

Parental origin-specific developmental defects in mice with uniparental disomy for chromosome 12

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SUMMARY

Genetic analysis has shown that the distal portion of mouse chromosome 12 is imprinted; however, the developmental roles of imprinted genes in this region are not known. We have therefore generated conceptuses with uniparental disomy for chromosome 12, in which both copies of chromosome 12 are either paternally or maternally derived (pUPD12 and mUPD12, respectively). Both types of UPD12 result in embryos that are non-viable and that exhibit distinct developmental abnormalities. Embryos with pUPD12 die late in gestation, whereas embryos with mUPD12 can survive to term but die perinatally. The mUPD12 conceptuses are invariably growth-retarded while pUPD12 conceptuses exhibit placentomegaly. Skeletal muscle maturation defects are evident in both types of

UPD12. In addition, embryos with paternal UPD12 have costal cartilage defects and hypo-ossification of mesoderm-derived bones. In embryos with mUPD12, the development of the neural crest-derived middle ear ossicles is defective. Some of these anomalies are consistent with those seen with uniparental disomies of the orthologous chromosome 14 region in humans. Thus, imprinted genes on chromosome 12 are essential for viability, the regulation of prenatal growth, and the development of mesodermal and neural crest-derived lineages.

Key words: Imprinting, Mouse development, Chromosome 12, Uniparental disomy, Fetal growth

INTRODUCTION

Parthenogenetic (PG; two maternal genomes) mouse embryos die around mid-gestation, are growth retarded and have poor development of their extra-embryonic tissues, while the reciprocal androgenetic (AG; two paternal genomes) conceptus can occasionally reach somite stages of development (Surani et al., 1984; Barton et al., 1984; McGrath and Solter, 1984). These phenotypes reflect functional differences between the parental genomes during development that are attributed to the differential expression of imprinted genes. Developmental properties of AG and PG cells have also been studied in chimaeras with wild-type cells. These analyses showed that chimaeras with PG cells are viable and fertile, though growth-retarded (Fundele et al., 1997). By embryonic day (E) 15.5, PG chimaeras lack PG cells in skeletal muscle (Fundele et al., 1990, 1994). By contrast, chimaeras with AG cells are growth-enhanced and exhibit striking overgrowth of the costal cartilage and hypo-ossification of mesoderm-derived bones (Barton et al., 1991; Mann and Stewart, 1991). These chimaeras have an increased contribution of AG cells to mesodermal lineages, notably skeletal muscle (Barton et al., 1991).

Robertsonian and reciprocal translocation heterozygote intercrosses have been used to generate uniparental disomies (UPD) and uniparental duplications of whole or selected chromosome regions, respectively (Cattanach and Beechey,

1997; Beechey et al., 1999). This has allowed the identification of eleven autosomal regions, which are associated with gross phenotypic abnormalities when their parental origin has been perturbed, and has generated a mouse 'imprinting map' (Beechey et al., 1999). Based on such genetic studies, it was observed that offspring with maternal duplication/paternal deficiency or paternal duplication/maternal deficiency of the distal portion of mouse chromosome 12 (MatDp.dist12 and PatDp.dist12, respectively) failed to be recovered postnatally (Cattanach and Rasberry, 1993). This indicated the presence of imprinted genes on chromosome 12 that are essential for normal development. The imprinted region from these studies cytogenetically maps distal to band B3. This large chromosomal region shares syntenic homology with human chromosome 14q12–qter. UPD involving human chromosome 14 is rare; nonetheless, several cases of maternal and paternal uniparental heterodisomy have been reported (mUPD14 and pUPD14) and are associated with distinct abnormalities, including growth defects (Temple et al., 1991; Wang et al., 1991; Cotter et al., 1997; Georgiades et al., 1998; Fokstuen et al., 1999).

In order to investigate the function of imprinted genes on mouse chromosome 12, we have generated conceptuses with either maternal or paternal uniparental disomy for chromosome 12 (mUPD12 and pUPD12, respectively). Both types of UPD12 offspring have distinct phenotypes, are non-viable and

exhibit developmental defects, some of which overlap with the phenotypes described in UPD14 patients. These mouse conceptuses will provide a valuable resource for the identification of new imprinted genes, the analysis of their developmental function and the mechanism of their imprinting.

MATERIALS AND METHODS

Generation of UPD12 conceptuses

To generate mUPD12 and pUPD12 offspring, we intercrossed mouse stocks, both of which were heterozygous for two Robertsonian translocation chromosomes that share monobrachial homology for chromosome 12. Stocks that were homozygous for these translocations were generously provided by Dr H. Winking (Lubeck, Germany), and these homozygotes were initially intercrossed to generate double heterozygote animals. Rb(8.12)5Bnr/Rb(6.12)3Sic double heterozygote females on a BALB/c background were crossed with Rb(8.12)5Bnr/Rb(4.12)9Bnr double heterozygote males on a C57BL/6J background. Predicted outcomes of the cross are shown in Fig. 1. Aneuploidy of the chromosomes involved in these translocations has been reported previously and results in lethality (Gropp and Kolbus, 1974; Gropp et al., 1983; Baranov, 1983; Magnuson et al., 1985; Epstein, 1986; Hernandez and Fisher, 1999). Pregnant females were dissected at embryonic day 9.5 (E9.5), E12.5, E13.5, E14.5, E15.5 and E18.5 (E0.5 was noon on the day of vaginal plug). Several litters were allowed to develop to term. All embryos were photographed and samples taken from tail and yolk sac for genomic DNA isolation. The PCR genotyping (see below), in conjunction with the reported outcomes associated with aneuploidy for the chromosomes involved in the translocations, allowed unambiguous identification of UPD12 and normal conceptuses at the stages analysed.

Genotyping of conceptuses

Genomic DNA was isolated from all tail and yolk sac specimens as described previously (Ferguson-Smith et al., 1993). PCR primers specifically amplify polymorphic BALB/c and C57BL/6 microsatellite markers on chromosome 12 (*D12Nds2* or *D12Mit20*), chromosome 4 (*D4Mit116*), chromosome 6 (*D6Mit15*) and chromosome 8 (*D8Mit41*) (Eppig et al., 1998; Dietrich et al., 1992). All DNAs were genotyped for BALB/c alleles and C57BL/6 alleles. Allele-specific polymorphic fragments were amplified in 1.5 mM MgCl₂, 0.25 mM dNTPs, 0.25 μM primers, 1 U Taq polymerase (Promega), 0.1 μl [³²P]-dCTP (3000 Ci/mmol, Amersham), in a volume of 10 μl. Amplification conditions for all primer pairs (except *D12Mit20*) were: 5 minutes at 94°C then 30 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute. Amplification conditions for *D12Mit20* were 93°C for 3 minutes then 30 cycles of 93°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute. 10 μl of loading buffer (100% formamide with 1 mg/ml xylene cyanol, 1 mg/ml Bromophenol Blue and 10 mM EDTA) was then added. Samples were heated at 98°C for 2 minutes then 5 μl sample was subjected to electrophoresis on a 6% polyacrylamide/7M urea gel. Gels were dried, exposed to Kodak X-ray film overnight and

genotypes for all tail and most yolk sac samples determined (data not shown). For each sample pair, there was complete concordance between the embryo and yolk sac DNA.

Embryo and placental mass determination

Wet masses were determined for all embryos and placentae at 5 and 4 gestational stages, respectively. Mass measurements were subjected to the non-parametric Mann-Whitney *U*-test, as described previously for similar sample sizes (Sturm et al., 1997), with *P*<0.05 for statistical significance.

Histology, immunohistochemistry and skeletal preparation

Material for analysis was fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and embedded in paraffin wax using standard protocols. 8–10 μm sections were either stained with Haematoxylin and Eosin, or with the MY32 monoclonal antibody (M4276 Sigma, 1:1600 dilution at 4°C overnight). Biotinylated goat anti-mouse IgG (Amersham, 1:2000 dilution) was used as the secondary antibody and the signal was visualised using the Elite kit (Vector Labs) according to the manufacturer's instructions; sections were counterstained with Haematoxylin. The MY32 antibody is specific for skeletal muscle myosin heavy chain molecules at the stages analysed, allowing accurate identification and measurement of all myofibres (Harris et al., 1989; Venuti et al., 1995). For staining of cartilage and bone, animals were skinned, eviscerated and the carcasses stained with Alcian Blue (cartilage) and/or Alizarin Red (bone) using standard procedures (McLeod, 1980).

Morphometric analysis of skeletal muscle

Comparative morphometrics were carried out on MY32-stained histological sections through the largest cross-sectional area of several muscles prepared from normal, pUPD12 and mUPD12 embryos at E13.5, E15.5 and E18.5. For each analysis, measurements were taken from two adjacent sections of at least two embryos of each type. Three skeletal muscle parameters were determined. These were mean myofibre number, mean myofibre cross-sectional area and the mean proportion of myofibres containing centrally located nuclei. Muscle images were digitally analysed using NIH Image 1.62 software (<ftp://zippy.nimh.nih.gov/pub/nih-image>). Values were calculated as mean ± s.e.m. The data were analysed statistically using the student's *t*-test (*P*<0.03).

RESULTS

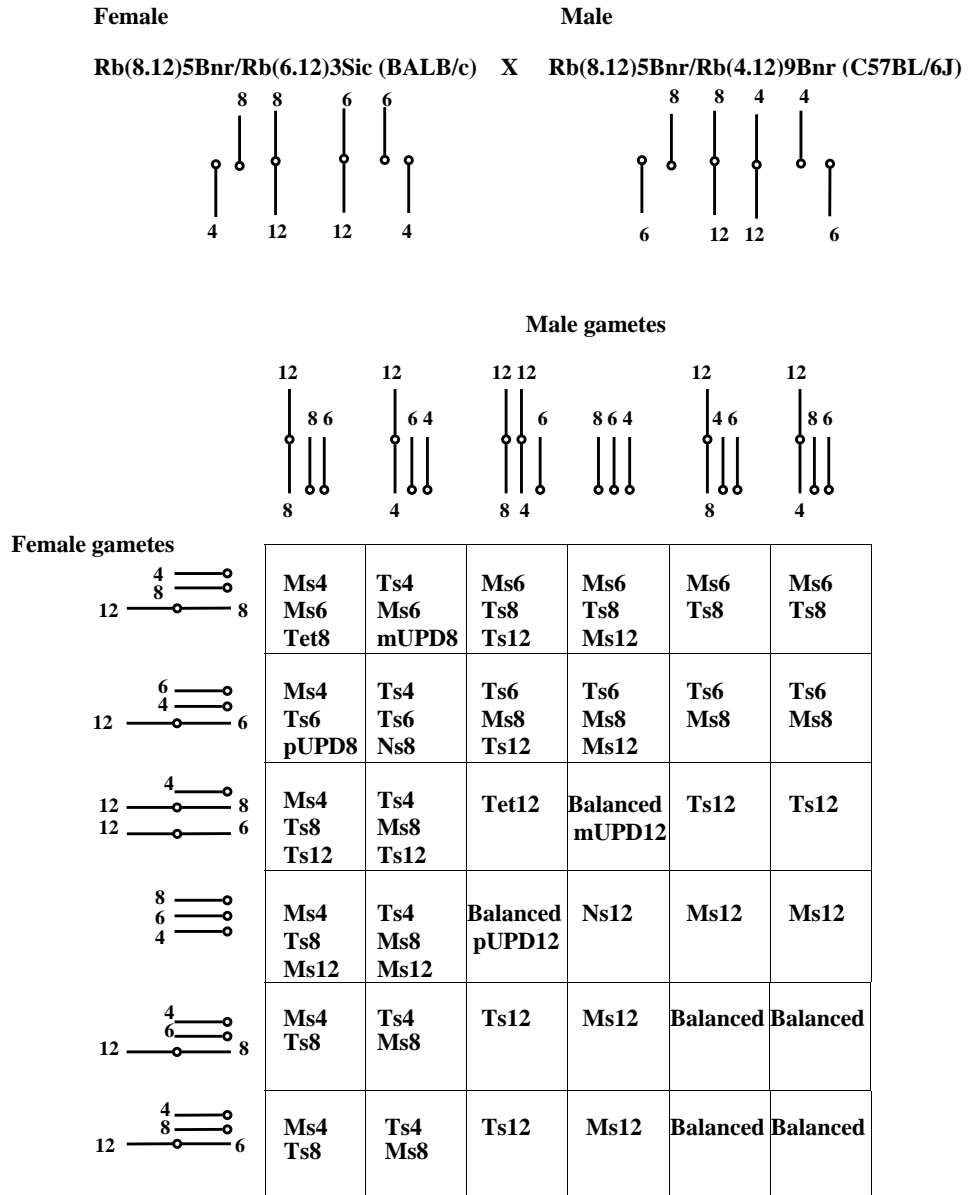
Viability and frequency of UPD12 conceptuses

Table 1 summarises the number and viability of UPD12 offspring generated, and shows their frequencies expressed as a percentage of the total number of implantation sites at all stages analysed. All pUPD12 embryos identified up to and including E15.5 were alive. Thereafter, the number of live pUPD12 embryos decreased, with approximately 50% dying between E15.5 and E18.5. None survived to term. In contrast, all mUPD12 embryos identified prenatally were alive. Two mUPD12 newborns were found alive. These exhibited severe

Table 1. Frequency and viability of pUPD12 and mUPD12 animals

Stage (E)	Number of implantation sites (litters)	pUPD12			mUPD12		
		Total number	(%)	Number alive	Total number	(%)	Number alive
9.5	156 (29)	5	(3.2)	5/5	4	(2.6)	4/4
12.5	282 (54)	6	(2.1)	6/6	5	(1.8)	5/5
13.5/14.5	306 (59)	10	(3.3)	10/10	8	(2.6)	8/8
15.5	166 (32)	5	(3.0)	5/5	2	(1.2)	2/2
18.5	253 (41)	13	(5.1)	7/13	4	(1.6)	4/4
Newborn	– (70)	6	–	0/6	4	–	2/4

Fig. 1. Schematic representation of the parental gamete genotypes and predicted zygotic outcomes that result from the Robertsonian heterozygote intercross used in this study. Only the chromosomes involved in the translocations are shown. Both parental mice have monobrachial homology for chromosome 12 (Rb5/Rb3 females and Rb5/Rb9 males). The majority of the predicted zygotic outcomes are very rare, unbalanced and are peri-implantation lethal. Ms, monosomy; mUPD, maternal uniparental disomy; Ns, nullisomy; pUPD, paternal uniparental disomy; Tet, tetrasomy; Ts, trisomy.



respiratory distress and did not survive. We therefore conclude that at least some mUPD12 embryos can survive birth and die perinatally.

Our frequency values (Table 1) assume that all UPD12 conceptuses are detected because there is no decrease in the frequency of detectable UPD12 conceptuses with increasing gestational time. The number of resorbed embryos remained relatively constant throughout gestation, consistent with the predicted perimplantation lethality of aneuploid conceptuses (Fig. 1). Despite surviving to term, in this cross mUPD12 conceptuses are generated at a lower mean frequency ($2.06 \pm 0.25\%$) than pUPD12 offspring ($3.38 \pm 0.41\%$) throughout gestation (Table 1). This difference is statistically significant (student's *t*-test, $P=0.02$). Although the expected generation frequency should be the same, differences in frequency between mUPD and pUPD offspring have been described previously for other chromosomes and were shown to be independent of imprinting effects. One possible explanation is that this may reflect non-random segregation of chromosomes to the polar bodies in the oocyte of the female (Cattanach and Beechey, 1994, 1997).

Growth of UPD12 conceptuses

The growth of UPD12 conceptuses was compared to their normal littermates from E12.5 to E18.5 (see Fig. 2). The mean masses of pUPD12 embryos did not show a statistically significant difference from normal littermates at any stage examined, although at E15.5 pUPD12 embryos tended to weigh more (Fig. 2C). At E18.5, pUPD12 placentae were significantly heavier than those of normal littermates (Fig. 2D), though at earlier stages, the mass and size of pUPD12 placentae did not differ significantly from normal. Detailed examination of pUPD12 placentae resulted in the

identification of defects in all the three major zones of the placenta (P. Georgiades, M. Watkins, G. Burton and A. Ferguson-Smith, unpublished). The fetomaternal interface in the labyrinth zone was disrupted and defects were observed in the properties of trophoblast cells in the junctional (spongiotrophoblast) zone. In addition, in the decidua basalis, the central maternal artery supplying the placenta exhibited anatomical abnormalities (P. Georgiades, M. Watkins, G. Burton and A. Ferguson-Smith, unpublished). These defects are likely to contribute to the progressive loss of pUPD12 fetuses after E15.5. The external appearance of surviving E18.5 pUPD12 embryos compared to normal (Fig. 2B) indicated a reduced crown-to-rump length, protruding thorax and abdominal distention (see below).

Beginning at E15.5, mUPD12 embryos and placentae were significantly growth-retarded compared to their normal littermates (Fig. 2). At E15.5 the mean mUPD12 embryo mass was 72% that of normal, and 63% of normal at E18.5. The mean

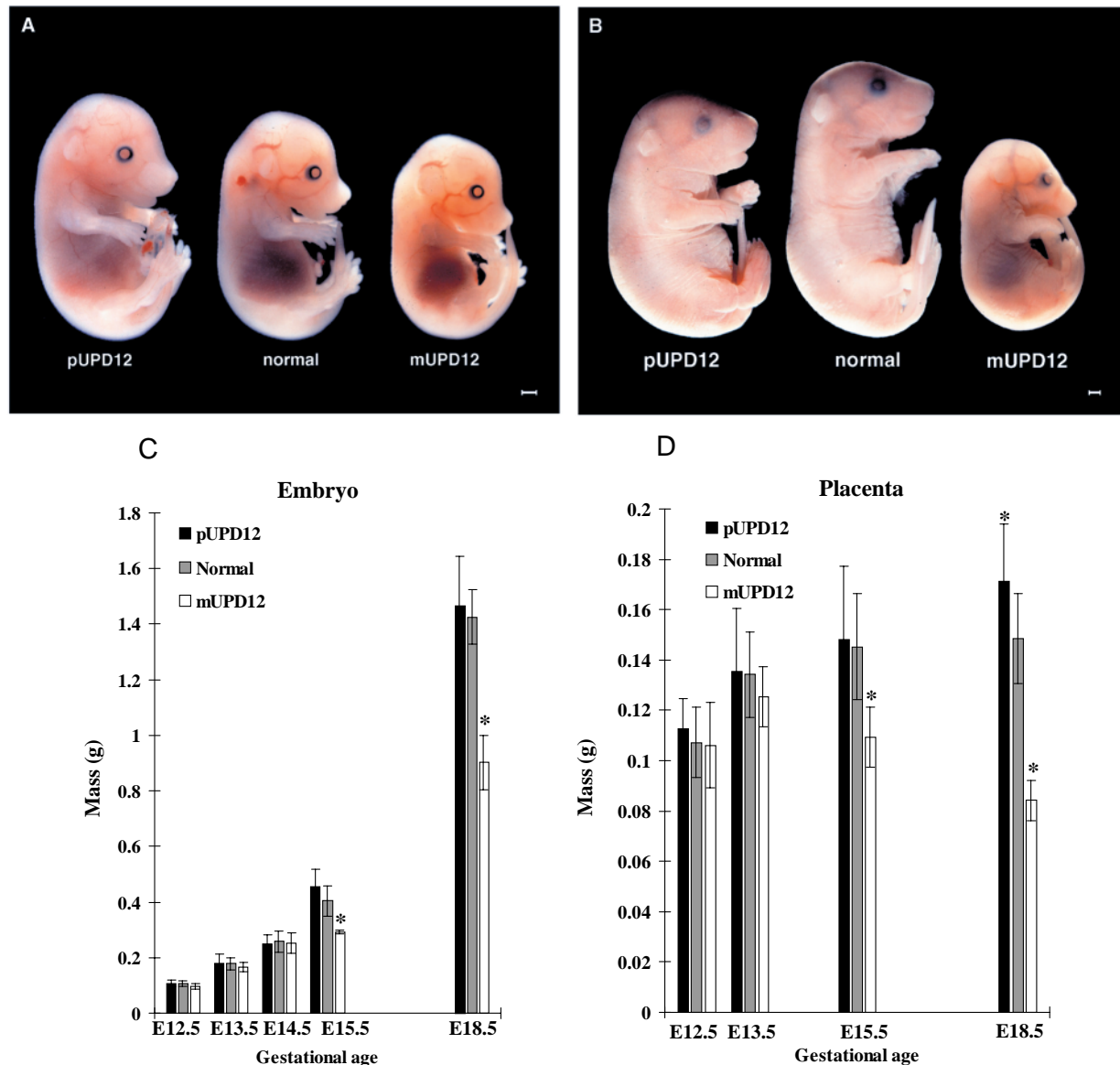


Fig. 2. Normal and UPD12 embryos at (A) E15.5 and (B) E18.5. Scale bars, 1 mm. (C,D) Graphical representation of fresh embryo (C) and placenta (D) masses throughout gestation. Values are means \pm s.d. (indicated by error bars). Statistically significant differences from normal are indicated by an asterisk (non-parametric Mann-Whitney *U*-test: $P < 0.5$). pUPD, paternal uniparental disomy; mUPD, maternal uniparental disomy.

placental mass was 75% of normal at E15.5 and 57% of normal by E18.5. Interestingly, the mean mUPD12 placental mass dropped between E15.5 and E18.5. Histological examination of these placentae revealed a proportional reduction in all regions and in situ analysis with anatomical markers showed no gross morphological defects (data not shown).

Skeletal muscle defects in UPD12 embryos

Gross histological examination of UPD12 embryos indicated parental origin-specific abnormalities in the growth and development of skeletal muscles (Fig. 3). pUPD12 embryos appeared to have a larger myofibre cross-sectional area and a disproportionately high number of myofibres with centrally located nuclei compared to normal. In contrast, inspection of mUPD12 embryos revealed an abnormally small muscle mass

and myofibre cross-sectional area. These observations were noted in all types of skeletal muscle.

To confirm and examine these findings in more detail, morphometric analysis was performed on four types of developing skeletal muscle. These were the extensor pollicis longus (EPL, a predominantly fast muscle), the extensor carpi radialis longus (ECRL, a mixed muscle) and the flexor digitorum superficialis (FDS, a predominantly slow muscle). These are hypaxial muscles of the forelimb. An example of an epaxial muscle, a paravertebral muscle, was also measured. Mean myofibre number, mean myofibre cross-sectional area and the percentage of fibres with centrally located nuclei were measured at E13.5, E15.5 and E18.5. At E13.5 there was no significant difference between mUPD12, pUPD12 and normal muscles for any of the parameters analysed (Fig. 4). The mean

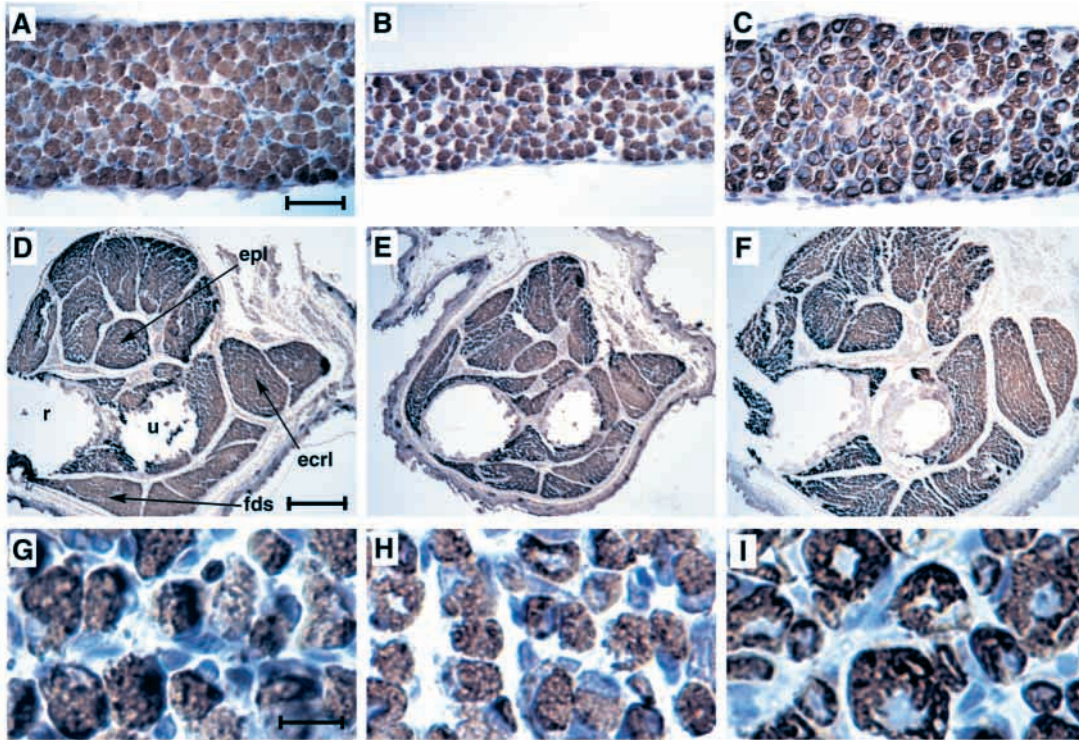


Fig. 3. Muscle morphology of normal (A,D,G), mUPD12 (B,E,H) and pUPD12 (C,F,I) embryos at E18.5 stained with the myofibril specific antibody MY32. (A-C) Comparable sections through the diaphragm and (D-F) forearm muscles. (G-I) High power views of the extensor pollicis longus muscle of normal (G), mUPD12 (H) and pUPD12 (I). ecrl, extensor carpi radialis longus; epl, extensor pollicis longus; fds, flexor digitorum superficialis; r, radius; u, ulna. Scale bars, 0.05 mm (A); 0.2 mm (D), 0.0125 mm (G).

number of myofibres was not significantly different between pUPD12 and normal embryos at any stage or in any muscle analysed (Fig. 4A).

At E15.5 and E18.5, however, the mean pUPD12 myofibril cross-sectional area was significantly increased (Fig. 4B). For mUPD12 embryos, there was a significant reduction in the number of myofibrils counted at E15.5 and E18.5 (Fig. 4A). In addition, there was a significant reduction in the mean myofibre cross-sectional area compared to normal embryos at E15.5 and E18.5 (Fig. 4B). After E15.5 in normal embryos, the mean number of myofibres with centrally located nuclei declines and reflects the maturation of the fibre as nuclei move to a peripheral location. In all pUPD12 muscles analysed, there was a dramatic retention of these centrally located nuclei. In contrast, the opposite was seen in mUPD12 embryos that had significantly fewer immature fibres (Fig. 4C).

Skeletal defects in UPD12 embryos

Examination of skeletal preparations of mUPD12 and pUPD12 embryos stained with Alcian Blue and Alizarin Red revealed a number of abnormalities. At E18.5, the thoracic cage of pUPD12 embryos protruded outwards and was bell-shaped (Fig. 5A,B). The angulation of the ribs relative to the sternum was abnormally wide and the 8th, 9th and 10th ribs were noticeably longer (Fig. 5A-D). Hypo-ossification of the sternbrae and xiphoid process was evident and, while normal embryos have seven ribs attached to the sternum, eight attachments were seen in the pUPD12 embryos (Fig. 5A-D). Hypo-ossification of the neural arch and centrum was seen in

all vertebrae. In some cases, the ossification centres of the long bones of E18.5 pUPD12 limbs were shorter and in others, they were abnormally wide (data not shown). An abnormally wide saggital suture was observed in the heads of pUPD12 embryos at this stage (Fig. 5E,F). Together these findings indicate that pUPD12 embryos exhibit costal cartilage defects and ossification abnormalities in many mesoderm-derived bones.

mUPD12 embryos at E18.5 had proportionally small skeletons, reflecting their small size, and did not exhibit a developmental delay in ossification (data not shown). However, skeletal abnormalities in the bones (ossicles) of the middle ear were observed. While ossification of the stapes appeared normal, ossification of the malleus and incus was absent (Fig. 5G,H). The fact that cartilaginous areas were detected where the malleus and incus were expected to reside suggests that these bones fail to ossify (Fig. 5G,H). This phenotype persisted in the newborn (data not shown).

Other defects

External features and subsequent examination of saggital sections of E18.5 pUPD12 embryos indicated an abnormal abdominal distention. This included protrusion of the liver, which may be associated with the reduction in body length due to the vertebral abnormalities and/or defects in the thoracic cage (Fig. 6). Closer inspection of the liver revealed an abnormally large number of blood-filled spaces (not shown). Examination of other structures in mUPD12 and pUPD12 embryos did not reveal obvious gross abnormalities; however, the presence of more subtle defects cannot be excluded.

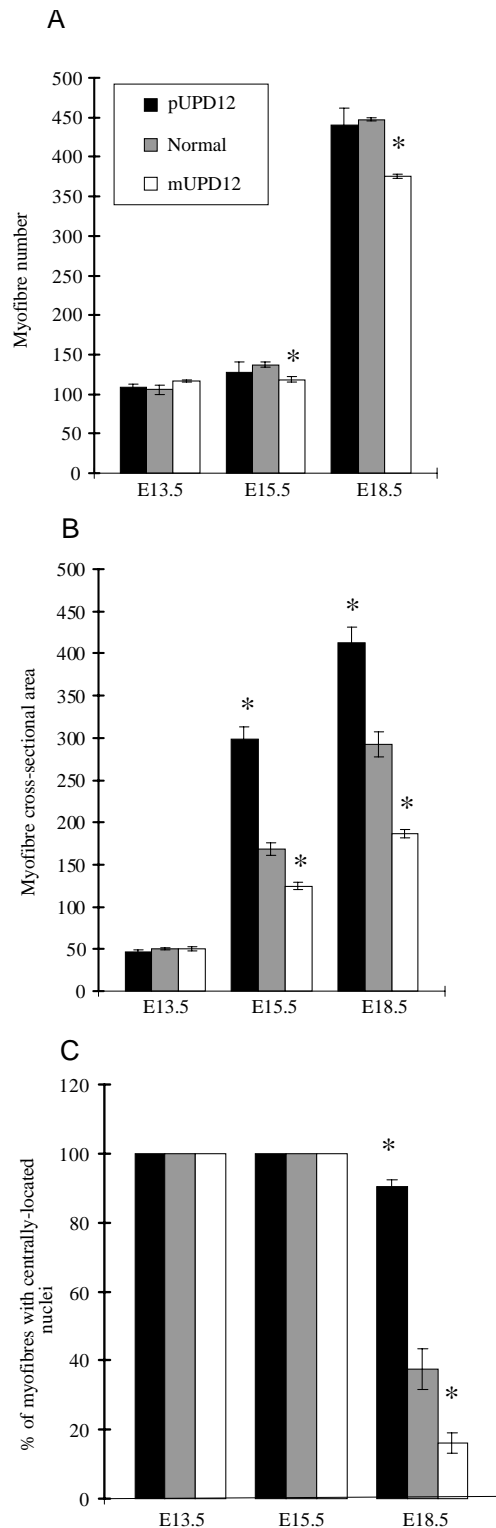


Fig. 4. Morphometric analysis showing differences in (A) mean myofibre number, (B) mean myofibre cross-sectional area (resolution is 280 pixels per linear inch) and (C) proportion of myofibres with centrally located nuclei between pUPD12, normal and mUPD12 embryos at E13.5, E15.5 and E18.5. Values shown are for the extensor pollicis longus and are representative of all muscles examined. The values are means \pm s.e.m. (indicated by error bars). Statistically significant differences are indicated by an asterisk (student's *t*-test; $P < 0.03$).

DISCUSSION

Our findings show that pUPD12 and mUPD12 animals die late in gestation and perinatally, respectively, and have defects in growth and in the development of particular lineages. This suggests a potential role of chromosome 12 imprinted genes during development, and that UPD12 conceptuses may contribute to the identification and functional analysis of these genes. Gross imprinting effects associated with the proximal portion of chromosome 12 have been ruled out (Cattanach et al., 1996), indicating that the lethality and defects we observe are most likely due to abnormal expression of imprinted genes on the distal half of chromosome 12.

Chromosome 12 imprinting is necessary for normal embryonic and placental growth. The concomitant growth retardation of mUPD12 embryos and placentae suggests placental involvement in this defect. The pUPD12 placentomegaly is associated with a larger cross-sectional area of the labyrinthine zone and other defects likely to affect placental function (P. Georgiades, M. Watkins, G. Burton and A. Ferguson-Smith, unpublished). The role of imprinted genes in growth control has been a recurrent theme in studies involving the function and evolution of imprinting. Many of the identified imprinted genes are involved in the control of prenatal growth in both mouse and human and are expressed in the placenta (Efstratiadis, 1998; Joyce and Ferguson-Smith, 1999). The growth defects described here are, in part, consistent with the 'genomic conflict' theory, which predicts that imprinted genes that promote growth and/or the utilisation of maternal resources are active on paternally inherited chromosomes and those expressed from maternally inherited chromosomes are negative regulators of growth (Moore and Haig, 1991; Haig and Trivers, 1995). Placentomegaly, in the absence of statistically significant embryonic growth enhancement in surviving pUPD12 fetuses, appears to be at odds with the conflict theory. However, one explanation for this may be that, in conjunction with the other defects, fetal overgrowth is incompatible with viability; this is consistent with the increase in fetal loss observed after E15.5 and might be tested in chimerae containing pUPD12 and normal cells, as has been shown for other paternally duplicated imprinted regions (Ferguson-Smith et al., 1991). Alternatively, the tissue-specific overgrowth seen in the skeletal muscle, and the defects associated with the maternal and fetal components of the placenta, might be considered to be consistent with the theory.

Defects in myogenesis are observed at and after E15.5; however, no comparative differences between mUPD12, pUPD12 were found at E13.5. In rodents, skeletal myofibre generation is a discontinuous process that occurs in two waves: a primary wave of myofibre formation commencing at around E12.5, followed by a secondary wave of myogenesis commencing at around E15 (Miller, 1992; Wigmore and Dungleison, 1998). Hence in UPD12 muscles, primary myogenesis appears normal and defects are associated with the secondary wave of myogenesis. These findings are consistent with evidence suggesting that factors regulating aspects of primary and secondary myogenesis are different (Wilson et al., 1988; Venuti et al., 1995). Myofibre growth appears enhanced in pUPD12 muscles, as suggested by the later gestation increase in mean myofibre cross-sectional area. The reduced number and mean cross-sectional area of myofibres in the

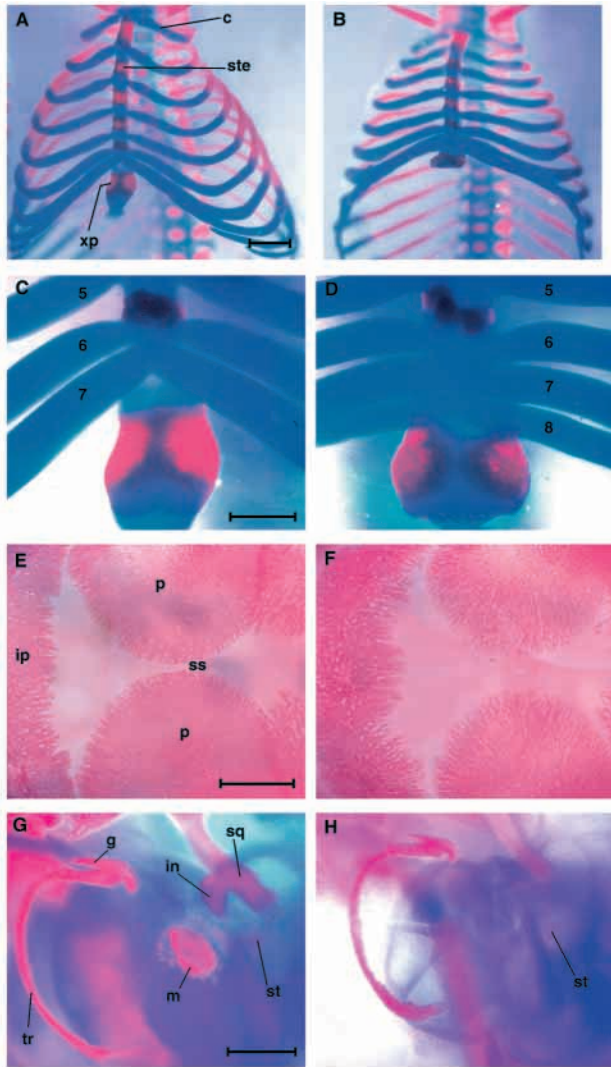


Fig. 5. Skeletal defects in E18.5 pUPD12 (B,D,F) and mUPD12 (H) embryos compared to normal (A,C,E,G). All preparations are stained with Alcian Blue/Alizarin Red except E and F, which are Alizarin Red preparations. Ventral view of the rib-cage (A-D) and dorsal part of the skull (E,F) are shown. (G) The area showing middle ear ossicles in normal embryos with the mUPD12 ossicle defect illustrated in (H). The numbers in C and D refer to the costal cartilages, with those of the anteriormost rib defined as 1. c, costal cartilage; g, gonial bone; ip, interparietal bone; in, incus; m, malleus; p, parietal bone; sq, squamosal bone; ss, sagittal suture; st, stapes; ste, ossification centre of one of the sternbrae; tr, tympanic ring; xp, xiphoid process. Scale bars, 1 mm (A); 0.25 mm (C,G); 1 mm (E).

mUPD12 embryos relative to control littermates may be due to defects in secondary myofibre formation, elongation or maintenance/viability. The presence of a significantly higher proportion of myofibres with centrally located nuclei in E18.5 pUPD12 muscles relative to normal, and the observation of an opposite phenotype in mUPD12 myofibres, suggests a reciprocal defect in myofibre maturation. Normally, as myofibres mature, the initially centrally located nuclei move to a sub-sarcolemmal location (Ontell and Kozeka, 1984; Furst et al., 1989; Zhang and McLennan, 1995). Hence, mUPD12

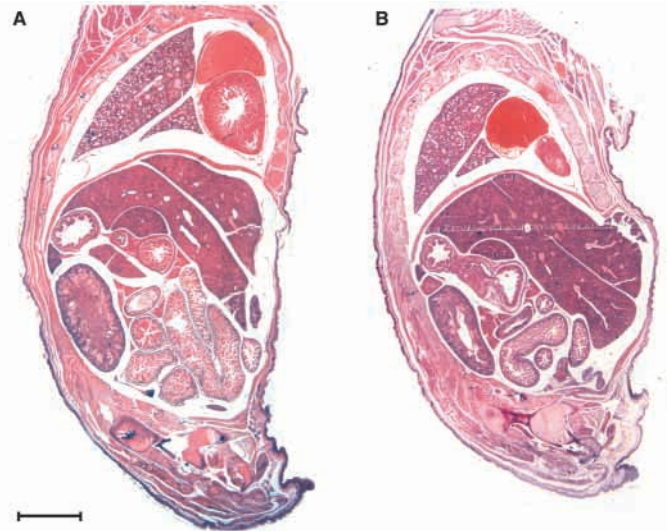


Fig. 6. Comparable sections of (A) E18.5 normal and (B) pUPD12 embryos stained with Haematoxylin and Eosin, illustrating the malformed thoracic cage and abdominal distension observed in pUPD12 embryos. Scale bar, 1 mm.

myofibres appear more mature than normal whereas a greater number of immature myofibres are present in pUPD12 muscles.

The present findings show that pUPD12 embryos have abnormalities in costal cartilage development and in endochondral ossification. These abnormalities are reminiscent though less extreme than the costal cartilage defects observed in AG-normal chimaeras (Mann et al., 1990; Barton et al., 1991; McLaughlin et al., 1997). Defects in endochondral ossification were also observed in the long bones of the limbs, vertebrae, sternbrae and the xiphoid process. Absence of ossification centres in the pUPD12 vertebrae at E15.5 suggests that these defects may be due to a developmental delay in ossification. Currently, it is not clear whether the costal cartilage defect and the hypo-ossification are caused by the same mechanism. The abnormally wide membranous sagittal suture in the skull of pUPD12 embryos at E18.5 was not accompanied by an obvious size defect in the adjacent parietal bones, which form via intramembranous ossification. It is possible that the wide sagittal suture reflects a subtle increase in brain volume or a defect in the suture membrane itself. Further experiments are required to determine the nature of this defect. Although mUPD12 embryos were growth-retarded, their mesodermal-derived bone and costal cartilage development was not developmentally delayed and was in proportion to the size of the embryo.

The heads of mUPD12 embryos lack ossification in the malleus and incus ossicles of the middle ear, which are neural crest-derived bones from the first branchial arch. Ossification of the third ossicle, the stapes, originating from the second branchial arch, was observed, consistent with the fact that its development is regulated differently from the malleus and incus (Clouthier et al., 1998; Kaufman and Bard, 1999). The presence of cartilagenous condensations at the sites of the malleus and incus suggests that the defect occurs after normal neural crest cell migration and chondrocyte differentiation.

Other derivatives of the first branchial arch (e.g. the gonial bone and tympanic ring) appeared normal.

Previous studies have shown that imprinted genes play a role in murine skeletogenesis. The study of developmental defects associated with the altered dosage of imprinted genes unlinked to chromosome 12 has identified some, though not all, of the imprinted genes involved in these pathways. Mutants in the maternally expressed *Igf2r* allele exhibit a subset of the skeletal defects seen in AG-normal chimaeras (Lau et al., 1994; Ludwig et al., 1996; Eggenschwiler et al., 1997). Embryos lacking the maternally expressed allele of the imprinted *p57^{kip2}* cyclin-dependent kinase inhibitor exhibit limb defects similar to those noted in pUPD12 embryos (Yan et al., 1997; Zhang et al., 1997). *Igf2* has also been implicated in the costal cartilage defects of AG-normal chimaeras (McLaughlin et al., 1997). *Igf2* is expressed at normal levels in mUPD12 and pUPD12 embryos (data not shown). The defects reported here suggest that imprinted genes on chromosome 12 also contribute to the development of the murine skeleton and it will be of interest to determine whether they interact with unlinked imprinted genes.

In the mouse, two transgene insertions into chromosome 12 with parental origin specific defects have been reported. First, a proximal insertion at 29 cM and distal to band B3 resulted in the acrodysplasia mutation (*Adp*). *Adp* mice are viable and exhibit vertebral, skull and limb deformities with variable expressivity and incomplete penetrance, which is manifest on paternal inheritance (DeLoia and Solter, 1990; Watanabe et al., 1994). The transgene insertion is differentially methylated on the two parental chromosomes. However, the imprinting phenotype is lost on some genetic backgrounds and the pre-integration site has not been cloned, so it is unclear whether the insertion has affected an imprinted gene or whether the parental-origin specific effects are conferred by the presence of exogenous sequences. Second, a gene-trap insertion into a more distal region of chromosome 12 causing parental origin effects has also been described; *Gtl2LacZ* (Schuster-Gossler et al., 1996). This insertion results in viable, growth-retarded offspring on paternal inheritance. The transgene inserted upstream of the *Gtl2* gene, which encodes multiple alternatively spliced transcripts, and is imprinted, being expressed predominantly from the maternally inherited chromosome (Schuster-Gossler et al., 1998; Miyoshi et al., 2000; Takada et al., 2000). There are no obvious open-reading frames associated with the sequence of this gene. Transcripts are absent from sclerotome derivatives but found in myogenic lineages as development proceeds, and later *Gtl2* is expressed in all skeletal muscles. *Gtl2* is also expressed in the brain and epithelial cells of the liver, pancreas and kidney. We have identified a second imprinted gene located 80 kb upstream of *Gtl2* called *Delta-like (Dlk)*. This gene is expressed from the paternally inherited allele, silent on the maternally inherited copy and co-expressed with *Gtl2* in most tissues during development (Takada et al., 2000). *Dlk* encodes a member of the Notch-Delta family of proteins involved in differentiation. The developmental expression of *Dlk* and *Gtl2* suggest that they may play a role in some but not all of the phenotypes we describe, suggesting the presence of other imprinted genes on chromosome 12.

An interesting muscle hyperplasia locus in sheep, called *Callipyge (CLPG)*, has been mapped to sheep chromosome 18

in a region of syntenic homology with mouse chromosome 12. *CLPG* exhibits parental origin specific defects. *CLPG/+* heterozygous lambs inheriting the mutant allele from the ewe are normal, but heterozygous lambs inheriting the *CLPG* allele from a ram have muscle hyperplasia. Intriguingly, *CLPG/CLPG* homozygous lambs are normal (Cockett et al., 1994, 1996). Comparative analysis of the genetic and molecular mechanisms causing the muscle defects in mouse and sheep should provide insight into the function and regulation of imprinted genes in this region.

Human chromosome 14q shares syntenic homology with the distal half of mouse chromosome 12. At least 20 cases of mUPD14 and 4 cases of pUPD14 have been reported (Temple et al., 1991; Wang et al., 1991; Cotter et al., 1997; Georgiades et al., 1998; Fokstuen et al., 1999). In cases of heterodisomy, where action of recessive alleles can be ruled out, distinct anomalies associated with mUPD14 and pUPD14 indicate that imprinting is involved. At birth mUPD14 patients are small for their gestational age and remain small thereafter. Most mUPD14 patients have muscular hypotonia, recurrent otitis media and obesity, and all patients old enough to be assessed had premature puberty. Some of these defects are consistent with the phenotypes we describe for the mUPD12 mice, notably growth defects and muscle abnormalities. pUPD14 patients are also small at birth, consistent with the size difference we observe in E18.5 pUPD12 embryos that survive. Consistent with our observations in the mouse, pUPD14 patients have skeletal abnormalities, which include short limbs and a small bell-shaped thoracic cage with upslanting ribs. Abdominal distention was also reported in three out of four pUPD14 patients. Three of the four pUPD14 patients were reported to have 'life-threatening complications' that required medical intervention. Nonetheless, the phenotypes we describe in UPD12 mice result in lethality and suggest an increase in severity compared to human; however, it is possible that these animals might be viable on other genetic backgrounds. Genetic background-specific differences have been observed for imprinting effects on chromosome 12 (Schuster-Gossler et al., 1996). Taken together, these studies suggest that imprinting of this region is at least partially conserved between mouse and man and that the mouse is a suitable model for investigating aspects of the UPD14 phenotype in human.

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