REPRODUCTION
RESEARCH

Evaluation of triploid ↔ diploid and trisomy-3 ↔ diploid mouse chimeras as models for investigating how lineage restriction occurs in confined placental mosaicism

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Abstract

Human confined placental mosaicism (CPM), where the placental trophoblast is mosaic for a chromosome abnormality but the fetus is chromosomally normal, can cause problems for prenatal diagnosis, but its causes are poorly understood. Tetraploid ↔ diploid chimeras provide a model for the development of one type of CPM, but animal models for other types of restricted mosaicism are needed. The objective of the present study was to evaluate triploid ↔ diploid and trisomy-3 ↔ diploid chimeric mouse conceptuses as new models for investigating the development of restricted mosaicism. Novel stocks of mice were generated to produce triploid and trisomy-3 embryos that could be identified by DNA *in situ* hybridisation to a chromosome 3 transgenic marker. Triploid ↔ diploid and trisomy-3 ↔ diploid mouse chimeras were produced by embryo aggregation, and the contribution of triploid or trisomy-3 cells was analysed in the fetus and extraembryonic tissues. Only two trisomy-3 ↔ diploid chimeras were analysed but trisomy-3 cells contributed well to all lineages, so these chimeras did not show restricted mosaicism. In contrast, triploid cells usually contributed poorly to all lineages in the ten 3n ↔ 2n chimeras analysed. They contributed more to the primitive endoderm derivatives than other lineages and were present in the primitive endoderm derivatives of all ten chimeras, but excluded from fetuses and trophectoderm derivatives in some cases. This pattern of restricted mosaicism differs from that reported for tetraploid cells in tetraploid ↔ diploid chimeras, and triploid ↔ diploid chimeras may provide a useful model for the development of some types of restricted mosaicism in human conceptuses.

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Introduction

A high proportion of human preimplantation embryos have chromosomal abnormalities but most are lost either during preimplantation stages or as later spontaneous abortions (Hassold *et al.* 1980, Hassold & Jacobs 1984). Some survive as mosaics and these include both generalised mosaics, where chromosomally normal and abnormal cells co-exist throughout the conceptus, and restricted (or confined) mosaics, where only some developmental lineages are mosaic. Type-I confined placental mosaicism (CPM) is where the placental trophoblast is mosaic for a chromosome abnormality, but the fetus and other epiblast derivatives are non-mosaic and usually chromosomally normal (Kalousek & Dill 1983, Kalousek 1990, 1994, Wolstenholme *et al.* 1994, Kalousek & Vekemans 1996, Wolstenholme 1996).

Human CPM usually involves mosaicism for a trisomic (Ts) cell line, but not all trisomies are equally associated with CPM (Wolstenholme 1996) and CPM involving polyploid mosaicism also occurs (Tuerlings *et al.* 1993, Kalousek 1994, Wolstenholme *et al.* 1994, Wilkins-Haug *et al.* 2006). CPM is a clinically significant condition that affects ~2% of conceptuses. It can produce a false-positive result after prenatal diagnosis by chorionic villus sampling and may be associated with prenatal or perinatal complications (Bennett *et al.* 1992, Kalousek & Vekemans 1996). There is a need to develop a range of realistic animal models of human CPM and other forms of restricted mosaicism to help understand how different types may arise.

Mouse tetraploid ↔ diploid (4n ↔ 2n) chimeras provide one useful animal model because tetraploid (4n) cells contribute poorly to derivatives of the fetus and other epiblast derivatives but well to the primitive endoderm and trophectoderm lineages (Tarkowski *et al.* 1977, 2001, Nagy *et al.* 1990, James *et al.* 1995, Goto *et al.* 2002) (In the mouse, the primitive endoderm tissue produces the endoderm layer of the visceral yolk sac and the parietal endoderm associated with Reichert's membrane. The equivalent lineage in human conceptuses is usually called the hypoblast). The 4n ↔ 2n animal model has been used to investigate the mechanisms responsible for the restricted

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distribution of chromosomally abnormal cells (James *et al.* 1995, Everett & West 1996, 1998, Goto & Takagi 1998, Everett *et al.* 2000, Tang *et al.* 2000, Goto *et al.* 2002, Eakin *et al.* 2005, MacKay & West 2005). However, human 4n/2n CPM is relatively uncommon, so new animal models are required. The aim of this study was to evaluate two other possible models of restricted mosaicism involving, respectively, another class of polyploid cells (triploid) and a class of aneuploid cells (trisomy-3) in mouse chimeras.

The origin of human triploid/diploid mosaics has been discussed by several authors (e.g. Daniel *et al.* 2003, Golubovsky 2003). Although most die, their fate is variable and may depend on whether triploidy is diandric or digynic, the relative proportions and distributions of triploid and diploid cells, the genetic background and the presence of other chromosomal anomalies. Some human triploid/diploid mosaics show CPM (Sarno *et al.* 1993, Tuerlings *et al.* 1993, Daniel *et al.* 2003) but others survive postnatally as mosaic individuals (van de Laar *et al.* 2002). The previous reports of mouse triploid↔diploid chimeras showed that 3n cells can contribute to extraembryonic, fetal and adult tissues (Azuma *et al.* 1991, Suwinska *et al.* 2005), but it is not yet known whether 3n cells contribute less readily to the fetus (and other epiblast derivatives) than to the primitive endoderm and trophectoderm derivatives.

The ideal model of human CPM would be mosaicism for a mouse trisomy that is equivalent to a human trisomy that is commonly involved in type-I CPM. Spontaneous trisomic/diploid (Ts/2n) mosaicism is very rare in mammals other than humans, so experimental trisomic↔diploid (Ts↔2n) mouse chimeras offer a better chance of developing a realistic model for CPM. Specific trisomic mouse embryos can be produced from mice heterozygous for particular Robertsonian translocations (Gropp *et al.* 1974, Gropp 1976), but because mouse and human chromosomes are not syntenic, it is difficult to decide which mouse trisomies are likely to be most relevant to the trisomies commonly involved in type-I human CPM.

The production of mouse Ts↔2n chimeras is technically demanding and relatively few have been produced (Magnuson *et al.* 1982, Cox *et al.* 1984, Epstein *et al.* 1984, Fundele *et al.* 1985, Epstein 1986). In Ts↔2n chimeras studied so far (Ts12, Ts15, Ts16, Ts17 and Ts19), there was little or no selection against Ts cells in the fetus. These previous studies were not intended to model CPM and the extraembryonic tissues were usually ignored or pooled for analysis. For the current study, we investigated the distribution of trisomy-3 cells in mouse Ts3↔2n chimeras. Mouse Ts3 fetuses die at early stage (10–11 days) with hypoplasia and developmental retardation (Gropp *et al.* 1974, 1983).

For this study, we developed special strains of mice to generate either digynic 3n or Ts3 embryos carrying a transgenic lineage marker on chromosome 3, which is highly reiterated and so readily detectable by DNA *in situ* hybridisation (Lo 1986, Lo *et al.* 1987, Katsumata & Lo 1988, Keighren & West 1993, Everett *et al.* 1994). DNA *in situ* hybridisation was used to detect chromosome 3, such that two hybridisation signals were produced in interphase nuclei of 3n or Ts3 cells but only one signal was seen in 2n nuclei. We have used this novel approach to assess the contribution of 3n and Ts3 cells to different developmental lineages of postimplantation stage mouse chimeras. Our results suggest that 3n↔2n chimeras may provide a useful new model system for investigating the development of restricted mosaicism.

Results

Frequency and physical characteristics of 3n↔2n chimeras

Three different strain combinations were used to produce 3n↔2n chimeras. The frequency of 3n↔2n chimeras (Table 1) was higher in series TrB and TrC (9/22 chimeras; 40.9%) than in series TrA (2/27 chimeras; 7.4%). Different frequencies were expected because of the nature of the strains used, but the frequency of 3n↔2n chimeras in TrA was lower than that expected. TRIP is an LT/SvKau congenic and so expected to produce about 40% 3n embryos, whereas derived strain PO2 was expected to produce ∼23% 3n embryos (West *et al.* 1993, Everett *et al.* 2004). The frequency of non-chimeric conceptuses varied among series and this may also partly reflect differences in strain combination. All were entirely NIH or FVB genotype (embryo 2) and some may have been 3n↔2n chimeras in which 3n cells were depleted to below the detectable level.

Physical parameters of the two E12.5 3n↔2n chimeras are shown in Table 2. Although 3n↔2n chimera TrA-16 had reached the average developmental stage (hind limb morphology index) for its age, it was well below the control average for conceptus weight, fetal weight, placental weight and crown-rump length, and the placental weight was below the minimum for the control group. Triploid↔2n chimera TrA-26 was within the control range for all physical parameters measured.

Figure 1 shows examples of sections of E9.5 3n↔2n and 2n↔2n chimeras and the hybridisation signals used to distinguish between chimeras containing *Tg/Tg/*− 3n cells and those containing *Tg/*− 2n cells. Abnormalities were seen in two of the E9.5 3n↔2n chimeras. Chimera TrC-26 was an empty gestational sac, without a fetus or amnion, and chimera TrB-3 had an abnormal allantoic vesicle, which had not fused normally with the chorion (Fig. 1F).

Contribution of 3n cells to different tissues of 3n↔2n chimeras

The percentages of glucose phosphate isomerase (GPI)1-A in the fetus and different extraembryonic tissues of the

Table 1 The number of chimeric conceptuses recovered from each chimera series analysed (see text for details of mouse strains).

Series	Age	Embryo 1 (GPI1-A)↔Embryo 2 (GPI1-B) (female×male)↔(female×male)	Number (%) of conceptuses						
			Implantations	Empty	Non-chimeric[a]	Unclassified	2n↔2n	3n↔2n	Ts3↔2n
3n↔2n chimeras									
TrA	E12.5	PO2×(BALB/c×A/J)F1↔NIH×NIH	34	0	6 (17.6[b])	1[c]	25 (92.6[d])	2 (7.4[d])	–
TrB	E9.5	TRIP×(BALB/c×A/J)F1↔NIH×NIH	8	1 (12.5)	1 (8.0)	0	4 (66.7)	2 (33.3)	–
TrC	E9.5	TRIP×(BALB/c×A/J)F1↔FVB×FVB	38	6 (15.8)	16 (50.0)	0	9 (56.3)	7 (43.8)	–
Ts3↔2n chimeras									
TS	E9.5	(BALB/c×A/J)F1×(TGA×RBT)↔FVB×FVB	51	8 (15.7)	9 (20.9)	0	32 (94.1)	–	2 (5.9)

[a]All non-chimeric conceptuses in all four groups were derived from diploid embryo 2 (NIH or FVB).
[b]Percentage of all conceptuses.
[c]Conceptus TrA-6 was chimeric in only the yolk sac mesoderm and was not classified as 2n↔2n or 3n↔2n.
[d]Percentage of chimeric conceptuses (excluding unclassified conceptuses).

E12.5 conceptus are shown in Table 3 for the two 3n↔2n chimeras in series TrA together with their 2n↔2n controls. Overall, the controls showed a balanced chimeric composition with the means for each tissue being ~50%. Triploid cells made no contribution to the fetus in either of the E12.5 3n↔2n chimeras, but contributed to the primitive endoderm lineage (visceral yolk sac endoderm and parietal endoderm) of both chimeras and also contributed to the extraembryonic epiblast tissues (amnion and visceral yolk sac mesoderm) and the trophectoderm (trophoblast cells overlying Reichert's membrane and placenta) of chimera TrA-16.

Table 4 shows the contribution of 3n cells to the fetus and different extraembryonic tissues of eight E9.5 3n↔2n chimeric conceptuses in series TrB and TrC and five of the E9.5 2n↔2n control chimeras (ordered by contribution to the fetus). 3n↔2n chimera TrC-25 was damaged and so was not analysed and TrC-26 was an incomplete conceptus with no fetus.

Overall, the 3n cells made a low contribution to the E9.5 3n↔2n chimeras. Grouping the different tissues into four lineages (excluding the allantois and chorion, which were only scored for TrB-3) showed that 3n cells contributed 7.8±3.9% to the fetus, 7.9±3.1% to the extraembryonic epiblast (amnion plus visceral yolk sac mesoderm), 16.8±4.6% to the primitive endoderm and 9.5±4.7% to the trophectoderm. The contribution of 3n cells varied significantly among these four lineages ($P=0.01$ by the Kruskal–Wallis test). Although the contribution of 3n cells was lowest in the fetus, Mann–Whitney *U*-tests revealed no significant differences between the fetus and the extraembryonic epiblast ($P=0.75$), the primitive endoderm ($P=0.09$) or the trophectoderm ($P=0.09$). However, the contribution of 3n cells was significantly lower in the epiblast as a whole (fetus plus extraembryonic epiblast) than the primitive endoderm ($P=0.03$) but not the trophectoderm lineage ($P=0.09$).

The contribution of 3n cells to the trophectoderm derivatives was significantly lower than in the primitive endoderm ($P=0.002$ by Mann–Whitney *U*-test) but variable. Two of the eight E9.5 3n↔2n chimeras had mean 3n contributions greater than 70% in one of the trophectoderm derivatives sampled but most had low 3n levels, and no 3n cells were detected in any trophectoderm derivatives of three out of the eight chimeras. Chimera TrB-3 had relatively high 3n contributions to all lineages, which might be associated with its apparently abnormal placental development (see above).

The histograms shown in Fig. 2 indicate that 3n contributions in the fetus did not differ markedly among the three different tissues scored (brain, heart and somites).

Figure 3A and B summarises the 3n contribution to the fetus and the mean contribution to each of the three extraembryonic lineages for both E12.5 and E9.5 3n↔2n chimeras. It is clear that 3n cells usually contributed poorly to all lineages of these 10 3n↔2n chimeras.

Table 2 Physical parameters of E12.5 3n↔2n and 2n↔2n chimeric conceptuses.

Chimera	Weight (mg)			Crown-rump length (mm)	Hind limb morphology index[a]
	Conceptus	Fetus	Placenta		
3n↔2n chimeras					
TrA-16	263.2	80.3	59.1	8.3	7.5
TrA-26	321.6	96.4	87.4	9.3	7.0
Mean±S.E.M.	292.4±29.2	88.4±8.1	73.3±14.2	8.8±0.5	7.3±0.3
2n↔2n chimeras (N=25)					
Mean±S.E.M.	338.0±6.4	105.3±3.1	88.8±1.9	9.4±0.1	7.3±0.2
Minimum	261.9	61.6	71.8	8.2	5.0
Maximum	397.3	132.4	106.1	10.2	8.0

[a]Hind limb morphology index: 7.0=early stage 7; 7.5=late stage 7.

Although 3n cells were only excluded completely from three out of the nine E9.5 and E12.5 fetuses in 3n↔2n chimeric conceptuses, only three out of the nine had more than 2% 3n cells in the fetus. This was significantly fewer than that for the combined tissues of the primitive endoderm lineage (9/10; P=0.02) but not significantly different from either the extraembryonic epiblast (4/10; P>0.99) or the trophectoderm lineage (5/10; P=0.65), by Fisher's exact test.

There are too few chimeras to evaluate whether 3n cells are depleted between E9.5 and E12.5. Although most 3n↔2n fetuses contained some 3n cells at E9.5 (6/7), none did at E12.5 (0/2), but this difference was not significant by Fisher's exact test (P=0.08). Also, the fetuses of three other E9.5 3n↔2n chimeras contained <2% 3n cells, and this may be below the level detectable by GPI electrophoresis method used to analyse E12.5 chimeras (West & Green 1983). There was less difference in the frequency of fetuses with >2% 3n cells at E9.5 and E12.5 (3/7 vs 0/2; P=0.50).

Both of the E12.5 and one of the E9.5 3n↔2n chimeras showed restricted chimerism where 3n cells were absent from the fetus but present in extraembryonic tissues. In TrA-16, 3n cells were absent from the fetus but present in the extraembryonic epiblast, trophectoderm and primitive endoderm lineages, and in TrA-26 and TrC-11 they were absent from the fetus, extraembryonic epiblast and trophectoderm but present in the hypoblast.

Ts3↔2n chimeras

One series of Ts3↔2n and 2n↔2n control chimeras (TS) were produced and analysed at E9.5 (Table 1). Ts3↔2n chimeras were identified retrospectively by DNA *in situ* hybridisation to the transgenic sequence (*Tg/Tg/−* Ts3 cells produce two hybridisation signals per nucleus). Only 2 out of 34 chimeras proved to be Ts3↔diploid (5.9%) but this is close to the expected frequency (see the Discussion section). The composition of the two E9.5 Ts3↔2n chimeras and two of the control 2n↔2n

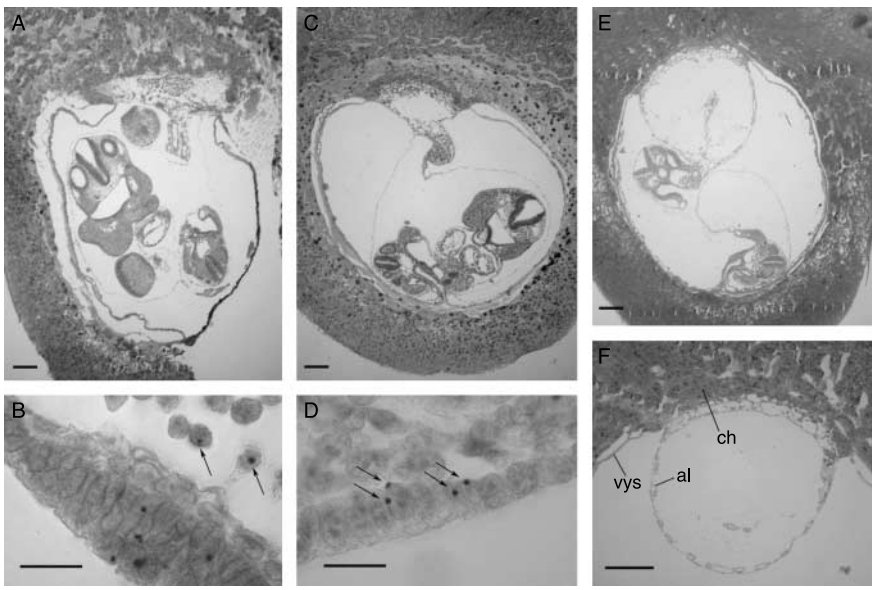

Figure 1 Triploid↔diploid chimeras. Histological sections and DNA *in situ* hybridisation of (A and B) E9.5 2n↔2n control chimera TrC-21, (C and D) E9.5 3n↔2n chimera TrC-16 and (E and F) E9.5 3n↔2n chimera TrB-3. Arrows show (B) examples of 2n (*Tg/−*) nuclei with single hybridisation signals and (D) examples of 3n (*Tg/Tg/−*) nuclei with two hybridisation signals. (F) An abnormal allantoic vesicle in E9.5 3n↔2n chimera TrB-3 (ch, chorion; al, allantoic vesicle; vys, visceral yolk sac). Scale bars=200 μm (A, C, E and F) and 20 μm (B and D).

Table 3 Composition (% GPI1-A) of E12.5 3n↔2n and 2n↔2n chimeric conceptuses.

	Epiblast derivatives			Primitive endoderm derivatives		Trophectoderm derivatives	
Chimera	Fetus	Amnion	YS mes	YS end	P end	Troph (RM)	Placenta
3n↔2n chimeras (uncorrected %GPI1-A)[a]							
TrA-16	0.0	6.7	10.9	50.7	50.9	27.7	14.4
TrA-26	0.0	0.0	0.0	28.3	25.7	0.0	0.0
Mean±S.E.M.	0.0±0	3.4±3.4	5.5±5.5	39.5±11.2	38.3±12.6	13.9±13.9	7.2±7.2
3n↔2n chimeras (corrected %GPI1-A)[a,b]							
TrA-16	0.0	4.6	7.5	40.7	40.9	20.3	10.1
TrA-26	0.0	0.0	0.0	20.8	18.7	0.0	0.0
Mean±S.E.M.	0.0±0	2.3±2.3	3.8±3.8	30.8±9.9	29.8±11.1	10.2±10.2	5.0±5.0
2n↔2n chimeras ($N=25$)[c]							
Mean±S.E.M.	47.0±3.6	47.1±3.8	45.8±3.6	56.5±3.3	62.2±4.8	45.1±7.7	42.1±7.1

YS mes, visceral yolk sac mesoderm; YS end, visceral yolk sac endoderm; P end, parietal endoderm; Troph (RM), trophoblast overlying Reichert's membrane.
[a]Recipient females were *Gpi1*$^{c/c}$, so any maternal contribution (GPI1-C) was excluded from the analysis.
[b]The corrected %GPI1-A$=\frac{2}{3}A\times100/(\frac{2}{3}A+B)$, where A=%GPI1-A and B=%GPI1-B. This allows for the expectation that 3n cells would produce 1.5 times the GPI1 activity of 2n cells and probably provides a closer estimate of the percentage 3n cells.
[c]Conceptus TrA-6 was not classified as 3n↔2n or 2n↔2n and is excluded from the table. (It had 15.9% GPI1-A in the yolk sac mesoderm and 0% GPI1-A in the other four tissues analysed.)

chimeras were analysed by DNA *in situ* hybridisation (Table 5). Figure 4 shows examples of sections of E9.5 Ts3↔2n and 2n↔2n chimeras and the hybridisation signals used to distinguish between chimeras containing *Tg/Tg/−* Ts3 cells and those containing *Tg/−* 2n cells. In contrast to the 3n↔2n chimeras, both Ts3↔2n chimeras had a high Ts3 cell contribution in all lineages (Table 5; Fig. 3C). Ts3↔2n chimera TS-10 was an incomplete conceptus, comprising an empty gestational sac without a fetus or amnion but the trophectoderm lineage and parietal endoderm consisted entirely of Ts3 cells. Ts3↔2n chimera TS-46 had a high contribution of Ts3 cells in all tissues analysed. The overall contribution to the fetus was 74.7% and high levels were seen in all the three fetal tissues scored (76%, 72% and 58% in the brain, heart and somites respectively). The high Ts3 fetal contribution indicated that Ts3↔2n chimeras are unlikely to provide a useful model of CPM.

Discussion

Our new genetic stocks successfully produced trisomy-3 and triploid embryos respectively without the need for experimental manipulations, but yields were low. The

Table 4 Corrected percentage contribution of *Tg*-positive cells in E9.5 3n↔2n and 2n↔2n chimeras.

	Epiblast derivatives				Primitive endoderm derivatives		Trophectoderm derivatives			
Chimera	Fetus	Amnion	YS mes	Allantois	YS end	P end	PT	PTGC	RMGC	Chorion
3n↔2n chimeras[a]										
TrC-26	Absent	Absent	9.5		64.5	3.0	0.0	0.0	0.0	
TrC-11	0.0	0.0	0.0		18.8	0.8	0.0	0.0	0.0	
TrC-8	0.6	ND	0.0		11.2	13.4	0.0	0.0	0.0	
TrB-6	1.8	0.0	0.0		12.0	12.7	14.3	0.0	0.0	
TrC-16	1.8	3.4	2.5		5.8	6.7	0.3	0.0	0.0	
TrC-37	10.7	ND	6.9		0.9	1.9	17.0	ND	3.0	
TrC-22	11.6	5.5	13.6		54.8	27.3	73.2	0.0	20.9	
TrB-3[b]	28.2	24.1	37.0	38.0[b]	14.9	20.2	ND	ND	71.2	88.0[b]
Mean±S.E.M.	7.8±3.9	6.6±4.5	8.7±4.4		22.9±8.3	10.8±3.3	15.0±10.1	0.0±0.0	11.9±8.8	
2n↔2n chimeras										
TrB-8	29.4	28.6	38.1		0.0	45.5	98.2	22.5	83.8	
TrC-5	31.0	7.5	31.9		20.6	28.7	40.0	0.0	14.4	
TrC-34	38.2	42.7	33.3		85.1	78.2	16.4	ND	30.9	
TrC-21	43.5	45.9	41.3		59.8	60.5	68.4	39.9	73.4	
TrB-9	92.0	93.4	77.1		12.8	22.2	18.0	0.0	0.0	
Mean±S.E.M.	46.8±11.6	43.6±14.2	44.3±8.4		35.7±15.9	47.0±10.3	48.2±15.7	15.6±9.7	40.5±16.4	

YS mes, yolk sac mesoderm; YS end, yolk sac endoderm; P end, parietal endoderm; PT, placental trophoblast; PTGC, placental trophoblast giant cells; RMGC, trophoblast giant cells overlying Reichert's membrane; ND, not done.
[a]Triploid↔2n chimera TrC-25 is not included in the table. It was damaged and so not analysed.
[b]Abnormal placental development; an allantoic vesicle was present, which had not fused normally with the chorion. These two tissues were scored separately, but uncorrected values are shown as there were no corresponding tissues in age-matched positive controls.

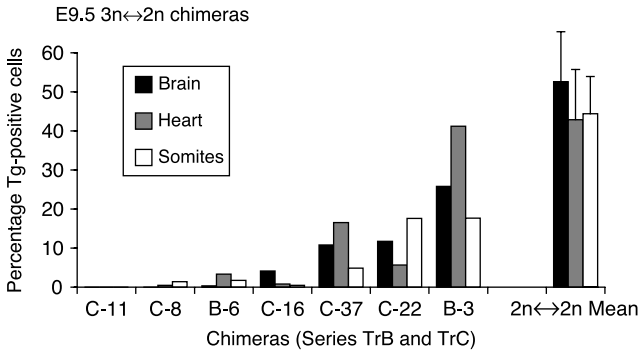

Figure 2 Histogram showing percentage Tg-positive cell contributions to three fetal tissues of seven E9.5 3n↔2n chimeras from series TrB and TrC (identified as B- and C- respectively) and mean contributions (±S.E.M.) for five 2n↔2n control chimeras.

reiterated chromosome 3 marker transgene provided a good means of identifying Ts3↔2n and 3n↔2n chimeras and quantifying the Ts3 and 3n contributions in histological sections. The yield of embryos from TRIP strain females was poor and hampered the production of 3n↔2n chimeras. The breeding performance was improved by further genetic crosses but only at the expense of a lower proportion of triploid embryos. Unless higher yields of triploid embryos can be obtained for future experiments (e.g. by selective breeding), it may be worth evaluating the use of cytochalasin B to induce triploidy (Niemierko 1975) in another strain carrying the chromosome 3 transgene.

Only two E9.5 series TS chimeric conceptuses proved to be Ts3↔2n, but this frequency (2/34; 5.9%) is close to that expected if meiotic non-disjunction in Rb1 heterozygotes produces ∼19% aneuploid zygotes (Cattanach & Moseley 1973, Gropp *et al*. 1974, Everett *et al*. 1996), comprising about 5% each of trisomy-3, monosomy-3, trisomy-1 and monosomy-1. If monosomic cells contribute infrequently to postimplantation stage chimeras (Magnuson *et al*. 1982), the expected frequency of Ts3↔2n chimeras is ∼5.6% (five Ts3↔2n chimeras per 90 overt *Tg*-positive↔−/− chimeras).

Although only one of these two Ts3↔2n chimeric conceptuses had a fetus, this fetus and the extraembryonic tissues of both chimeras had high levels of Ts3 cells, implying that Ts3 cells can survive well in all tissues of chimeric conceptuses. The absence of the fetus in one Ts3↔2n chimera with a very high Ts3 contribution may have been a result of too few diploid cells to correct the abnormal Ts3 phenotype, which is normally lethal around E10–11 days (Gropp *et al*. 1974, 1983). As Ts3 cells contributed well to the only Ts3↔2n chimeric fetus and to the extraembryonic epiblast lineage in both chimeras, Ts3↔2n chimeras are unlikely to provide a useful model of CPM. This follows the pattern established by earlier studies on other trisomy↔diploid chimeras, involving Ts12, Ts15, Ts16, Ts17 and Ts19, where Ts cells contributed reasonably well to embryos and adults (Magnuson *et al*. 1982, Cox *et al*. 1984, Epstein *et al*.

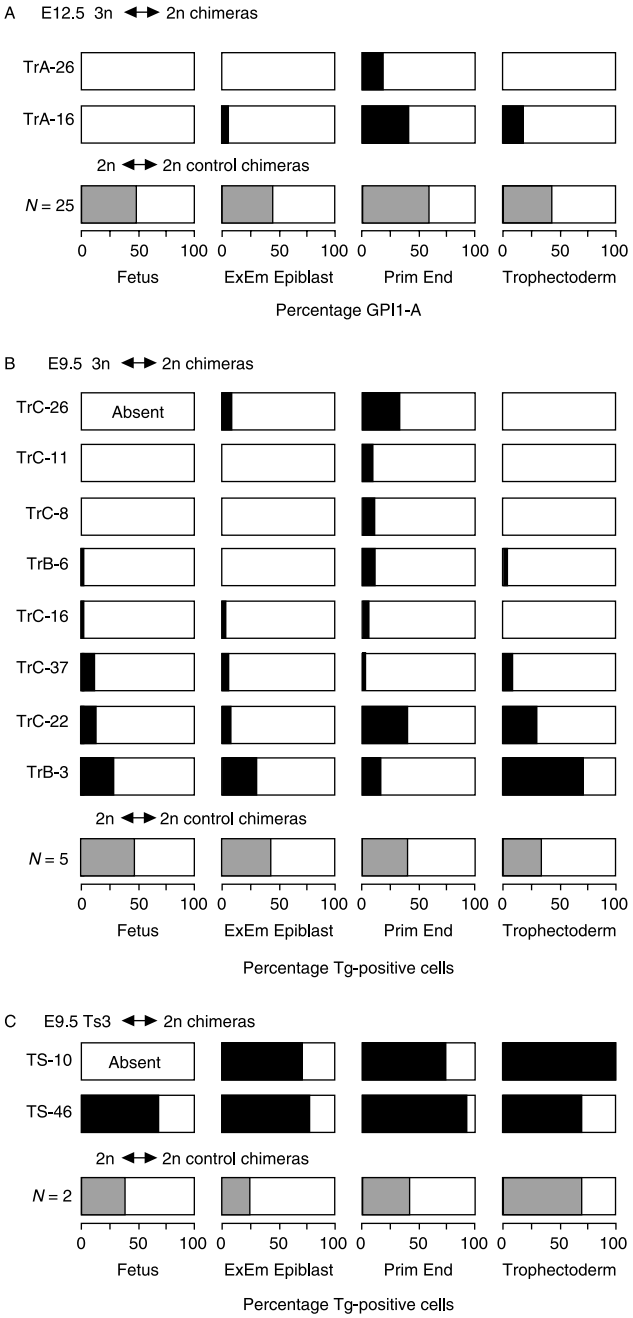

Figure 3 Diagrammatic summary of the composition of the fetus and three extraembryonic lineages in (A) E12.5 3n↔2n chimeras, (B) E9.5 3n↔2n chimeras and (C) E9.5 Ts3↔2n chimeras. Shaded regions represent the percentage of Tg-positive cells in the different lineages (shading is black for individual 3n↔2n and Ts3↔2n chimeras and grey for means of 2n↔2n control chimeras). The extraembryonic epiblast values are means of amnion and visceral yolk sac mesoderm; primitive endoderm values are means of visceral yolk sac endoderm and parietal endoderm; E12.5 trophectoderm values are means of the placenta (predominantly placental trophoblast) and the trophoblast overlying Reichert's membrane; E9.5 trophectoderm values are means of separate estimates for the placental trophoblast, placental trophoblast giant cells and trophoblast giant cells overlying Reichert's membrane (Tables 3–5). Triploid↔2n chimera TrC-25 is not included because it was damaged and so not analysed. Abbreviation: ExEm, extraembryonic.

1984, Fundele *et al.* 1985, Epstein 1986). None of the trisomy↔diploid chimeras studied so far has been identified as a good model of human CPM.

Some digynic triploid↔diploid chimeras showed morphological abnormalities. E9.5 chimera TrC-26 had no fetus or amnion, but this may not be attributable to the presence of 3n cells as they were not particularly abundant in this chimera. The chimera with the highest contribution of 3n cells (E9.5 chimera TrB-3) had an abnormal allantoic vesicle that was not fused normally with the chorion. This also occurs in some fully digynic triploids (Wróblewska 1971), parthenogenetic↔tetraploid chimeras (Spindle *et al.* 1996) and several mutants and genetic knockouts (Copp 1995). E12.5 chimera TrA-16 had a small placenta which may reflect poor placental trophoblast growth associated with digynic imprinting abnormalities (Surani *et al.* 1986).

Unlike Ts3 cells in Ts3↔2n chimeras, digynic triploid cells generally contributed poorly to all lineages of 3n↔2n chimeras. This cannot be attributable to the marker transgene as both Ts3 and 3n cells had an additional copy of chromosome 3 carrying the marker transgene. Only five out of nine (56%) of the 3n↔2n chimeras had any 3n cells in the fetus, and this contribution only exceeded 2% in three out of nine (33%) cases. All of the non-chimeras in the 3n↔2n series were derived from the diploid *Gpi1*$^{b/b}$ (FVB or NIH strain) embryo, and it seems likely that the 3n cell population was preferentially lost because the 2n↔2n control series showed no such bias in non-chimeric genotype.

Two other studies have demonstrated that 3n cells can contribute to adult tissues of 3n↔2n chimeras. Azuma *et al.* (1991) identified triploid cells in 24 out of 89 (27%) potential adult 3n↔2n chimeras in two reciprocal series, where digynic triploidy was induced by suppressing extrusion of the second polar body with cytochalasin B. The frequency and genotype of non-chimeras was consistent with loss of 3n cells from the fetal lineage. Suwinska *et al.* (2005) produced 3n↔2n mouse chimeras by two methods and showed that 3n cells could survive at postimplantation stages and in some adults. The low overall level of 3n contribution estimated by *in situ* hybridisation in the present study was similar to the cytogenetic results for adult 3n↔2n chimeras reported by Azuma *et al.* (1991), but rather lower than estimates made by GPI electrophoresis for embryonic, fetal and adult 3n↔2n chimeras reported by Suwinska *et al.* (2005). Differences in genetic background and methods of production and analysis could each contribute to these differences.

In our 3n↔2n chimeras, 3n cells contributed less to the epiblast (including fetus) and trophectoderm derivatives than to the primitive endoderm derivatives. Triploid cells were present in the primitive endoderm derivatives of all ten 3n↔2n chimeras, but their contribution to the trophectoderm was variable, being absent from three out of ten chimeras but contributing over 70% in one chimera. This variability is consistent with previous analyses of 2n↔2n chimeric placentas (West *et al.* 1995). Suwinska *et al.* (2005) also found that 3n cells tended to contribute more to the visceral yolk sac than to the fetus or placenta of 3n↔2n chimeras, but it was unclear whether 3n cells were specifically more abundant in the yolk sac endoderm layer because it was not analysed separately.

In two of our chimeras, 3n cells were absent from the fetus and confined to the primitive endoderm lineage. Although this is not equivalent to CPM, it is not known whether this class of restricted mosaicism occurs spontaneously in humans because the primitive endoderm (hypoblast) lineage is rarely investigated in human conceptuses. While some 3n↔2n chimeras showed some sort of restricted chimerism, 3n cells were not rigorously excluded from the fetus and the extra-embryonic epiblast to the extent that tetraploid cells are excluded from these lineages in 4n↔2n chimeras (James *et al.* 1995). Instead, the low contribution of 3n cells more closely resembles the consistently low contribution of one cell population in genotypically unbalanced 2n↔2n chimeras, where cells of one strain or genotype tend to predominate in all lineages (West & Flockhart 1994). It seems likely that the cases of restricted chimerism, where 3n cells were excluded from the fetus but not from the other tissues, may have arisen partly by chance following a more generalised depletion of 3n cells from all lineages and partly because of a tendency for 3n cells to survive better in the primitive endoderm. It is not yet known how lineage restriction in human CPM occurs, but different mechanisms may apply to different types of restricted mosaicism and different chromosome abnormalities (Wolstenholme 1996). For some types of human restricted mosaicism, 3n↔2n mouse chimeras may provide a more appropriate model than 4n↔2n chimeras.

Materials and Methods

Mice

Four series of chimeras (TrA, TrB, TrC and TS) were made by aggregating embryos from genetically distinct stocks of mice (Table 1). One of each pair of aggregated embryos carried a highly reiterated β-globin transgene (abbreviated to *Tg*) that is located on chromosome 3 (Lo *et al.* 1992, Everett *et al.* 1994) and can be identified readily by DNA *in situ* hybridisation in histological sections (Lo *et al.* 1987, Katsumata & Lo 1988, Keighren & West 1993). The pairs of aggregated embryos also differed at the albino locus (*Tyr*) and *Gpi1* (glucose phosphate isomerase). Genetic crosses with specially developed strains of mice were designed to produce some chromosomally abnormal embryos with either a complete extra set of maternal chromosomes (digynic triploid) or an extra paternal copy of chromosome 3 (trisomy-3) carrying the chromosome 3 transgenic marker. All animal work was carried out in accordance with the UK Home Office regulations. Mice were bred and maintained under conventional conditions in the Centre for Reproductive Biology, University of Edinburgh. (For cross-referencing, TrA, TrB, TrC and TS were originally designated

Table 5 Corrected percentage contribution of *Tg*-positive cells in E9.5 Ts3↔2n and 2n↔2n chimeras.

	Epiblast derivatives			Primitive endoderm derivatives		Trophectoderm derivatives		
Chimera	Fetus	Amnion	YS mes	YS end	P end	PT	PTGC	RMGC
Ts3↔2n chimeras								
TS-10	Absent	Absent	71.0	50.3	100[a]	100[a]	100[a]	100[a]
TS-46	68.4	82.1	74.7	96.8	91.0	72.4	43.3	75.6
2n↔2n chimeras								
TS-42	13.9	25.5	18.4	90.6	54.1	71.6	71.1	99.0
TS-6	61.8	27.8	26.8	17.4	10.8	95.1	41.6	52.1

YS mes, yolk sac mesoderm; YS end, yolk sac endoderm; P end, parietal endoderm; PT, placental trophoblast; PTGC, placental trophoblast giant cells; RMGC, trophoblast giant cells on outside of Reichert's membrane.
[a]Corrected values that exceeded 100% are shown as 100%.

XU, C9B, C9H and C9I respectively.) The female is shown first in each genetic cross.

Strains TRIP, PO2, TGA and RBT are all pigmented ($Tyr^{+/+}$), $Gpi1^{a/a}$ and homozygous for the reiterated β-globin marker transgene, *Tg/Tg* (Everett *et al.* 1994, Keighren & West 1994). Inbred strains NIH and FVB are albino ($Tyr^{c/c}$), $Gpi1^{b/b}$ and *Tg* negative; (BALB/c × A/J)F1 hybrids are $Tyr^{c/c}$, $Gpi1^{a/a}$ and *Tg* negative. Strains TRIP and PO2 were derived from inbred strain LT/SvKau and designed to produce *Tg/Tg/−* triploid embryos (see below). RBT is homozygous for both the Rb(1.3)1Bnr Robertsonian translocation (abbreviated to *Rb1*) and the reiterated transgene: *Rb1/Rb1*, *Tg/Tg* (Everett *et al.* 1994). Heterozygous *Rb1/+* males undergo meiotic non-disjunction and produce elevated frequencies of sperm with aneuploidy for chromosomes 1 or 3, and so produce some Ts-1 and Ts-3 embryos (Gropp *et al.* 1974). Hybrid (TGA×RBT)F1 males are *Rb1/+*, *Tg/Tg* and their trisomy-3 offspring are *Tg/Tg/−*.

Production of 3n↔2n chimeras

LT/SvKau females spontaneously ovulate up to 40% of oocytes as diploid primary oocytes which, on fertilisation, produce digynic triploid zygotes (Kaufman & Howlett 1986, Kaufman & Speirs 1987, O'Neill & Kaufman 1987, Speirs & Kaufman 1988). TRIP is an LT/SvKau congenic strain but is homozygous for the chromosome 3 transgenic marker (*Tg/Tg*) and was developed to generate *Tg/Tg/−*, digynic 3n embryos for chimera production. However, TRIP females produced a low yield of embryos, so a different strategy was used to produce *Tg/Tg/−* triploid embryos for the first series of triploid↔diploid chimeras (series TrA). *Tg/Tg* TRIP males were outcrossed to *Tg/Tg* TGA females and the *Tg/Tg* F1 hybrid females (designated PO1: 'primary oocyte ovulation stock 1') were backcrossed to *Tg/Tg* TRIP males to produce *Tg/Tg* PO2 mice with 75% TRIP (~75% LT/Kau) genetic background. PO2 females produced a better yield of embryos but a lower proportion were triploid, which proved to be even more of a problem. Thus, *Tg/Tg* TRIP females were used to produce *Tg/Tg/−* triploid embryos in series TrB and TrC.

TRIP and PO2 females were superovulated at 3–4 weeks to maximise the yield of digynic, *Tg/Tg/−* triploid embryos (Speirs & Kaufman 1990) using a standard protocol (West & Flockhart 1994). Other females were superovulated at 6–8 weeks old. Eight-cell stage embryos were collected at E2.5 (vaginal plug date designated E0.5) in M2 handling medium (Quinn *et al.* 1982). A mixture of *Tg/Tg/−* 3n and *Tg/−* 2n

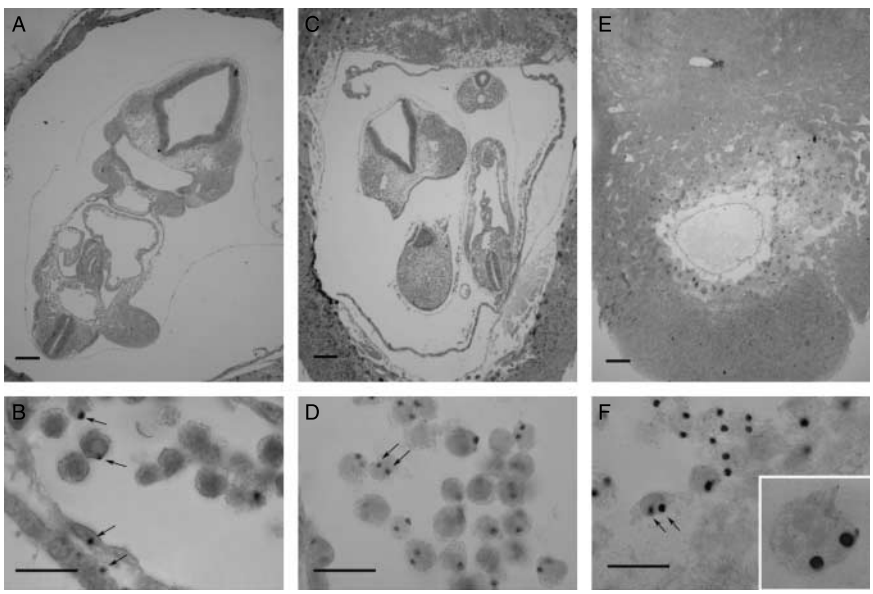

Figure 4 Trisomy-3↔diploid chimeras. Histological sections and DNA *in situ* hybridisation of (A and B) E9.5 2n↔2n control chimera TS-42, (C and D) E9.5 Ts3↔2n chimera TS-46 and (E and F) E9.5 Ts3↔2n chimera TS-10. Arrows show (B) examples of 2n (*Tg/−*) nuclei with single hybridisation signals and (D and E) examples of Ts3 (*Tg/Tg/−*) nuclei with two hybridisation signals. Inset in F shows trophoblast giant cell at the same magnification. Two hybridisation signals are not seen in all sections of Ts3 nuclei. Scale bars = 200 μm (A, C and E) and 20 μm (B, D and F).

embryos were produced from both the PO2×(BALB/c×A/J) and TRIP×(BALB/c×A/J) crosses and aggregated (without identifying the 3n embryos) with either FVB or NIH embryos using methods described previously (James *et al.* 1993, West & Flockhart 1994), based on the original method of Tarkowski (1961). NIH strain embryos were used as partner 2n embryos for series TrA and TrB, but FVB embryos were used for series TrC because FVB females responded better to our superovulation protocol. Homozygous *Gpi1*$^{c/c}$ females (hybrid stock 'CF1'; West & Flockhart 1994) were used as recipients at E2.5 and pregnancies timed according to the recipient female. Series TrA was analysed at E12.5 but series TrB and TrC were analysed at E9.5. Each series produced its own diploid↔diploid control chimeras. DNA *in situ* hybridisation was used to identify *Tg/Tg/−* cells in triploid↔2n chimeras and distinguish them retrospectively from control 2n↔2n chimeras.

Production of Ts3↔2n Chimeras

Female (BALB/c×A/J)F1 mice were superovulated and mated to (TGA×RBT)F1 males and embryos were collected and aggregated with FVB embryos as described above. Aggregates were cultured and transferred to pseudopregnant females and conceptuses were analysed at E9.5. Ts3↔2n and 2n↔2n chimeras were distinguished retrospectively by DNA *in situ* hybridisation to the transgene. *Tg/Tg/−* Ts3 cells produce two hybridisation signals per nucleus but *Tg/−* 2n cells produce only one. Ts1 and Ms1 cells that were disomic for chromosome 3 (*Tg/−*) would also produce single hybridisation signals, whereas Ms3 cells (−) would produce no hybridisation signal.

Analysis of E12.5 chimeras

E12.5 conceptuses were dissected to provide seven tissue samples: placenta, trophoblast overlying Reichert's membrane (plus maternal decidual tissue), parietal endoderm, yolk sac endoderm, yolk sac mesoderm, amnion and fetus (West & Flockhart 1994). The whole conceptus, placenta and fetus were weighed, crown-rump length measured and morphological index assessed by hind limb development (McLaren & Buehr 1990, Palmer & Burgoyne 1991). Tissue preparation, electrophoresis, GPI1 staining and densitometry are described elsewhere (James *et al.* 1993, West & Flockhart 1994).

The proportions of the two cell populations in the chimeric tissue were estimated from the proportions of GPI1-A and GPI1-B allozymes. The recipient females were *Gpi1*$^{c/c}$ and produced only GPI1-C enzyme; so any maternal contamination could be excluded from the analysis. The percentage GPI1-A contribution was also corrected to allow for the expectation that triploid cells would produce 1.5 times the activity of diploid cells. The corrected value was calculated as $^{2}/_{3}A\times100/(^{2}/_{3}A+B)$, where A=%GPI1-A and B=%GPI1-B, and this probably provides a closer estimate of the percentage 3n cells. (However, triploid cells do not always produce the predicted 1.5-fold increase in gene product (Epstein 1986), so both uncorrected and corrected GPI1-A levels are presented.) A piece of yolk sac was fixed in acetic alcohol (3 ethanol: 1 acetic acid) and used to distinguish E12.5 3n↔2n and 2n↔2n chimeras by DNA *in situ* hybridisation on 7 μm histological sections (see below). 3n↔2n chimeras were identified by the presence of *Tg/Tg/−* triploid cells with two hybridisation signals per nucleus.

Analysis of E9.5 chimeras

E9.5 conceptuses were fixed in acetic alcohol and sections were cut at 7 μm. Probe pMβδ2 was labelled using digoxygenin-labelled deoxyuridine triphosphate (non-radioactive DNA Labelling and Detection Kit; Boehringer Mannheim), DNA–DNA *in situ* hybridisation was performed and hybridisation signals scored as described previously (Keighren & West 1993). *In situ* hybridisation of Ts3↔2n and 3n↔2n chimeras produced two hybridisation signals in most *Tg*-positive nuclei, so they were distinguishable from 2n↔2n control chimeras with one signal per nucleus. Conceptuses with no hybridisation signals were excluded from the analysis, and considered to be non-chimeric, although in series TS some may have been Ms3↔2n chimeras.

Three longitudinal sections of each conceptus were scored (middle plus two flanking sections, approximately midway from the middle to the sides). Fetal brain, heart, somites, amnion, yolk sac mesoderm, yolk sac endoderm, parietal endoderm, placental trophoblast and giant cells were analysed separately and a mean fetal value was calculated from the results for the brain, heart and somites. (The term 'fetus' is used at both E9.5 and E12.5 to avoid confusion with the preimplantation embryo, which produces both the fetus and extraembryonic tissues.) The percentage of *Tg*-positive nuclei was estimated for each tissue by counting positive and negative nuclei in a sample of ∼250–300 cells. Not all 2n nuclei produce the expected (single) hybridisation signal and not all 3n and Ts3 nuclei produce two signals because only part of the nucleus may be included in the section (Keighren & West 1993). Thus, once a chimera had been identified as Ts3↔2n or 3n↔2n, all nuclei with hybridisation signals were scored as *Tg* positive (and therefore Ts or 3n) irrespective of whether one or two signals were present. The percentages of *Tg*-positive cells in *Tg/Tg/−*↔*−/−* (Ts3↔2n or 3n↔2n) and *Tg/−*↔*−/−* (2n↔2n) chimeras were corrected using tissue-specific means from two homozygous *Tg/Tg* and two hemizygous *Tg/−* control conceptuses respectively.

Statistical analysis

Statistical tests were performed on an Apple Macintosh computer using StatView software (Abacus Concepts Inc., Berkeley, CA, USA).

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