980; Georgiades et al., 2000). Haematoxylin and Eosin staining of the sagittal sections was used to assess thoracic abnormalities in ΔIG-DMR/+ mutants.

Histology and immunohistochemistry

Immunostaining of muscle was conducted according to Georgiades et al. (Georgiades et al., 2000) with the following modifications. Whole embryo or placenta samples were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and embedded in paraffin wax using standard protocols. Sections of 7-10 μm were either stained with Haematoxylin and Eosin (Fig. 3), or with MY32 monoclonal antibody (M4276, Sigma; Fig. 4). The MY-32 antibody is specific for skeletal muscle fibre myosin heavy chain molecules at the stage analysed, allowing accurate identification and measurement of all myofibres (Harris et al., 1989; Venuti et al., 1995). The modified protocol for myosin (MY-32) staining used in this study was as follows. The cross-sectionally cut 7 μm paraffin-embedded limb sections were first softened on a hot plate (rotate 360°) for 1 minute then dehydrated in xylene for 10 minutes. The slides were then rehydrated through ethanol for 5 minutes, 70% methanol for 2 seconds, 50% methanol for 2 seconds, and 50% methanol for 2 minutes. The slides were then incubated for 5 minutes. Antigen retrieval was conducted by incubating slides in 0.01 M sodium citrate (pH 6.0) for 4 minutes in a pressure cooker. The hot slides were then washed four times (10 minutes each) in Phosphate Buffer Saline (PBS). Preblocking took place in 3% H2O2 (in tap water) for 10 minutes, 5% H2O2 in methanol (H1009, Sigma) for 90 minutes, then slides were washed three times in PBS for 10 minutes each before blocking in 3%...
BSA (A7888, Sigma) in PBS for 1 hour. Slides were then incubated in the first antibody solution (Fast Myosin MY-32: M4276, Sigma; 1:1000 dilution) for 2-3 days at room temperature. After washing three times in PBS0.01 M Tween20 (P1379, Sigma) for 10 minutes, slides were incubated with the secondary antibody (anti-mouse IgG; Sigma, B7264: 1:50 in 1% BSA in PBS) for 90 minutes at room temperature followed by three 10 minute washes in PBS/Tween. A peroxidase treatment step was then introduced by incubating the slides in Elite Vectastain for 30 minutes at room temperature (the Elite-Vectastain solution was freshly made according to manufacturer’s instructions). This was followed by a wash in PBS/Tween for 2 minutes. Finally, they were developed for up to 10 minutes in DAB (Sigma Fast 3’3’diaminobenzidine; D4293, Sigma; one tablet was allowed to dissolve in 5 ml PBS for 30 minutes and urea tablet added 2 minutes before use). The slides were rinsed twice with PBS (15 minutes each) and counterstained lightly with GillsIII Haematoxylin (352015M, BDH(Gurr)-VWR) before mounting with DPX mounting medium (360294H, BDH).

**Morphometric analysis of skeletal muscle**

Comparative morphometrics were carried out on MY32-stained histological sections through the largest cross-sectional area of the forelimb. Comparable sections were carefully selected from the mutants and wild-type littermates and 3-4 sections (7 μm) with 10-section intervals were selected for each embryo. For example, if section 300 contained the largest cross-sectional area for an embryo, sections 285, 295, 305 and 315 would be chosen. Two skeletal muscle parameters were determined with the Computer Assisted Stereology Toolbox (CAST) 2.0 system from Olympus Denmark. These were the mean myofibre cross-sectional area and the mean proportion of myofibres containing centrally located nuclei. Measurements were made by randomly selecting the fields across the extensor carpi radialis longus (ecrl) and values were calculated as means ± s.e.m. Other muscles, including extensor pollicis longus (epl) and flexor digitorum superficialis (fds) were also measured and the trend found to be the same. The Bartlett’s test was performed to evaluate whether raw data obtained were homogenous. If this was shown to be the case, an unpaired t-test was used to compare results from mutants with wild-type littermates with P<0.05 for statistical significance as described for the myofibre area. Conversely, if the raw data were not homogenous, as for the percentage of centrally located nuclei per field, the Mann-Whitney U test was introduced to compare results from the mutants and the wild-type littermates, also with P<0.05 for statistical significance.

**Placenta stereology**

Weights were taken for all selected embryos and placenta (n=5 for each genotype). Placentae were then hemisectioned using a double-edged razor blade, each half weighed, and then immediately fixed. The stereology work was carried out using methods described previously (Coan et al., 2004).

**Imprinting and expression levels in placentas isolated from normal and ΔIG-DMR conceptuses**

Total RNA from the placenta samples without the maternal decidua was extracted by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). PolyA+ RNA was extracted from total RNA using the Dynabeads Oligo (dT)25 Kit (Dynal). Total RNA (120 μg) was used as the starting material and all procedures and reagents were as described in the manufacturer’s protocol.

Quantitative northern blot analyses were used to detect Dlk1, Gtl2, Rtl1, C/D snoRNAs and Dio3 from the mutant and wild-type placenta samples as described previously (Lin et al., 2003); primer extension was applied to the microRNAs (Setz et al., 2004). Quantification of multiple northern blots and primer extensions, in addition to those shown in Fig. 5A, were used to generate the histograms shown in Fig. 5B.

The biallelic expression of Dlk1 and Rtl1 from heterozygous placentas with a maternally inherited deletion [embryonic day (E) 16] was demonstrated using a single nucleotide polymorphism (SNP) identified between C57BL/6 and DBA/2 for Dlk1, and between C57BL/6 and Mus molossinus for Rtl1, as described previously (Lin et al., 2003). Dio3 imprinting was not assessed because allelic differences in the expression of Dio3 in the placenta have previously been shown to be less pronounced (Tsai et al., 2002; Yevtodiyenko et al., 2002).

**Methylation status of differentially methylated regions in the placenta**

DNA was isolated, digested with restriction enzymes including those specific for methylated DNA, and assessed by Southern blot analysis with the following enzymes and probes: for the exon 5 region in Dlk1 (Dlk1-DMR), DNA was digested with Nhel and HpaII or Hhal and hybridised with probe D2 as described in Takada et al. (Takada et al., 2000). For the IG-DMR, DNA was digested with Stul and MspI or HpaII or Hhal and hybridised with a Not-Apal fragment probe (M4) as described in Takada et al. (Takada et al., 2002). For the GtI2-DMR, DNA was digested with HincII and with either MspI, HpaII or Hhal and hybridised with probe G1 as described in Takada et al. (Takada et al., 2000). The additional placentall-specific Dlk1-DMR0 was identified using DNA digested with PstI along with MspI, HpaII or Hhal and hybridised to a 986 bp PstI fragment located approximately 7.4 kb upstream from the Dlk1 promoter in a CpG-rich region conserved between mouse and human.

**RESULTS**

**ΔIG-DMR/+ mice die pre-/peri-natally and demonstrate skeletal and muscle defects**

Deletion of the unmethylated IG-DMR from the maternal chromosome results in lethality commencing after E16 (Table 1) (Lin et al., 2003). The overall growth performance of the ΔIG-DMR/+ mutants was similar to wild-type littermates, except that a shorter body length and a shorter, broader neck was observed in many of the mutant mice (Fig. 1). The lethality, short body length and neck phenotypes have been observed in PatDi(12) and pUPD14 patients (Georgiades et al., 2000; Kamnasaran and Cox, 2002).

By contrast, the reciprocal +/ΔIG-DMR animals inheriting the deletion paternally did not exhibit lethality or significant growth abnormalities when measured up to the ninth week postnatally. This is consistent with a lack of any effect on the expression or imprinting of genes on the paternally inherited chromosome in the presence of this deletion (Lin et al., 2003).

At E19, skeletal defects were observed in ΔIG-DMR/+ embryos. These included a bell-shaped thorax with abnormally wide angulation of the ribs relative to the sternum and longer 8th, 9th and 10th ribs (Fig. 2), with the deformed thoracic cage associated with the protrusion of the abdominal organs (Fig. 3). Hypo-ossification of the centra and the neural arches of the vertebrae were also observed in ΔIG-DMR/+ embryos in relation to their wild-type littermates (10.3±0.29 ossification centres below the pelvis in ΔIG-DMR/+, compared with 12.7±0.59 in wild-type littermates). Abnormal ossification was also observed in the sternum of ΔIG-
DMR/+ embryos (6 out of 7 embryos having the 5th ossification centres on the sternum protruding to the 6th sternebra, compared with 2 out of 11 in the +/- littermates). The extra ossification centre observed in the 6th sternebra of ΔIG-DMR/+ embryos could be associated with the inappropriate elongation and attachment of the 8th rib to the sternum, suggesting that this abnormal attachment might signal ectopic ossification (Fig. 2). In addition to the defects in endochondral ossification, abnormalities in intramembranous ossification in the skull were seen. The sagittal sutures of the ΔIG-DMR/+ embryos, though variable, were generally wider than in the wild-type littermates, suggesting delayed ossification of the skull (data not shown). The above mentioned skeletal defects are similar to those reported in both human pUPD14 patients and PatDi(12) mice (Georgiades et al., 2000; Sutton et al., 2003), suggesting that deregulation of imprinted genes in the Dlk1-Dio3 locus is the cause of these skeletal abnormalities.

Reciprocal skeletal muscle defects have been demonstrated in MatDi(12) and PatDi(12) embryos. In PatDi(12) embryos, a larger myofibre cross-sectional area (muscle hypertrophy) and a disproportionately high number of myofibres with centrally located nuclei, have been described (Georgiades et al., 2000). Normally, the number of myofibres with centrally located nuclei declines after E16, reflecting the maturation of the fibres as nuclei move to a peripheral location within the fibre. The abnormally high percentage of centrally located nuclei found in myofibres of late gestational PatDi(12) embryos suggests muscle immaturity. Reciprocal defects are observed in MatDi(12) muscles.

In ΔIG-DMR/+ mice, we observed that 24.76% of the muscle cells contained centrally located nuclei as compared with the significantly lower 3.51% present in wild-type littermates (P<0.0001; Mann-Whitney U test; Fig. 4G). Muscle hypertrophy was also observed in ΔIG-DMR/+ embryos, as indicated by the significantly larger mean myofibre area of 79.7 \( \mu \text{m}^2 \) compared with 60.17 \( \mu \text{m}^2 \) in +/- littermates (P<0.0001; unpaired t-test; data from individual litters are shown in Fig. 4H). The similarities in the lethality and in the skeletal and muscle phenotypes observed in ΔIG-DMR/+ and PatDi(12) mice indicates that these are caused by the overexpression of one or more of the three protein-coding genes and/or by the absence of expression of the non-coding RNAs in both mouse models.

**Fig. 1. Gross phenotype of E18 ΔIG-DMR/+ embryos.** Comparison of (A) wild-type littermate (+/+) with (B) an embryo with the maternally inherited IG-DMR deletion (KO/+). Scale bar: 1 mm.

**Fig. 2. Skeletal defects of E19 ΔIG-DMR/+ embryos.** Comparison of skeletal defects between E19 maternally transmitted IG-DMR knockout embryos (B,D, KO/) and wild-type littermates (A,C, +/+). Preparations are stained with Alcian Blue for cartilage and Alizarin Red for bone. An upward slanting thoracic cage, with wider angulation of the ribs relative to the sternum, is observed in B as compared with A. Eight ribs (instead of 7) were attached to the sternum for IG-DMR knockout embryos (compare D with C). The green arrow indicates the extra-ossification observed in KO/+ embryos at the site where the 6th to 8th ribs attach to the sternum (D). The numbers in C and D refer to the costal cartilages, where the anterior-most rib is defined as 1. c, costal cartilage; ste, ossification centre of one of the sternebrae; xp, xiphoid process. Scale bar: 1 mm.

**Absence of placental defects in the ΔIG-DMR/+ conceptuses**

Whereas all of the embryonic phenotypes observed in the PatDi(12) conceptuses were recapitulated in the ΔIG-DMR/+ conceptuses in which the maternal chromosome had acquired a paternal epigenotype, this was not the case for the placenta. In PatDi(12) conceptuses, placentomegaly (an increase of 20%), and defects in all three placental layers – the labyrinthine zone, the junctional zone and the decidua basalis – were identified. These included discontinuities and disruption of the trophoblast-endothelial interface within the labyrinthine zone and shallow/delayed migration of glycogen cells from the junctional zone into the maternal decidua, a process normally commencing at around E12 of gestation. As all of the imprinted genes located in the Dlk1-Dio3 imprinted cluster are imprinted in the placenta (Takada et al., 2000; Tsai et al., 2002; Seitz et al., 2003) (Fig. 5), perturbation of their dosage might be expected to contribute to the abnormal placental phenotypes in the PatDi(12).

In the maternally transmitted ΔIG-DMR/+ conceptuses, however, there were no significant placental weight differences as compared with the placentas of wild-type littermates. Indeed, the total placental volume was also similar in all genotypes (P=0.7) and no genotype-specific morphological defects were observed in the placentas of either maternally or paternally inherited mutants (data not shown).
More detailed stereological measurements were applied. These included percentage volume fractions of junctional and labyrinthine zones, the surface areas within the labyrinthine zone (fetal capillaries and maternal blood spaces), and the interhemal membrane thickness. These measurements were compared between the $\Delta$IG-DMR/+/ placentae and that of wild-type littermates. The labyrinthine zone surface areas and the interhemal membrane thickness are of physiological importance, as these placental components govern the physical determinants for exchange in gestation (Coan et al., 2004). No significant differences were observed in any of the criteria tested between five $\Delta$IG-DMR/+/ placentae and those from five wild-type littermates ($P=0.24-0.85$; ANOVA with Fisher’s PLSD test). We therefore conclude that the $\Delta$IG-DMR/+ conceptuses do not have the same placental phenotype as described in the PatDi(12) conceptuses.

There are at least two possible explanations for this striking discrepancy between the PatDi(12) and the $\Delta$IG-DMR/+ placentas: First, the placenta phenotypes observed in PatDi(12) placentas may be caused by a currently unidentified imprinted gene or cluster of imprinted genes on mouse chromosome 12 that is not controlled by the IG-DMR. Alternatively, the IG-DMR may not confer the same epigenetic control over the Dlk1-Dio3 imprinted domain in the placenta as it does in the embryo. These two hypotheses are not mutually exclusive.

To date, no imprinted genes located outside the Dlk1-Dio3 domain have been identified on chromosome 12. Multiple candidate genes, selected through array-based expression profiling of MatDi(12) and PatDi(12) placentas and through in silico and functional predictions, have shown bi-allelic expression in attempts to experimentally validate imprinting (D. Gray and A.F.S., unpublished). Nevertheless, we currently cannot rule out the presence of other imprinted genes outside the Dlk1-Dio3 imprinted domain on mouse chromosome 12 that might cause the placental defects in uniparental disomy conceptuses. In order to address whether imprinting in the placenta was governed by the IG-DMR in the same way as in the embryo, expression levels of all the imprinted transcripts were measured in the placenta.

Deletion of the IG-DMR results in activation of protein-coding genes but only partial repression of the non-coding RNAs in placenta

Upregulation of paternally expressed genes (Dlk1, Rtl1, Dio3) was observed in the $\Delta$IG-DMR/+ placenta samples (Fig. 5A). However, the effect was different to that seen in embryos (compare to the inserted panel of Fig. 5B). The expression levels of the paternally expressed Dlk1, Rtl1 and Dio3 from the $\Delta$IG-DMR/+ placenta samples were 148.2%, 181.3% and 150.7%, respectively, relative to the expression levels from the placenta of wild-type littermates. The 48.2% further activation of Dlk1 in placentas with a maternally inherited IG-DMR deletion was from activation of the normally repressed maternal allele, as
similar loss of imprinting was also seen for Dio3, demonstrated by allele-specific RT-PCR sequencing (Fig. 5C). A similar loss of imprinting was also seen for Rtl1 (data not shown). Because Dio3 already exhibits significant biallelic activity in the normal placenta (Tsai et al., 2003; Yevtodiyenko et al., 2004), allelic changes were not assessed.

In contrast to the Dlk1-Dio3 domain, the normally paternally expressed protein-coding genes on the maternal chromosome resulting in their overexpression, the non-coding RNAs expressed from the maternal chromosome are not fully repressed but rather display significant activity. Therefore, IG-DMR deletion from the maternally inherited chromosome does not cause a complete maternal to paternal epigenotype switch in placentas.

We demonstrated previously that, in embryos, the absence of microRNAs antisense to Rtl1 is associated with increased Rtl1 transcript levels (Lin et al., 2003). Normally, a direct trans-

interaction between Rtl1 transcripts and the microRNAs results in RNAi-mediated degradation of a proportion of (but not all) Rtl1 transcripts (Davis et al., 2005). Compared with the 450% increase in Rtl1 transcripts in ΔIG-DMR/+ embryos in the absence of the microRNAs expressed in the placenta, unlike in the normal placenta, the expression of the non-coding RNAs in the placenta, unlike in the normal placenta, is exclusively from the paternal allele.

Differential methylation in the placenta and embryos

Previous IG-DMR deletion studies in the embryo indicated that the unmethylated locus on the maternal chromosome was required for expression and hypomethylation at the maternally inherited Gtl2 promoter. In its absence, the associated repression of Gtl2 was correlated with hypermethylation of the promoter. Recently, maintenance methylation was shown to be dispensable for imprinting at the Kcnq1 domain on distal chromosome 7 in the mouse placenta (Lewis et al., 2004; Umlauf et al., 2004). Results presented here suggest that the IG-DMR is not alone responsible for expression of the non-coding RNAs in the placenta, unlike in the

Fig. 5. Expression of imprinted genes in placentas as a consequence of the IG-DMR deletion. (A) Northern blot analysis for imprinted genes in the Dlk1-Dio3 domain (Gtl2, Dlk1, Rtl1, Dio3), and primer extension assay (for micro-RNAs mir-127 and mir-410). MBII-48, MBII-49 and MBII-78 are snoRNA genes. Gapdh and U3 (Rnu3 – Mouse Genome Informatics) are controls. Gene symbols in blue and red denote those expressed from the paternally and maternally inherited chromosomes, respectively. Each genotype is represented by duplicate tracks of RNA isolated from different placentas. (B) Bar chart comparing expression levels between E16 placentas carrying the maternally-derived IG-DMR deletion (red; KO/+), with the paternally derived deletion (blue; +/KO), and wild-type littermates (yellow; +/+). Error bars represent s.e.m. Values were calculated from data generated for each gene using control (n=5-8) and mutant (n=2-5) placentas from different litters (n=2-4). Statistically significant differences from normal are: *, P<0.05; **, P<0.005; ***, P<0.0005; ANOVA + Fisher’s PLSD test. Values were normalised against Gapdh except the small RNAs which were normalised against the unlinked, non-imprinted snoRNA U3. For comparison, the inserted panel shows the equivalent expression analysis in E16 embryos (see Lin et al., 2003). (C) Biallelic expression of Dlk1 in placentas of conceptuses with a maternally transmitted IG-DMR deletion. Sequence analysis of RT-PCR products from control littermate and heterozygote placenta upon maternal transmission of the IG-DMR deletion. Activation of Dlk1 from the normally repressed maternal allele is only observed when the IG-DMR deletion is maternally transmitted. The Dlk1 expression in the normal placenta is exclusively from the paternal allele.
embryo. We therefore assessed the placental methylation status of previously characterised DMRs in the region to determine whether this differed from the embryo. We used mice with uniparental duplications of the distal portion of mouse chromosome 12 (Tevendale et al., 2006) and their normal littermates to compare the two parental chromosomes. Our results show a different methylation pattern at the Dlk1-Dio3 domain in placentas and embryos (Fig. 6) (Takada et al., 2000). Importantly, the methylation profile of the germ-line derived IG-DMR was the same in the placenta as in the embryo (Fig. 6A). However, the other differentially methylated regions in the placentas had different methylation profiles than in the embryo. Overall, this profile indicates a reduction in the methylation differences between the maternal and paternal chromosomes and suggests that, with the exception of the germ-line mark, differential DNA methylation at other DMRs at this locus may not be as relevant as in the embryo (Fig. 6B-E). Methylation at these regions was not analysed in the IG-DMR mutant as the differences between the two parental chromosomes in the placenta is so small. Despite this reduction in differential methylation in the normal placenta, Dlk1 and Gtl2 are imprinted (Takada et al., 2000).

In addition to the previously identified Dlk1-DMR, IG-DMR and Gtl2-DMR, an additional differentially methylated region, Dlk1-DMR0, was found upstream of Dlk1. This DMR was differentially methylated in the placenta exhibiting hypermethylation on the paternal chromosome and less methylation on the maternal allele, but was completely unmethylated on both chromosomes in the embryo (Fig. 6D,E). Any function for this element remains to be determined. These findings suggest that either there is a different set of imprint marks in the placenta and/or that the placenta possesses a different mechanism for interpreting imprinted marks than in the

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**Fig. 6. Methylation studies of the Dlk1-Gtl2 imprinted domain in placenta.** (A) The IG-DMR region is fully methylated on the paternal chromosome and unmethylated in the maternal chromosome in placenta. The Dlk1-DMR (B) and Gtl2-DMR (C) are partially methylated on both parental alleles in the placentas, with methylation level being slightly higher on the paternal allele. (D) A placenta-specific differentially methylated region, Dlk1-DMR0, is identified ~7.4 kb upstream of the Dlk1 transcriptional start site. (E) Summary of the methylation status of the Dlk1-Gtl2 domain in embryos, sperm and placentas. White and black circles represent unmethylated and fully methylated regions, respectively. One, two and three quarters-filled circles represent alleles methylated by approximately 25%, 50% and 75%, respectively. M, maternal allele; P, paternal allele.
Deletion of the unmethylated IG-DMR on the maternal chromosome causes the 1 Mb Dlk1-Dio3 imprinted locus on that chromosome to undergo an epigenotype switch and behave like the paternal one in the mutant embryos. This involves inappropriate activation of three normally repressed protein-coding genes and the repression of a series of non-coding RNAs on the maternal paternal chromosome that are likely to play important roles. The consequences of this epigenotype switch are that mutant embryos are no longer viable, and that they share most of the abnormal developmental phenotypes previously described for animals with paternal uniparental duplication of the entire chromosome 12. This finding suggests that the 1 Mb Dlk1-Dio3 imprinted domain is the key imprinted domain on chromosome 12 that is responsible for the developmental defects and lethality of the PatDi(12) conceptuses. One exception to this is the placenta. Mutant phenotypes described for PatDi(12) placentas were not recapitulated in the IG-DMR knockout in placenta. This finding indicates that the developmental abnormalities in the fetus, and not the placenta, are responsible for the prenatal lethality in both genetic models.

It is not yet clear which, if any, of the imprinted genes in the Dlk1-Dio3 imprinted locus is the primary cause of the embryonic lethality described here. Overexpressing Dlk1 to 200% alone does not cause lethality in hemizygous Dlk1 transgenic mice. However, homozygous Dlk1 transgenic conceptuses die late in gestation and exhibit a similar skeletal phenotype to that described for PatDi(12) and ΔIG-DMR/+ embryos (S.T.R., S.P.L., and A.F.S., unpublished). This is consistent with the expression of Dlk1 during skeletogenesis (Abdallah et al., 2003; Yevtodyienko and Schmidt, 2006). Targeted deletion of Dlk1 in mice results in skeletal malformations that include rib fusions and asymmetrical fusion of the ribs to the sternum (Moon et al., 2002), indicating that Dlk1 is indeed required for normal skeletal development. Transgenic mice overexpressing the soluble Pref-1/hFc fusion protein (the soluble form of Dlk1 fused to the human immunoglobulin-gamma constant region) under the control of the adipocyte fatty acid-binding protein (aP2) promoter, also have skeletal abnormalities, primarily in the distal vertebrae. The severity of these abnormalities correlates with the levels of circulating Pref-1/hFc, and includes a smaller thoracic cavity with short ribs, and fused and disorganised vertebrae resulting in scoliosis (Lee et al., 2003). In addition to its role in skeletal development, Dlk1 is also implicated in myogenesis. Dlk1 is strongly expressed during myogenesis (Yevtodyienko and Schmidt, 2006) and ectopic expression of an ovine Dlk1 cDNA in muscle results in post-natal muscular hypertrophy (Davis et al., 2004).

The upregulated Dio3 in ΔIG-DMR/+ embryos may also contribute to the lethality phenotype by downregulating the active form of thyroid hormone (T3), thyroid hormone being important in controlling many cellular, metabolic and physiological functions (Forhead et al., 2003; Fowden, 1995; Fowden and Silver, 1995; Harakawa et al., 1989; Oppenheimer et al., 1987). Upregulation of Dio3 in the ΔIG-DMR/+ and PatDi(12) embryos may contribute to the skeletal phenotypes also through downregulating the active form of thyroid hormone. Indeed, disrupted utilization of thyroid hormones has previously been shown to retard growth and bone maturation (Gothe et al., 1999). A contribution from Dio3 in the muscle phenotypes observed in PatDi(12) and ΔIG-DMR/+ mice also cannot be ruled out. Thyroid hormone has been demonstrated to be important in inducing myoblasts to exit the cell cycle, to differentiate and to express a muscle-specific phenotype, prenatally (Muscat et al., 1995). As a negative regulator of thyroid hormone, the excess level of Dio3 in PatDi(12) and ΔIG-DMR/+ embryos could reduce the contribution of thyroid hormone to the promotion of myoblast differentiation, consistent with the defects observed. The developmental effects of overexpressing Rtl1 and silencing the maternally expressed non-coding RNAs from the Dlk1-Dio3 locus have not as yet been demonstrated.

In addition to mouse, the contribution of the DLK1-GTL2 imprinted locus towards a mutant muscle phenotype has also been demonstrated in sheep. A single nucleotide substitution occurring between DLK1 and GTL2 (around 12 kb upstream of the IG-DMR) causes overexpression of several imprinted genes in the cluster without affecting their parental-allele-specific gene expression pattern. Overexpression of the genes expressed on the paternal chromosome, leads to a post-natal muscle hypertrophy phenotype termed Callipyge (Georges et al., 2003). This muscular hypertrophy is characterised by a rostro-caudal gradient (i.e. it is more pronounced in the hind quarters), and manifests itself only after three to four weeks of age (Georges et al., 2003). This phenotype only occurs when there is net upregulation of DLK1 and PEG11/RTL1 expression in +/Callipyge animals. No muscle hypertrophy phenotype is observed when the non-coding RNA genes (GTL2, anti-PEG11 and MEG8) are upregulated on the paternal chromosome (Callipyge+/-) (Bidwell et al., 2004; Charlier et al., 2001). In homozygous Callipyge animals, transcripts from all of the above mentioned genes are upregulated on both the maternal and paternally inherited chromosomes. Interestingly, the muscle defect manifest in paternal heterozygotes is abrogated in the homozygous animals, suggesting that maternally expressed non-coding RNAs might be negatively regulating the paternally expressed protein-coding genes post-transcriptionally, at least in muscle.

Such a mechanism may explain the absence of the defective placenta in the IG-DMR knockout conceptuses. Interestingly, although biallelic, the measured transcript levels of the paternally expressed protein-coding genes are not as high as in the embryo, correlating with the expression of the maternally expressed non-coding RNAs. It is known that the microRNAs expressed antisense to Rtl1 from the maternally inherited chromosome are able to target Rtl1 post-transcriptionally and guide its degradation by an RNAi-mediated mechanism (Davis et al., 2005). It is not known whether
the other paternally expressed imprinted genes might also be negatively regulated by the non-coding RNAs on the maternally inherited chromosome at this locus. Nonetheless, these results indicate that there is an inverse relationship between the levels of protein-coding genes and non-coding RNAs in more than one animal model and organ system, and the placental model here provides another experimental system in which to consider the relationship between protein-coding genes and non-coding RNAs at this locus.

Although all the imprinted genes in the distal 12 cluster are expressed in the placenta, the IG-DMR deletion experiment demonstrated that this imprinting control element is not sufficient for full control of imprinting at this domain in the placenta. This may be the reason that we did not observe placental abnormalities in ΔIG-DMR/+ conceptuses, in contrast to the PatDi(12) conceptuses. Consistent with this idea is the finding that a slightly different pattern of methylation is observed in the placenta than in the embryo. Our data suggest that the IG-DMR is necessary and sufficient for imprinting in the embryo. In the placenta, however, the IG-DMR is necessary for the repression of the protein-coding genes, but not for the activity of the non-coding RNAs. It is possible that chromatin is regulated differently in the placenta than in the embryo.

Several findings indicate that epigenetic control in extraembryonic tissues might be different from that in embryos (Chapman et al., 1984; Wagschal and Feil, 2006), and several imprinted genes show tissue-specific imprinting only in the placenta (Coan et al., 2005). Although maintaining DNA methylation at imprinted domains is required for imprinting maintenance in the embryo, it has been shown for one imprinted domain that placenta-specific imprinting does not require maintenance methylation (Lewis et al., 2004). These findings are consistent with results presented here. In placenta, although the germ-line-derived IG-DMR shows clear and comparable differential methylation to the embryo, the methylation state of those secondary DMRs is different, with the two parental chromosomes appearing more similar in their methylation state. This suggests that methylation at the non-germ-line DMRs may not be important in the placenta. The identification of an additional placenta-specific DMR 5′ of Dlk1 in a region that is hypomethylated in embryos again emphasises the difference in the epigenetic profiles of embryonic versus extraembryonic tissues. More extensive epigenetic profiling of this domain in the placenta is underway.

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