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Systematic elimination of parthenogenetic cells in mouse chimeras

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Summary

The developmental potential of primitive ectoderm cells lacking paternal chromosomes was investigated by examining the distribution of parthenogenetic cells in chimeras. Using GPI-1 allozymes as marker, parthenogenetic cells were detected in most organs and tissues in adult chimeras. However, these cells were under severe selective pressure compared with cells from normal fertilized embryos. In the majority of chimeras, parthenogenetic cells in individual animals were observed in a limited number of tissues and organs and, even in these instances, their contribution was substantially reduced. Nevertheless, parthenogenetic cells were detected more consistently in some organs, especially the brain, heart, kidney and spleen. In contrast, there was apparently a systematic selection against parthenogenetic cells in some tissues, most notably in skeletal muscle, liver and pancreas. These results suggest that paternally derived genes are probably required not only for the development of extraembryonic structures but also for subsequent development of embryonic tissues derived from the primitive ectoderm lineage.

Key words: parthenogenesis, chimeras, genomic imprinting, cell selection, primitive ectoderm.

Introduction

Recent investigations have demonstrated that both parental genomes are needed for normal development in the mouse (Surani *et al.* 1984; McGrath & Solter, 1984; Surani *et al.* 1986*a*). Furthermore, genetic studies have detected chromosomal regions in a number of autosomes whose influence on development is dictated by their parental origin (Cattanach & Kirk, 1985; Searle & Beechey, 1985; Cattanach, 1986). Therefore the functional differences between parental genomes observed can be attributed to germline origin of these chromosomal regions. It is likely that these chromosomal domains harbor the putative germline-specific imprinted genes which are crucial for normal development (Surani *et al.* 1986*b*). Interestingly, modification and expression of some transgenes is dependent on their parental origin which is consistent with the predicted behaviour of endogenous imprinted genes (Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987). However, no endogenous genes subject to germline-specific imprinting have yet been identified. Nevertheless, it is possible to study the consequences of imprinted genes on development. For example, the influence of imprinted genes on embryonic cells can be assessed from the developmental potential of androgenetic and parthenogenetic cells in chimeric fetuses and adults.

In previous studies it was demonstrated that parthenogenetic cells are eliminated progressively, first from the trophoblast followed by the yolk sac mesoderm and yolk sac endoderm, so that they are virtually absent from the extraembryonic tissues by about the midgestation stage (Nagy *et al.* 1987; Surani *et al.* 1988; Clarke *et al.* 1988; Thomson & Solter, 1988). However, at this time parthenogenetic cells persist and proliferate normally in embryonic tissues derived from the primitive ectoderm cells (Nagy *et al.* 1987; Surani *et al.* 1988). It was shown that parthenogenetic cells come under selection in the embryo between day 12 and 19 of fetal development; however, their precise fate in the differentiating tissues has not been analysed (Nagy *et al.* 1987). Their fate thereafter is also not known in any detail except that, in adult chimeras, parthenogenetic cells can be detected in many tissues, including the germ cells (Surani *et al.* 1977; Stevens *et al.* 1977; Stevens, 1978; Anderegg & Markert, 1986).

The purpose of this study was to carry out a detailed analysis of the distribution and contribution of parthenogenetic cells in adult chimeras. Our results show that parthenogenetic cells come under severe selective pressure between day 10 of gestation and birth and thereafter. It is likely that paternally derived chromosomal imprints are needed to sustain continued proliferation and differentiation of a variety of embryonic cell types.

Materials and methods

Animals

CFLP albino outbred mice (AFRC colony from Bantin and Kingman stock) homozygous for the glucose phosphate isomerase allozyme GPI-1A (*Gpi-1*^{a}/*Gpi-1*^{a}) and nonalbino (C57BL/6J×CBA/Ca)F_1 (from Bantin and Kingman stock, referred to as F_1) mice (*Gpi-1*^{b}/*Gpi-1*^{b}) were used in these experiments. Four- to six-week-old females were injected with 7·5 i.u. pregnant mare's serum (Intervet Ltd, Cambridge) followed 42–48 h later by 7·5 i.u. human chorionic gonadotrophin to induce superovulation (Fowler & Edwards, 1957).

Normal embryos

Embryos were obtained following fertilization *in vivo* from $F_1 \times F_1$ or CFLP × CFLP matings. The embryos were retrieved on day 2 or day 3 of pregnancy (day 1 = day of vaginal plug).

Parthenogenetic embryos

Unfertilized eggs from F_1 mice were obtained at 16·5–17·5 h post-hCG. The cumulus cells were removed by incubation with 300 i.u. ml^{-1} hyaluronidase (ovine testis type V Sigma) in PB1 + BSA (Whittingham & Wales, 1969) for 3 min and washed twice with medium T6 + BSA (Howlett *et al.* 1987). The eggs were activated in T6+BS which contained 7 % ethanol for 4·5 min at room temperature (Kaufman, 1982; Cuthbertson, 1983), washed six times with T6 + BSA and cultured in this medium with 5 μg ml^{-1} cytochalasin B (in 0·1 % dimethylsulphoxide) for 3·5–4 h at 37·8 °C in 5 % CO_2 in air. The eggs were then washed nine times in T6 + BSA and cultured for a further period of 2 h at 37·8 °C in 5 % CO_2 in air. After this time all the diploid eggs containing two pronuclei produced by the suppression of second polar body extrusion by cytochalasin B (Niemierko, 1975) were separated (60–90 %) from haploid, fragmented and other abnormal eggs and cultured for 2 days in T6 + BSA.

Preparation of aggregation chimeras and transfer to the uterus

Asynchronous parthenogenetic ↔ fertilized aggregation chimeras (PFCs) were made using 2-cell fertilized embryos and 4-cell parthenogenetic embryos. The zona pellucida was first removed by a brief exposure (5–20 s) to acid Tyrode's medium pH 2·0 (Nicholson *et al.* 1975) and washed six times in T6 + BSA. The two types of embryos to be aggregated were then placed in separate 10 μl drops of T6 + BSA medium under liquid paraffin oil. Each pair of embryos was aggregated by pushing the two embryos into small wells made with a blunt needle in the bottom of the plastic Petri dish and cultured in T6 + BSA in 5 % CO_2 in air. The chimeric embryos were then left in culture for 48 h. Control fertilized ↔ fertilized chimeras (FFCs) were made using two day-3 8-cell embryos. These aggregated embryos were cultured for 24 h. All the aggregated embryos that developed into morphologically normal blastocysts were transferred to day-3 pseudopregnant recipient F_1 females obtained by mating with vasectomized males of proven sterility. The recipients were left until day 20 of gestation for development of the embryos to term.

Analysis of chimeras

Adult chimeras were injected intraperitoneally with approximately 700 USP-K-1 units of heparin (Sigma, St Louis, MO) and exsanguinated approximately 30 min later by opening the hepatic vein under ether anaesthesia in order to eliminate contamination of tissues with blood. Either whole organs (heart ventricle, kidneys, gonads, spleen, bladder) or 40–60 mg samples of tissues (skeletal muscle, liver, brain, thymus, lung, gut, pancreas) were collected. Liver samples were taken from the left lobe and from the left and right side of the median lobe; gut samples were from the duodenum and the descending colon. Three to four definite regions of the brain were dissected for analysis; medulla oblongata, cerebellum and both the cerebral hemispheres without thalamus. Between five and ten samples of skeletal muscle from different sites were taken. From one chimera three additional tissues, lymph node, adipose tissue and adrenals were dissected out.

GPI-electrophoresis

GPI analysis of chimeras was carried out first on cellulose acetate using Helena Titan III electrophoresis plates (Eicher & Washburn, 1978). Tissue extracts that showed parthenogenetic contribution were analysed further by electrophoresis on cellulose acetate gel (Chemetron, Milan, Italy) with subsequent semiquantitative determination of relative allozyme activity (Bücher *et al.* 1980). Electrophoresis was carried out for 80 min at 300 V at 18 to 20 °C in Tris–glycine buffer (Eicher & Washburn, 1978). Staining was carried out as described before (Fundele *et al.* 1985).

DNA analysis

DNA was isolated from tissues of chimeric animals as described previously (Reik *et al.* 1987), restricted with *Bam*HI, and Southern blots were hybridized with a Immunoglobulin Heavy Chain probe. Polymorphic *Bam*HI fragments at the Ig-H locus that are characteristic for the CFLP, CBA and C57BL/6J genotypes, respectively, were used to determine the relative contributions in chimeric tissues.

Phosphoglycerate kinase (PGK) and phosphoglycerate mutase (PGAM) electrophoresis

PGK and PGAM isozymes were used as markers for spermatogenesis (VandeBerg *et al.* 1976; Fundele *et al.* 1987). Electrophoresis was carried out as described earlier (Fundele *et al.* 1987; Bücher *et al.* 1980).

Statistical analysis

In view of the frequency of zero values in the PFC group, a classical parametric hypothesis test (PFCs *vs* FFCs) using the standard errors would not have been appropriate. Consequently the significance probabilities were derived from an application of Fisher's nonparametric randomization test. In comparing the incidence of parthenogenetic cells in various tissues Fisher's randomization test for a contingency table was used.

Results

Table 1 summarizes the results of the aggregation experiments. Fifteen PFCs were obtained and of these five males and eight females were analysed. One PFC was killed together with its nine litter-mates by the foster mother at birth. This chimera was considerably smaller than its litter-mates and had substantial contribution from parthenogenetic cells as judged from GPI-1B activity of 22 % (determined after homogenization of the whole body). The contribution of parthenogenetic cells to various tissues of thirteen PFCs is given in

Table 1. *Results of fertilized↔fertilized (FFC) and parthenogenetic↔fertilized (PFC) aggregations (percentages of total number of aggregations in parentheses)*

Number of experiments	Number of aggregations	Number of embryos transferred	Mice born	Chimeras
PFC 7	375 (100)	176 (47)	40* (11)	15† (4)
FFC 1	32 (100)	32 (100)	21 (66)	16 (50)

* In most cases non-chimeric albino offspring were killed without any further analysis to avoid competition of the large CFLP newborns against the usually much smaller PFCs.

† One chimera not analysed, one killed by foster mother after birth and analysed after homogenization of the entire body.

Table 2 and compared to the fate of fertilized F_2 cells in tissues of six randomly selected FFCs. A reduced panel of tissues was analysed in six additional chimeras (Table 3).

When all chimeras are considered, parthenogenetic cells were apparently not excluded from any of the embryonic cell lineages as they were found in ectodermal, mesodermal and endodermal derivatives. However, when distribution of parthenogenetic cells is examined in individual animals, these cells were present in a few tissues only, where they contributed a minor proportion of cells (PFCs 1,3,4,7,8,11,12,13). Nevertheless, four PFCs had parthenogenetic cells in the majority of the tissues with a relatively high proportion of parthenogenetic cells in some tissues (PFCs 2,5,6,10). PFC9 was an exceptional chimera with only marginal coat chimerism and three positive tissues, but two of these had a high proportion of parthenogenetic cells. By contrast, FFCs showed a much more balanced distribution of CFLP and F_2 cells. Only FFC5 had a few tissues that were derived from only one of the aggregated embryos, in this case from the F_2 embryo.

Besides the overall reduction of parthenogenetic cells in all chimeras, an additional tissue-specific selection seems to take place. For instance, brain, heart, duodenum and kidneys were frequently found to contain parthenogenetic cells. In contrast, low proportions and/or rare occurences of parthenogenetic cells were observed in skeletal muscle, pancreas and liver. Participation of parthenogenetic cells in skeletal muscle was observed only in one case, PFC5 (maximum value 23%, minimum value 4%). The presence of heterodimers (GPI-1AB) showed that myoblast fusion between fertilized and parthenogenetic cells had taken place. The other PFCs, including PFCs 2, 6 and 10, which had a considerable contribution from parthenogenetic cells, showed no GPI-1B activity in skeletal muscle.

In the case of PFC10, a number of tissues (skeletal muscle, heart, kidney, thymus, pancreas, liver) were further assayed by DNA analysis in order to exclude that fusion between parthenogenetic and fertilized myoblasts had taken place but the parthenogenetic genome failed to be expressed. In all tissues including skeletal muscle, the results of DNA analysis were identical with those obtained by GPI-1 analysis (data not shown). In FFCs a reduced participation of F_2 myoblasts in skeletal muscle formation was not observed. In the six FFCs analysed we determined a mean GPI-1B value in skeletal muscle of 68%, while the mean total contribution to all other tissues was 55%.

Pancreas was another tissue in which decreased contribution of parthenogenetic cells was observed. Only PFC2 had GPI-1B activity in this organ. Tables 2 and 3 show that there is no selection against F_2 cells in pancreas of FFCs. It is however, noteworthy that, in FFCs 3, 10 and 11, pancreas showed a rather unbalanced mosaicism with percental GPI-1B values 18, 19 and 82 compared to mean values of 55 ± 3, 38 ± 3 and 61 ± 4 for the other tissues given in Table 3 ($\bar{x} \pm$ S.E.M.).

The contribution of parthenogenetic cells to the liver was also low. Only PFCs 2, 6, 9 and 10 were positive and of the three samples analysed, only one was positive in PFCs 6 and 9 (3% and 24%) and two were positive in PFC10 (4% and 2%). All three liver samples were positive for GPI-1B (3%, 4% and 9%) in PFC2. Contamination of liver samples with blood can be ruled out as a source of GPI-1B, as PFC6 was positive for GPI-1B in blood but negative in liver, PFC9 showed the opposite distribution (Table 2). In livers of FFCs, F_2 cells were not selected again (Table 2).

Two of the male chimeras, PFC5 and PFC10, had GPI-1B activity in the testes showing that female parthenogenetic cells can participate in testis differentiation. Using the sperm-specific isozymes PGK-2 and PGAM-B as markers, no spermatogenesis took place in the testes of PFC5, whereas PFC10 showed PGK-2 and PGAM-B in both testes. The germ cells of PFC10 were derived exclusively from the fertilized CFLP embryo, as judged from the allelic PGK-2 form (data not shown). Testes weights were 1·6, 1·5, 33·8 and 38·2 mg for PFCs 5 and 10, respectively. In PFC6, three additional tissues, lymph node, adrenals and adipose tissues were analysed. All samples were positive for GPI-1B with values of 12%, 3% and 4%, respectively.

When parthenogenetic cells were present in the majority of tissues it resulted in marked reduction in size of the chimera. In case of PFC2, three non-chimeric litter-mates were alive when the animal was killed. PFC2 had a body weight of 4·58, compared to 7·05, 7·28 and 6·68 g of its litter-mates, on day 8. In the case of PFCs 6, 7 and 8, which were litter-mates, body weights were determined on day 20 of age prior to autopsy. PFC6, which had an overall contribution of 10% parthenogenetic cells detected in twelve out of fourteen tissues, weighed 6·1 g. PFCs 7 and 8, both of which had mean contributions of 1% parthenogenetic cells detected in three tissues, weighed 10·0 and 11·0 g, respectively. The weight of PFC5 was not determined before autopsy but from the heart weight of 27 mg, a body weight of approximately 4 g can be estimated. For comparison PFCs 6, 7 and 8 had heart weights of 37, 58 and 65 mg. Mean body weights of approximately 13 g and 12 g were determined for 20-day-old CFLP and F_2 mice, respectively.

Table 2. *Participation of parthenogenetic and fertilized F_2 cells in aggregation chimeras given in % GPI-1B*

	PFC														FFC						
Tissue	1	2	3	4	5	6	7	8	9	10	11	12	13	$\bar{x} \pm$ S.E.M. of PFCs	1	2	3	4	5	6	$\bar{x} \pm$ S.E.M. of FFCs
Brain*	12	19	7	16	23	11	0	0	1	10	7	1	4	9 ± 2	39	78	42	49	79	26	52 ± 9
Muscle†	0	0	0	0	13	0	0	0	0	0	0	0	0	1 ± 1	68	68	70	70	82	47	68 ± 5
Heart	3	8	7	6	22	16	0	0	0	13	7	4	3	7 ± 2	50	70	82	57	78	44	63 ± 7
Kidneys	2/2	4/4	0/0	0/0	13/21	9/13	3/0	0/3	0/0	7/9	0/4	4/3	0/0	4 ± 1	31/32	49/53	47/58	61/55	81/72	44/35	52 ± 6
Spleen	0	22	10	8	43	13	0	0	0	28	10	0	10	11 ± 4	42	76	66	61	77	46	61 ± 6
Blood	9	17	n.d.	0	35	9	0	12	0	24	4	5	0	10 ± 3	51	53	38	47	65	40	49 ± 4
Thymus	0	5	0	0	39	5	0	0	0	10	8	0	0	5 ± 3	33	44	47	54	73	28	47 ± 7
Pancreas	0	7	0	0	0	0	0	0	0	0	0	0	0	1 ± 1	34	68	18	63	100	19	51 ± 13
Liver	0	5	0	0	0	1	0	0	8	2	0	0	0	1 ± 1	31	64	58	61	100	23	56 ± 11
Duodenum	10	25	26	17	29	30	5	0	33	4	0	0	0	14 ± 4	40	81	73	69	82	31	63 ± 9
Colon	0	12	0	0	19	9	0	0	n.d.	7	0	0	0	4 ± 1	47	69	50	58	87	45	59 ± 7
Lung	0	5	0	0	0	11	0	0	0	20	3	2	0	3 ± 2	41	78	40	56	75	20	52 ± 9
Bladder	n.d.	12	0	0	12	11	0	5	0	0	0	n.d.	0	4 ± 2	49	62	61	57	81	47	59 ± 5
$\bar{x} \pm$ S.E.M.	3 ± 1	10 ± 2	4 ± 2	3 ± 2	9 ± 4	10 ± 2	1 ± 0	1 ± 1	3 ± 2	10 ± 2	3 ± 1	1 ± 1	1 ± 1	–	42 ± 3	65 ± 3	54 ± 5	57 ± 2	81 ± 3	36 ± 3	–
Gonads‡	n.d.	n.d.	n.d.	n.d.	20/21	7/11	5/3	0/0	0/0	7/8	0/0	0/0	5/2	–	10/11	20/17	39/30	83/85	100/100	23/24	
Sex	♀	♀	♀	♀	♂	♀	♀	♂	♂	♂	♂	♀	♀		♂	♂	♂	♂	♂	♂	
Age at autopsy (days)	8	8	9	9	20	20	20	20	22	26	51	51	66		20	29	53	54	72	74	

* Cerebellum was not analysed in PFCs 1 to 5.

† Ten muscle samples were analysed from PFCs 1 and 6, five from PFCs 1–4 and from all other chimeras six samples were dissected and analysed.

‡ For the determination of mean GPI-1B percentages per chimera values determined in gonads were not used, because of the selection against female cells in the gonads of an XX↔XY chimera developing as a male. Where more than two values were measured per tissue, e.g. brain, skeletal muscle and liver, mean values were used.

The differences between GPI-1B contribution in PFCs compared to FFCs are statistically significant for all the tissues shown in Table 2 ($P < 0{\cdot}001$). Comparing incidence of parthenogenetic cells in the tissues of PFCs, differences between brain and pancreas, and cardiac and skeletal muscle were significant at the $P < 0{\cdot}001$ level.

n.d. = tissue not analysed.

Table 3. *Percental values of GPI-1B in tissues of twelve control CFLP↔F_2 chimeras (FFCs)*

	FFC1*	FFC2*	FFC3*	FFC4*	FFC5*	FFC6*	FFC7	FFC8	FFC9	FFC10	FFC11	FFC12	x ± S.E.M.†
Heart	50	70	82	57	78	44	70	48	45	28	62	57	57 ± 5
Kidneys	31	49	47	61	81	44	59	36	37	45	60	69	51 ± 4
	32	53	58	55	72	35	67	43	45	42	44	67	
Lung	41	78	40	56	75	20	78	44	54	31	60	64	53 ± 6
Spleen	42	76	66	61	77	46	73	57	83	44	76	68	64 ± 4
Blood	51	53	37	47	65	40	60	52	73	37	66	53	53 ± 3
Pancreas	34	68	18	63	100	19	n.d.	n.d.	62	19	82	75	54 ± 9

* FFCs 1–6 correspond to FFCs in Table 2.
† Values are means ± standard error of the mean.

Discussion

This study demonstrates that the proportion of parthenogenetic cells is substantially reduced in adult chimeras. While contributing normally to midgestation embryos (Surani *et al.* 1988) parthenogenetic cells subsequently come under severe selective pressure between day 12 and 19 (Nagy *et al.* 1987). It was previously demonstrated that parthenogenetic embryos in ectopic sites can generate teratomas with a variety of differentiated tissues (Iles *et al.* 1975). Therefore it was expected that parthenogenetic cells can also give rise to a variety of differentiated cells in adult chimeras, although they fail to proliferate normally.

There is non-uniformity in the severity of selection against parthenogenetic cells in various tissues. For instance in skeletal muscle, parthenogenetic cells are eliminated much more consistently than in other mesodermal derivatives such as cardiac muscle ($P < 0{\cdot}001$). In the one chimera where parthenogenetic cells participated in differentiation of skeletal muscle, GPI-1AB heterodimers were observed. This shows that fusion of parthenogenetic and normal myoblasts had occurred in this instance. It is possible that the parthenogenetic genome becomes functionally repressed in the presence of normal nuclei in myotubes. However, this possibility was largely ruled out by employing a DNA polymorphic marker which failed to detect the presence of parthenogenetic nuclei in skeletal muscle of PFC10.

Amongst the endodermal derivatives there was a particular paucity of parthenogenetic cells in liver and the pancreas, compared to other organs with endodermal derivatives such as thymus, lung and especially duodenum. In the pancreas of control chimeras (fertilized↔fertilized) there was a tendency (unlike in most other organs) for wide variations in contribution to this organ by the cells of the two genotypes, it is therefore possible that in F_2↔CFLP chimeras, pancreas is derived from a small pool of progenitor cells. Hence, parthenogenetic cells may be excluded in the pancreas as a sampling artifact. Alternatively, the pancreas which consists predominantly of endoderm-derived exocrine cells (Wessels & Evans, 1968) and to a lesser extent of endocrine cells (possibly of neuroectodermal origin; Pearse, 1977; Alpert *et al.* 1988) may be subject to selection against parthenogenetic cells similar to that observed in the liver. Most tissues analysed here are composed of a variety of differentiated cell types. It is possible that selection affects specific subpopulations of parthenogenetic cells in various organs, which cannot be determined by using the GPI-1 marker. Detailed analysis with *in situ* markers is essential to identify the specificity of selection against the different cell types derived from parthenogenetic cells.

Parthenogenetic cells participated in the formation of testis where, being female, they were probably restricted to differentiation into Leydig cells (Burgoyne *et al.* 1988). This is supported by our findings on spermatogenesis in PFC10 where germ cells were exclusively of fertilized origin. The fact that no spermiogenesis took place in testes of PFC5, as shown by the absence of PGK-2 and PGAM-B, was probably due to PFC5 being extremely runted.

We also observed that chimeras in which there was a significant contribution by parthenogenetic cells tended to have reduced body weight. Similar findings have been reported previously in chimeras both with parthenogenetic as well as with uniparental gynogenetic embryos (Stevens *et al.* 1977; Stevens, 1978; Anderegg & Markert, 1986). In this context, it is interesting to note that animals with maternal disomy with respect to several chromosomal regions (e.g. 11, 2) suffer from growth retardation and reduced viability (Cattanach & Kirk, 1986; Cattanach, 1986).

Overall it appears that parthenogenetic cells are eliminated sequentially from the chimeric conceptus. This occurs first in the trophoblast tissue followed by other extraembryonic tissues in the yolk sac. Accordingly, parthenogenetic cells contribute negligibly to these tissues after midgestation while still being detectable in the embryo at the same relative frequency and proportion as normal cells (Nagy *et al.* 1987; Surani *et al.* 1988; Clarke *et al.* 1988; Thomson & Solter, 1988). As shown both in this study and previously (Nagy *et al.* 1987), parthenogenetic cells are subsequently eliminated from the chimeric embryo as well. What controls the timing of their elimination is not known at present nor is it clear why there is non-uniformity of selection in different tissues. However, our results point to possible roles of parental chromosomes and imprinted genes in influencing differentiation and growth of embryonic cells. Further studies have to elucidate more systemati-

cally at what time in fetal development selection begins in various tissues, as this may provide some indications on the temporal and spatial expression of imprinted genes in normal development.

It is also of interest to determine if the phenotypic variations and embryonic lethality encountered in mice with duplications and deficiencies of particular chromosomal regions (reviewed by Searle & Beechey, 1985; Cattanach, 1986), can be attributed to an abnormal development of specific tissues. Identification of the precise cell types affected in these disomic mice could help to link different imprinted chromosomal domains with the effects on specific embryonic tissues. If this is the case, it would enhance our understanding of the role of imprinted genes in mouse development and help towards identification of these genes.

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