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Size regulation does not cause the composition of mouse chimaeras to become unbalanced

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ABSTRACT Mouse chimaeras made by aggregating two 8-cell stage embryos undergo size regulation shortly after implantation. Thus chimaeric pups are approximately normal size at birth despite their origin from two complete embryos. Chimaeras of some strain combinations are genotypically unbalanced such that cells of one strain almost always predominate. For example, the BALB/c inbred strain often makes a low contribution to chimaeras. This genotypic imbalance in the composition could arise by selection against BALB/c cells. Selection may be particularly acute at the time of size regulation. To investigate if the mechanism(s) responsible for size regulation could cause the low contribution of BALB/c cells, we compared the composition of an unbalanced series of chimaeras, produced by aggregating two complete 8-cell stage embryos, with a similar series of chimaeras made by aggregating two half 8-cell stage embryos. In each case the unbalanced strain combination was BALB/c↔[(C57BL × CBA/Ca)F_1 × TGB] and parallel studies were undertaken with a genotypically balanced strain combination. For each chimaera, the composition of the fetus, placenta and extraembryonic membranes were determined at E12.5. When two half embryos were aggregated the BALB/c strain still made a poor contribution to all the tissues of the mid-gestation conceptus. This implies that this strain combination remained unbalanced even when size regulation was absent or minimal. Therefore, size regulation did not play a major role in reducing the contribution of BALB/c cells and producing the phenotypic imbalance in the chimaeras.

KEY WORDS: *Chimaera, chimera, size regulation, mouse, embryo aggregation.*

Introduction

Experimental mouse chimaeras have been widely used in developmental biology for over thirty years (reviews include Mintz, 1971; McLaren, 1976a; Le Douarin and McLaren, 1984; Gardner, 1998; Rossant and Spence, 1998; Tarkowski, 1998; West, 1999). The original method of producing chimaeric embryos involved aggregation of two complete cleavage stage embryos (Tarkowski, 1961; Mintz, 1964) and although several alternative methods have been introduced, they almost all increase the total number of cells in the embryo.

It was soon realised that a regulatory mechanism must adjust the body size of chimaeric embryos, made by aggregating two complete 8-cell stage embryos, because abnormally large offspring were not born after transferring the aggregated embryos to foster mothers. Several experiments have shown that regulation of body size can occur in either a downward or an upward direction but only downward size regulation is relevant here and this occurs around E5.5-E6 days (Tarkowski, 1963; Buehr and McLaren, 1974). Although it is possible that whatever mechanism causes size regulation also acts on normal embryos, it is clear that it has a much greater effect on double-sized chimaeric embryos. Rands (1986b) speculated that downward size regulation could involve a delay in the normal increase in growth rate that occurs soon after implantation. Lewis and Rossant (1982) considered three possible mechanisms: enhancing cell death, increasing the population of non-dividing cells and increasing the cell cycle length. Of these, they favoured the idea that downward regulation was achieved by a lengthened cell cycle but Gardner (1996) argued that cell death may also play a role. Gardner (1996) also pointed out that the size and composition of a chimaera could be simultaneously affected by whatever mechanism caused size regulation. If lengthening of the cell cycle and/or cell death affected the two aggregated embryos unequally, size regulation would also create an acute selection pressure which would alter the balance of the two component embryos in the chimaera.

It is well established that, for some strain combinations, chimaeras produced by aggregating two 8-cell stage embryos are consistently

Abbreviations used in this paper: YsE, yolk sac endoderm; YsM, yold sac mesoderm.

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genotypically unbalanced in favour of one strain (Mullen and Whitten, 1971). For example, Dvorak *et al.* (1995) found that two series of chimaeras became genotypically unbalanced between 24h after aggregation and E7.5. Similarly, BALB/c strain cells were poorly represented in the fetuses, placentas and extraembryonic tissues of E12.5 BALB/c↔(C57BL×CBA/Ca)F_2 chimaeric conceptuses (West and Flockhart, 1994). If cell selection was involved, the low contribution of BALB/c cells could result either from a continuous selection, reducing their contribution with time, or an acute selection at a specific 'bottleneck' before E12.5 (probably before E7.5). One possible 'bottleneck' is when chimaeric embryos undergo size regulation at E5.5-E6. Whatever mechanism is involved in size regulation, it could act unequally on the two aggregated embryos and so create an acute selection pressure which would reduce the overall contribution of BALB/c cells to the chimaera. For example, more BALB/c cells might die or their cell cycle might be lengthened preferentially or to a greater extent.

If the mechanism(s) responsible for size regulation simultaneously played a major role in reducing the contribution of BALB/c cells to most chimaeras in a genotypically unbalanced strain combination, it should be possible to convert the strain combination to a phenotypically balanced one if size regulation is avoided. The aim of this study was to test whether the poor contribution of BALB/c cells was dependent on size regulation by comparing two series of chimaeras of the same unbalanced, BALB/c↔[(C57BL×CBA/Ca)F_1 × TGB] strain combination. The first was made in the normal way by aggregating two complete 8-cell stage embryos, so size regulation would be expected to occur. The second series was made by aggregating two half 8-cell stage embryos and, because the total cell number was 8 rather than 16, size regulation would be absent or minimal. The contribution of BALB/c cells was analysed at E12.5 in the fetus, placenta and extraembryonic membranes. A similar comparison was made between two control series of chimaeras made from the genotypically balanced strain combination (BALB/c × A/J)F_2↔[(C57BL × CBA/Ca)F_1 × TGB]. The results showed that BALB/c cells still contributed poorly to chimaeras made from two half embryos. This implies that the reduction of BALB/c cells in chimaeric conceptuses was largely independent of size regulation.

Results

Production of aggregation chimaeras

The composition of the four series of chimaeras is shown in Table 1 and described in the Materials and Methods section. The unbalanced strain combination was similar to that used in previous experiments (West and Flockhart, 1994) except that [(C57BL x CBA)F_1 x TGB] embryos were used in place of (C57BL x CBA)F_2 embryos. The TGB stock carries a reiterated transgene but this marker was not used in the analysis, which was entirely based on GPI electrophoresis. Comparisons were made between balanced (B) and unbalanced (U) strain combinations made in the same way and between chimaeras of the same strain combination produced by aggregating different numbers of cells, ($^8/_8$↔$^8/_8$) and ($^4/_8$↔$^4/_8$). The recovery of the four different series of chimaeras is shown in Table 2. The implantation frequencies were higher for the two balanced series of chimaeras and the difference between series B($^8/_8$↔$^8/_8$) and U($^8/_8$↔$^8/_8$) was significant (χ^2=8.08, P=0.0045). Differences in the frequencies of postimplantation failures (resorbing moles) were

TABLE 1

STRAIN COMBINATIONS AND CONSTRUCTION OF THE FOUR SERIES OF *Gpi1$^{a/a}$*↔*Gpi1$^{b/b}$* AGGREGATION CHIMAERAS

Series	Strain combination	Embryos aggregated
U($^8/_8$↔$^8/_8$)	BALB/c ↔ (C57BL × CBA/Ca)F_1 × TGB	8-cell ↔ 8-cell
U($^4/_8$↔$^4/_8$)	BALB/c ↔ (C57BL × CBA/Ca)F_1 × TGB	half an 8-cell ↔ half an 8-cell
B($^8/_8$↔$^8/_8$)*	(BALB/c × A/J)F_2 ↔ (C57BL × CBA/Ca)F_1 × TGB	8-cell ↔ 8-cell
B($^4/_8$↔$^4/_8$)	(BALB/c × A/J)F_2 ↔ (C57BL × CBA/Ca)F_1 × TGB	half an 8-cell ↔ half an 8-cell

Abbreviations: U, genotypically unbalanced strain combination; B, genotypically balanced strain combination; $^8/_8$, whole 8-cell stage embryo; $^4/_8$, four cells from an 8-cell stage embryo. *Data for balanced series B($^8/_8$↔$^8/_8$) have been reported elsewhere (Tang and West, 2000).

TABLE 2

RECOVERY OF FOUR SERIES OF *Gpi1$^{a/a}$*↔*Gpi1$^{b/b}$* CHIMAERAS

	Number (percentage) of each type of conceptus			
Type of conceptus	U($^8/_8$↔$^8/_8$)	U($^4/_8$↔$^4/_8$)	B($^8/_8$↔$^8/_8$)	B($^4/_8$↔$^4/_8$)
Total aggregates transferred	122	163	70	109
Aggregates transferred to females that became pregnant	71	98	48	65
Implantations	44* (62%)§	59 (60%)	42 (88%)	46 (71%)
Resorbing moles	8 (18%)#	10 (17%)	9 (21%)	3 (7%)
Normal conceptuses	37	49	33	43
Chimaeric conceptuses	33*	36	30	40
Non-chimaeric GPI1-A conceptuses	0	0	2 (6%)†	1 (2%)
Non-chimaeric GPI1-B conceptuses	4 (11%)†	13 (27%)	1 (3%)	2 (5%)

*Includes two chimaeric fetuses within one yolk sac (counted as one implantation but two conceptuses) that were excluded from further analysis. § Percentage of those transferred to females that became pregnant # Percentage of implantations † Percentage of normal conceptuses.

TABLE 3

COMPARISONS OF PHYSICAL PARAMETERS (MEAN ±SEM) IN DIFFERENT SERIES OF E12.5 *Gpi1$^{a/a}$*↔*Gpi1$^{b/b}$* CHIMAERAS AND CONTROLS

Series	N (Nc)*	Conceptus weight (mg)	Fetal weight (mg)	Placental weight (mg)	Fetal length (mm)	Hind limb score
Chimaeras						
U($^8/_8$↔$^8/_8$)	31 (30)	302.5 ± 9.9^{†‡b}	89.8 ± 3.5^{†ab}	86.7 ± 3.0^{†‡b}	9.28 ± 0.15 ^{†‡b}	6.87 ± 0.17^{†a}
U($^4/_8$↔$^4/_8$)	36 (33)	269.6 ± 5.3^{†ab}	83.3 ± 2.4^{†ab}	71.9 ± 1.4^{†a}	8.85 ± 0.10^{ab}	6.97 ± 0.11$^{† ab}$
B($^8/_8$↔$^8/_8$)	30 (30)	356.7 ± 7.7$^{‡ ac}$	113.9 ± 3.7^{‡c}	94.5 ± 2.2^{‡c}	9.95 ± 0.13 ^{‡c}	7.72 ± 0.12^{‡c}
B($^4/_8$↔$^4/_8$)	40 (36)	303.2 ± 5.2^{c}	92.4 ± 2.0^{ac}	82.9 ± 1.9^{ac}	9.01 ± 0.08^{a}	7.38 ± 0.09
Controls						
BALB/c	14 (13)	244.8 ± 11.2^{a}	71.7 ± 4.5^{a}	71.0 ± 2.2^{a}	8.41 ± 0.19^{a}	6.54 ± 0.25
(C57BL × CBA/Ca)F_1 × TGB	24 (19)	323.7 ± 11.1	110.1 ± 5.0	90.6 ± 3.5	9.63 ± 0.14	7.46 ± 0.19^{b}
(BALB/c × A/J)F_2	33 (31)	266.3 ± 7.7^{a}	80.1 ± 3.3^{a}	66.6 ± 1.6^{a}	8.81 ± 0.14^{a}	7.13 ± 0.16^{b}

*: N= number of samples except conceptus weights; Nc= number of conceptus weights. †: significant differences between strain combinations, U($^8/_8$↔$^8/_8$) versus B($^8/_8$↔$^8/_8$) or U($^4/_8$↔$^4/_8$) versus B($^4/_8$↔$^4/_8$); P< 0.05 ‡: significant differences between cell numbers, U($^8/_8$↔$^8/_8$) versus U($^4/_8$↔$^4/_8$), or B($^8/_8$↔$^8/_8$) versus B($^4/_8$↔$^4/_8$); P< 0.05 ^{a}: significantly different from (C57BL×CBA/Ca)F_1×TGB controls; P< 0.05 ^{b}: significantly different from BALB/c controls; P< 0.05 ^{c}: significantly different from (BALB/c × A/J)F_2 controls; P< 0.05 Hind limb scores were compared by Mann-Whitney U-tests; other parameters were compared by Student's t-tests.

not significant. The frequency of non-chimaeric U($^4/_8\leftrightarrow^4/_8$) normal conceptuses was significantly higher than in the B($^4/_8\leftrightarrow^4/_8$) series ($\chi^2$=4.81; P=0.03).

Physical parameters

The weights of conceptuses, fetuses and placentas, the crown/rump length (fetal length) and hind limb morphological index of the four chimaeric series and three non-chimaeric controls series are shown in Table 3. (The weights of conceptuses whose yolk sacs were broken during dissection were ignored, because of fluid loss, but the other physical parameters were included for these conceptuses.) The [(C57BL x CBA)F_1 x TGB] conceptuses were significantly larger than the other two control strains which did not differ significantly from one another in size. BALB/c fetuses were developmentally less advanced than the other two control strains, which did not differ significantly from one another (using hind limb morphology as the criterion for developmental stage).

All parameters measured indicated that the genotypically unbalanced chimaera combination, U($^8/_8\leftrightarrow^8/_8$), was smaller and developmentally less advanced than the balanced combination B($^8/_8\leftrightarrow^8/_8$). Similar differences were seen between U($^4/_8\leftrightarrow^4/_8$) and B($^4/_8\leftrightarrow^4/_8$). The mean measurements for U($^8/_8\leftrightarrow^8/_8$) and U($^4/_8\leftrightarrow^4/_8$) chimaeras were all intermediate between the means for the constituent BALB/c and [(C57BL × CBA/Ca)F_1 × TGB] control strains. Likewise, the B($^4/_8\leftrightarrow^4/_8$) measurements were intermediate between (BALB/c × A/J)F_2 and [(C57BL × CBA/Ca)F_1 × TGB] control values. The mean B($^8/_8\leftrightarrow^8/_8$) values all exceeded those for both (BALB/c × A/J)F_2 and [(C57BL × CBA/Ca)F_1 × TGB] controls but differences with the larger control strain were mostly non-significant (Table 3).

Although series B($^4/_8\leftrightarrow^4/_8$) differed significantly from series B($^8/_8\leftrightarrow^8/_8$) for each parameter measured, Fig. 1 shows that size regulation was virtually complete in both U($^8/_8\leftrightarrow^8/_8$) and B($^8/_8\leftrightarrow^8/_8$). The ratio of the 'double-sized' aggregates, U($^8/_8\leftrightarrow^8/_8$) and B($^8/_8\leftrightarrow^8/_8$), and the 'normal-sized' aggregates, U($^4/_8\leftrightarrow^4/_8$) and B($^4/_8\leftrightarrow^4/_8$), was much closer to 1 : 1 than 2 : 1.

Composition of chimaeras made from two complete embryos: series U($^8/_8\leftrightarrow^8/_8$) and B($^8/_8\leftrightarrow^8/_8$)

The frequencies of chimaeric and non-chimaeric U($^8/_8\leftrightarrow^8/_8$) and B($^8/_8\leftrightarrow^8/_8$) conceptuses are shown in Table 2. Non-chimaeric and twin conceptuses were not included in the analysis. The composition of five samples from each *Gpi1*$^{a/a}\leftrightarrow$*Gpi1*$^{b/b}$ chimaera was determined by GPI electrophoresis. Fig. 2A and Table 4 show that, overall, series B($^8/_8\leftrightarrow^8/_8$) had the characteristics of a balanced strain combination whereas series U($^8/_8\leftrightarrow^8/_8$) was genotypically unbalanced according to the criteria used by West *et al.* (1995b). In Table 4, distributions for different tissues were classified as balanced or unbalanced and 'typical' or 'atypical'. In an unbalanced distribution the number of samples with <50% GPI1-A differs significantly from the number with >50% GPI1-A. In a 'typical' distribution, the number of balanced samples (25-75% GPI1-A) is equal to or greater than that in either of the two types of unbalanced samples (<25% or >75%). An 'atypical' distribution could be skewed or bimodal. For example, the distribution of 30 fetuses in chimaera series B($^8/_8\leftrightarrow^8/_8$) was considered to be balanced because the number of fetuses with <50% GPI1-A was not significantly different from the number with >50% GPI1-A (15 in each group). The distribution was also considered to be 'typical' because more had 25-75% GPI1-A (15 fetuses) than <25% GPI1-A (8 fetuses) or >75% GPI1-A (7 fetuses).

Each of the five tissues in series U($^8/_8\leftrightarrow^8/_8$) was genotypically unbalanced (significantly more samples with <50% than >50% GPI1-A) and four of these were also 'atypical', reflecting the skewing towards a low contribution of BALB/c cells (most samples had <25% GPI1-A). Fig. 2A shows that series B($^8/_8\leftrightarrow^8/_8$) was visibly more balanced than series U($^8/_8\leftrightarrow^8/_8$). The criteria used in Table 4 confirm that the fetal, amnion and yolk sac mesoderm, B($^8/_8\leftrightarrow^8/_8$) distributions were all balanced and 'typical'. Fig. 2A shows that many of the yolk sac endoderm samples had 40-50% GPI1-A and so the distribution is 'typical' but not completely balanced by the criterion used. The

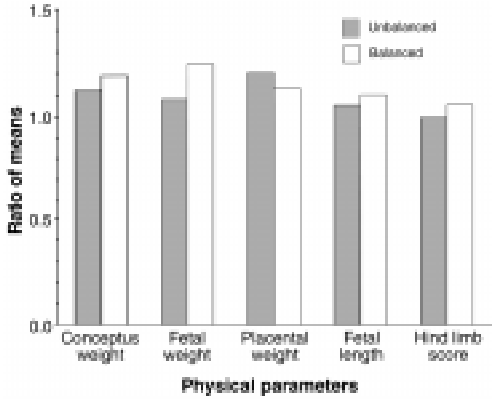

Fig. 1. Physical parameters for *Gpi1*$^{a/a}\leftrightarrow$*Gpi1*$^{b/b}$ chimaeras from whole-embryo aggregates relative to those from the equivalent half-embryo aggregates. *Ratio of means = mean for whole embryo aggregates / mean for half embryo aggregates. A ratio of 1.0 indicates that size regulation in the whole embryo aggregates is complete and a ratio of 2.0 would imply that no size regulation had occurred. The unbalanced series are shown by grey bars and the balanced series by white bars.*

TABLE 4

COMPOSITION (% GPI1-A) OF FOUR SERIES OF *Gpi1*$^{a/a}\leftrightarrow$*Gpi1*$^{b/b}$ CHIMAERAS GROUPED IN TWO WAYS TO DETERMINE WHETHER THE DISTRIBUTION OF % GPI1-A IS UNBALANCED OR 'ATYPICAL' IN DIFFERENT TISSUES

			Number of chimaeras grouped by % GPI1-A	
Chimaera series	Tissues	N†	< 50 : > 50	< 25 : 25 -75 : > 75‡
Balanced and 'typical' distributions				
B($^8/_8\leftrightarrow^8/_8$)	Fetus	30	15 : 15	8 : 15 : 7
B($^8/_8\leftrightarrow^8/_8$)	Amnion	30	13 : 17	7 : 15 : 8
B($^8/_8\leftrightarrow^8/_8$)	YsM	30	14 : 16	7 : 13 : 10
B($^4/_8\leftrightarrow^4/_8$)	YsE	40	24 : 16	12 : 19 : 9
B($^4/_8\leftrightarrow^4/_8$)	Placenta	40	23 : 17	16 : 18 : 6
Unbalanced but 'typical' distributions				
B($^8/_8\leftrightarrow^8/_8$)	YsE	30	25 : 5***	4 : 26 : 0
B($^4/_8\leftrightarrow^4/_8$)	Fetus	40	28 : 12*	15 : 21 : 4
B($^4/_8\leftrightarrow^4/_8$)	Amnion	39	29 : 10**	16 : 18 : 5
B($^4/_8\leftrightarrow^4/_8$)	YsM	40	33 : 7***	17 : 19 : 4
U($^8/_8\leftrightarrow^8/_8$)	YsE	31	28 : 3***	11 : 19 : 1
U($^4/_8\leftrightarrow^4/_8$)	YsE	36	28 : 8***	15 : 18 : 3
Unbalanced and 'atypical' distributions				
B($^8/_8\leftrightarrow^8/_8$)	Placenta	30	21 : 9*	13 : 10 : 7
U($^8/_8\leftrightarrow^8/_8$)	Fetus	31	26 : 5***	19 : 11 : 1
U($^8/_8\leftrightarrow^8/_8$)	Amnion	31	28 : 3***	22 : 9 : 0
U($^8/_8\leftrightarrow^8/_8$)	YsM	31	26 : 5***	21 : 10 : 0
U($^8/_8\leftrightarrow^8/_8$)	Placenta	31	25 : 6***	21 : 6 : 4
U($^4/_8\leftrightarrow^4/_8$)	Fetus	36	30 : 6***	21 : 10 : 5
U($^4/_8\leftrightarrow^4/_8$)	Amnion	36	30 : 6***	24 : 7 : 5
U($^4/_8\leftrightarrow^4/_8$)	YsM	36	30 : 6***	23 : 8 : 5
U($^4/_8\leftrightarrow^4/_8$)	Placenta	36	29 : 7***	19 : 13 : 4

YsM, yolk sac mesoderm; YsE, yolk sac endoderm. † N= Total number of chimaeras analysed. ‡ The distribution is classified as 'typical' if the middle of the three classes (25-75% GPI1-A) is the most frequent.*P<0.05; **P<0.005; ***P<0.001. Tested (by chi-square) against the expectation of equal proportions of < 50% and > 50% GPI-1A (only significant differences are noted).

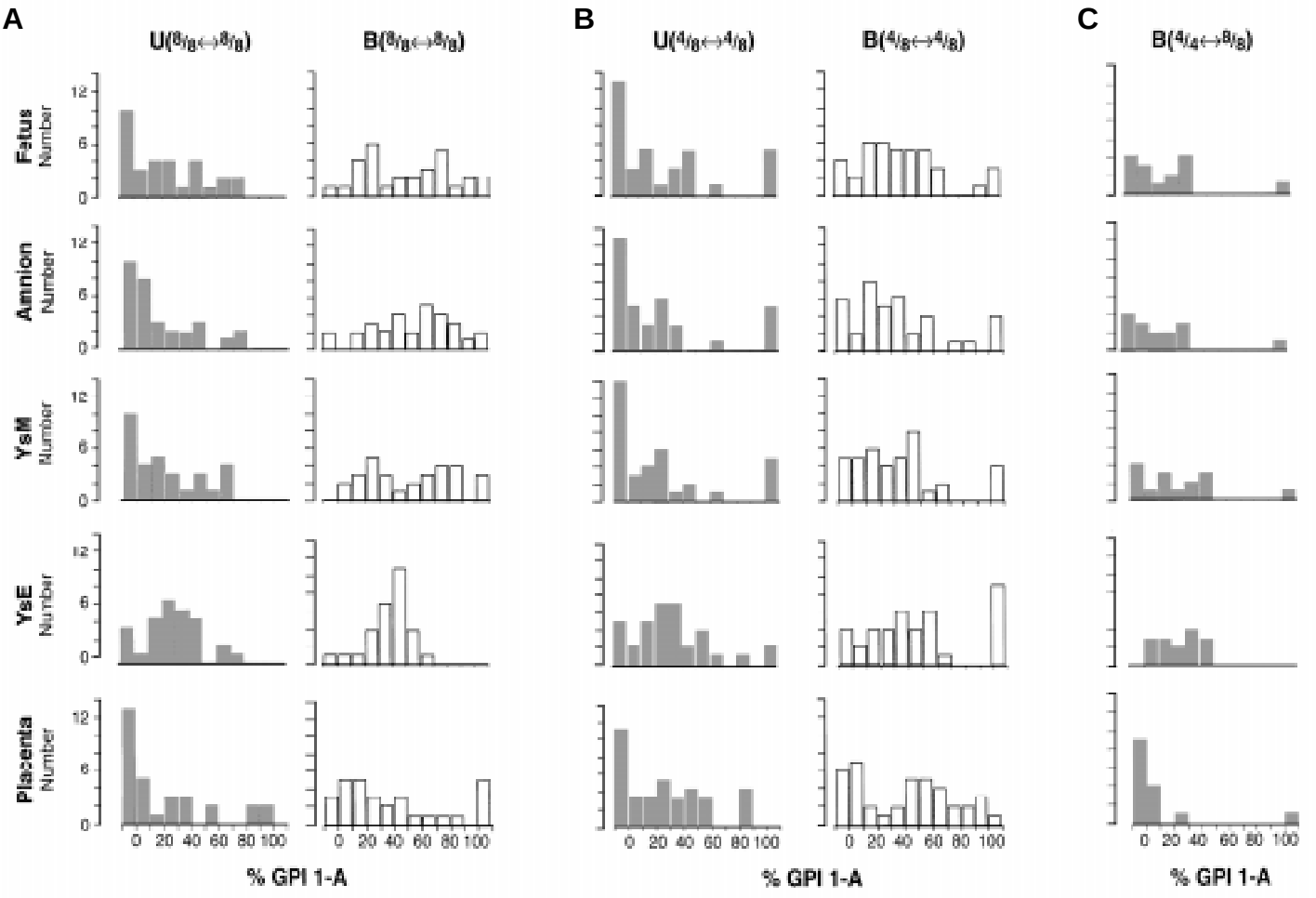

Fig. 2. Distribution of % GPI1-A in the five tissues analysed in five series of *Gpi1*$^{a/a}$↔*Gpi1*$^{b/b}$ chimaeric conceptuses. *Tissues with either 0% or 100% GPI1-A are shown separately at either end of the distributions. The three shaded series of histograms are less well balanced than the two unshaded series.* **(A)** *U($^8/_8$↔$^8/_8$) and B($^8/_8$↔$^8/_8$) chimaeric conceptuses;* **(B)** *U($^4/_8$↔$^4/_8$) and B($^4/_8$↔$^4/_8$) chimaeric conceptuses;* **(C)** *B($^4/_4$↔$^8/_8$) chimaeric conceptuses (data from Tang and West, 2000).*

B($^8/_8$↔$^8/_8$) placental distribution, was also slightly unbalanced and 'atypical'. It is probably unimportant that this is 'atypical' because other studies have shown that placental distributions are often bimodal (West *et al.,* 1995a, b).

The mean % GPI1-A for each tissue in these two series are listed in Table 5, where the five tissues analysed are grouped by their developmental origins. Mann-Whitney U-tests (Table 5) showed that

TABLE 5

COMPARISONS OF THE MEAN % GPI1-A AMONG THE TISSUES ANALYSED IN EACH SERIES OF *Gpi1*$^{a/a}$↔*Gpi1*$^{b/b}$ CHIMAERAS

Series of Chimaeras	N	Epiblast Lineage*			Primitive Endoderm*	Trophectoderm*
		Fetus	Amnion	YsM	YsE	Placenta
U($^8/_8$↔$^8/_8$)	31	22.91 ± 4.34†	18.05 ± 4.06†	20.35 ± 4.17†	28.91 ± 3.56†	21.80 ±5.61†
B($^8/_8$↔$^8/_8$)	30	49.98 ± 5.57	53.65 ± 5.11‡	53.03 ± 5.54‡	38.23 ± 2.65	38.68 ± 6.47
U($^4/_8$↔$^4/_8$)	36	28.10 ± 5.65†	25.29 ± 5.67†	25.70 ± 5.66†	32.79 ± 4.45	27.00 ± 4.76†
B($^4/_8$↔$^4/_8$)	40	38.01 ± 4.38	34.03 ± 4.81	33.84 ± 4.51	46.28 ± 5.41	40.62 ± 5.26

*Tissues are grouped by their developmental origins; YsM, yolk sac mesoderm; YsE, yolk sac endoderm; N, number of chimaeras analysed. †: balanced versus unbalanced comparisons (Mann-Whitney U-tests) between series U($^8/_8$↔$^8/_8$) and B($^8/_8$↔$^8/_8$), or U($^4/_8$↔$^4/_8$)and B($^4/_8$↔$^4/_8$); P< 0.05 ‡: whole versus half embryo comparisons (Mann-Whitney U-tests) between series U($^8/_8$↔$^8/_8$) and U($^4/_8$↔$^4/_8$), or B($^8/_8$↔$^8/_8$) and B($^4/_8$↔$^4/_8$); P< 0.05.

the % GPI1-A of each tissue studied was significantly lower in the unbalanced series U($^8/_8$↔$^8/_8$) than in the balanced series B($^8/_8$↔$^8/_8$), thus confirming the basic difference in composition between these two series of chimaeras.

Composition of chimaeras made from two half embryos: series U($^4/_8$↔$^4/_8$) and B($^4/_8$↔$^4/_8$)

The frequencies of chimaeric and non-chimaeric U($^4/_8$↔$^4/_8$) and B($^4/_8$↔$^4/_8$) conceptuses are shown in Table 2. The non-chimaeric conceptuses were not included in the analysis but their genotypes suggested that series U($^4/_8$↔$^4/_8$) was unbalanced (all 13 were GPI1-B). Fig. 2B indicates that most of the U($^4/_8$↔$^4/_8$) distributions were skewed towards a low % GPI1-A. Table 4 shows that, as in series U($^8/_8$↔$^8/_8$), the U($^4/_8$↔$^4/_8$) yolk sac endoderm distribution was classified as unbalanced but 'typical' whereas all other tissues were both unbalanced and 'atypical'. These observations imply that halving the number of cells to avoid the effects of size regulation failed to prevent the tissue composition from becoming unbalanced.

Fig. 2B and Table 4 indicate that the B($^4/_8$↔$^4/_8$) distributions were also slightly skewed towards a low % GPI1-A. Although only two distributions (placenta and yolk sac endoderm) were classified as completely balanced by the criterion used, all five distributions were

classified as 'typical'. Each tissue distribution had a high proportion of balanced samples (25-75% GPI1-A) but because there were more fetal, amnion and yolk sac mesoderm samples with <25% than with >75% GPI1-A these distributions were classified as unbalanced. All the B($^8/_8\leftrightarrow^8/_8$) and B($^4/_8\leftrightarrow^4/_8$) distributions, except for the B($^8/_8\leftrightarrow^8/_8$) placenta distribution discussed above, were classified as 'typical' because a high proportion of individual samples had a balanced composition (25-75% GPI1-A), whereas only the yolk sac endoderm distributions from U($^8/_8\leftrightarrow^8/_8$) and U($^4/_8\leftrightarrow^4/_8$) were classified as 'typical'. Table 5 shows that the mean % GPI1-A is lower in all five U($^4/_8\leftrightarrow^4/_8$) tissues than in series B($^4/_8\leftrightarrow^4/_8$) and this difference is statistically significant in all but the yolk sac endoderm. Thus, after halving the number of cells in the chimaeric aggregates, the difference between the composition of the balanced and unbalanced series is maintained for most tissues.

Comparisons between the ($^8/_8\leftrightarrow^8/_8$) and ($^4/_8\leftrightarrow^4/_8$) series

Comparisons between U($^8/_8\leftrightarrow^8/_8$) and U($^4/_8\leftrightarrow^4/_8$) showed no significant differences in composition in any of the five tissues studied (Table 5). This confirms that the effect of halving the number of cells in the aggregate did not convert the unbalanced series into a balanced one. Comparisons between B($^8/_8\leftrightarrow^8/_8$) and B($^4/_8\leftrightarrow^4/_8$) showed that, in two tissues, the % GPI1-A was higher in the B($^4/_8\leftrightarrow^4/_8$) series but, for three tissues, it was lower. It was significantly lower in the amnion and yolk sac mesoderm samples (Table 5).

Fig. 2C (data from Tang and West, 2000) shows results for another series of chimaeras produced by aggregating 4-cell stage (BALB/c × A/J)F_2 embryos and 8-cell stage (C57BL × CBA/Ca)F_1 × TGB embryos. This is equivalent to a B($^4/_4\leftrightarrow^8/_8$) series. It shows that the genotypically balanced strain combination can produce chimaeras with phenotypically unbalanced distributions if the two aggregated embryos differ significantly in developmental stage.

One striking feature of the chimaeras made by aggregating two half embryos is the higher frequency of chimaeric conceptuses with non-chimaeric fetuses or whole epiblasts (Table 6). If the non-chimaeric conceptuses are included, the frequency of non-chimaeric fetuses in series U($^4/_8\leftrightarrow^4/_8$) is significantly greater than in series U($^8/_8\leftrightarrow^8/_8$), (31/49 versus 14/35; $\chi^2 = 4.44$; P=0.035).

Sixteen chimaeric conceptuses in series U($^4/_8\leftrightarrow^4/_8$) had non-chimaeric epiblasts and five of these had epiblasts that were entirely composed of BALB/c cells (GPI1-A). In two of these five cases the yolk sac endoderm was also entirely BALB/c (Fig. 2B). This does not, however, reflect a failure of selection against BALB/c cells, because it may be accounted for by allocation of entirely BALB/c cells to the inner cell mass (ICM) or, more specifically, to the epiblast. Clearly, if only BALB/c cells were allocated to the epiblast, even stringent selection against BALB/c cells could not reduce the proportion below 100%. Despite these five chimaeras with entirely BALB/c epiblast tissues, series U($^4/_8\leftrightarrow^4/_8$) appeared to be unbalanced just like U($^8/_8\leftrightarrow^8/_8$).

TABLE 6

FREQUENCY OF CHIMAERISM IN DIFFERENT DEVELOPMENTAL LINEAGES

GPI1 composition (mixed or single)			Number of conceptuses			
Epiblast	Primitive endoderm	Trophectoderm	U($^8/_8\leftrightarrow^8/_8$)	U($^4/_8\leftrightarrow^4/_8$)	B($^8/_8\leftrightarrow^8/_8$)	B($^4/_8\leftrightarrow^4/_8$)
Chimaeric fetus						
(a) mixed	single	single	1	1	1	3
(b) mixed	single	mixed	0	1	0	8
(c) mixed	mixed	single	7	4	5	4
(d) mixed	mixed	mixed	13	12	21	18
Non-chimaeric fetus (chimaerism confined to extraembryonic tissues)						
(e) mixed	mixed	mixed	0	0	2	0
(f) mixed	mixed	single	0	2	0	0
(g) single	single	mixed	3	5	0	2*
(h) single	mixed	single	5	4	1	0
(i) single	mixed	mixed	2	7	0	5
Total chimaeric fetuses (a-d)			21	18	27	33
Total non-chimaeric fetuses (e-i)			10	18	3	7
Total chimaeric conceptuses (a-i)			31	36	30	40
Non-chimaeric conceptuses			4	13	3	3

*In both B($^4/_8\leftrightarrow^4/_8$) chimaeras in row (g), the composition of the epiblast lineage was different from the yolk sac endoderm (in one case the epiblast was GPI1-A but the yolk sac endoderm was GPI1-B; the other chimaera had a GPI1-B epiblast and a GPI1-A yolk sac endoderm). In the other eight chimaeras in row (g), the epiblast and yolk sac endoderm were of the same GPI1 type.

Discussion

Comparison of the physical parameters of the different series of chimaeras indicates that downward size regulation of the chimaeras made by aggregating two intact 8-cell stage embryos was essentially complete by E12.5, as expected from previous studies (Tarkowski, 1963; Buehr and McLaren, 1974; Lewis and Rossant 1982; Rands 1986a). These chimaeras initially had twice as many cells as those made by aggregating two half embryos yet by E12.5 the largest difference was that B($^8/_8\leftrightarrow^8/_8$) fetuses were, on average, only 1.23 times the weight of B($^4/_8\leftrightarrow^4/_8$) fetuses. This difference is smaller than that between [(C57BL × CBA/Ca)F_1 × TGB] fetal weights and those of either of the other two control strains, all of which were derived from single embryos. The observation that the mean B($^8/_8\leftrightarrow^8/_8$) physical parameters all exceeded the means of both constituent control strains (which, like the chimaeras, were transferred to CF_1 foster mothers) might reflect a combination of incomplete size regulation and vegetative heterosis, as described for adult body weight (Falconer *et al.*, 1981). However, the differences between the B($^8/_8\leftrightarrow^8/_8$) chimaeras and the [(C57BL × CBA/Ca)F_1 × TGB] controls were mostly non-significant.

Mixed populations of cells were less frequently found in the fetuses and other epiblast derivatives of chimaeras made by aggregating two half embryos than those made by aggregating two whole embryos. This difference was predicted by previous authors (e.g. Mintz, 1971) who argued that the proportion of non-variegated fetuses should be inversely correlated with the number of cells allocated to the fetal lineage. It is doubtful, however, whether the frequency of non-chimaeric fetuses could be used to estimate reliably the number of fetal progenitor cells, because the two cell populations in a chimaeric aggregate are not randomly distributed (see McLaren, 1972; West, 1978).

As previously noted (West *et al.*, 1995b), mixed populations of cells were also less frequently found in the fetuses and other epiblast derivatives of the unbalanced series than the balanced series of chimaeras. Although BALB/c cells also tended to be reduced in the yolk sac endoderm (primitive endoderm lineage) they were less often completely excluded from this tissue than from the fetus or other epiblast derivatives. Thus, when chimaeras are produced from unbalanced strain combinations or by aggregating two half embryos, chimaerism is more likely to be confined to primitive endoderm and/or trophectoderm lineages. An understanding of the mechanisms

involved may also shed light on the aetiology of human confined placental mosaicism. This is an important clinical condition that can cause problems when chorionic villus samples are used for prenatal diagnosis of cytogenetic disorders (Kalousek, 1994).

The present study shows that although the composition of the balanced strain combination was slightly affected by aggregating two half embryos rather than two whole embryos, the unbalanced strain combination remained unbalanced even when size regulation was absent or minimal. This leads us to conclude that, while size regulation may still have some effect on the composition of the chimaera, it does not play the main role in reducing the contribution of BALB/c cells in the unbalanced strain combination. It should be noted, however, that the reason for genotypic imbalance may differ for chimaeras of different strain combinations.

This leaves several possibilities to be considered for the unbalanced strain combination studied here. The late blastocyst comprises four developmental lineages (epiblast, primitive endoderm, polar trophectoderm and mural trophectoderm). In the unbalanced series of chimaeras made by aggregating two whole embryos, BALB/c cells were under-represented in the derivatives of the epiblast, primitive endoderm and polar trophectoderm but the mural trophectoderm was not analysed because it makes little contribution to E12.5 conceptuses. As previously discussed (West *et al.,* 1995b) it is possible either that the BALB/c cells were preferentially allocated to the mural trophectoderm or that they were depleted by selection.

Selection could act at the level of the conceptus such that chimaeras with higher proportions of BALB/c cells were less viable. In the present study slightly fewer aggregates from the unbalanced strain combination implanted but this is not a consistent feature of chimaeric embryos from unbalanced strain combinations involving BALB/c (West *et al.,* 1995b). Postimplantation losses were not significantly higher in the unbalanced strain combination so there is no evidence that differential viability plays a role in eliminating chimaeras with a higher proportion of BALB/c cells.

Selection could act at the cellular level and involve competition, between the two component genotypes, which depletes the contribution of BALB/c cells in the chimaeras. If so, this would have to be sufficiently generalised to affect the epiblast, primitive endoderm and polar trophectoderm lineages. The present study provides evidence against any significant competition between BALB/c and [(C57BL × CBA/Ca)F_1 × TGB] cells that depends on the downward size regulation that occurs in chimaeras produced by the aggregation of two whole embryos. It is still possible that cell selection could act continuously to gradually reduce the contribution of BALB/c cells so that by E12.5 they are significantly depleted. BALB/c preimplantation embryos tend to develop rather slowly (Goldbard and Warner, 1982) and Table 3 shows that by E12.5 they lag behind [(C57BL × CBA/Ca)F_1 × TGB] conceptuses in size and developmental stage. Also, Fig. 2C shows that a balanced strain combination can produce unbalanced chimaeras if one of the embryos in the aggregate is developmentally delayed. If, for example, BALB/c embryos lagged behind [(C57BL × CBA/Ca)F_1 × TGB] embryos by several hours throughout development, this would represent a larger difference, in terms of cell divisions, after implantation when the mitotic rate increases (McLaren, 1976b). In this way, a small difference in cell numbers between the two embryos aggregated might be amplified at later stages of development to produce an unbalanced chimaera, even if the actual rates of cell division did not differ.

Previous comparisons of preimplantation development of embryos from different inbred strains have shown that both BALB/c and C3H/He develop more slowly than C57BL/10 embryos. The difference between C3H/He and C57BL/10 is attributable to different alleles at the *Ped* (preimplantation embryo development) locus whereas the slow development of BALB/c embryos (which carries the fast *Ped* allele) is attributable to other uncharacterised genetic background effects (Verbanac and Warner, 1981; Goldbard and Warner, 1982; Goldbard *et al.,* 1982; Warner *et al*., 1998).

There is evidence that the slow development of both BALB/c and C3H strain embryos correlates with a poor contribution to aggregation chimaeras, even though reports on the composition of C3H↔BALB/c chimaeras are contradictory. Mullen and Whitten (1971) showed C3HeB/FeJ cells made a poor contribution to the coats of most C3HeB/FeJ↔(SJL/J × 129/Rr)F_1 aggregation chimaeras and that BALB/c cells tended to contribute poorly to the coats of C57BL/10GnDg↔BALB/cGnDgWt chimaeras. Dvorak *et al*. (1995) found that C57BL/6N cells predominated in C3H/HeN↔C57BL/6N chimaeras by E7.5. They also reported that C3H/HeN predominated in C3H/HeN↔BALB/cA chimaeras, whereas Tachi *et al*, (1991) found that BALB/cA predominated in the coats of adult chimaeras of the same strain combination.

Although the slow or delayed early development of BALB/c embryos seems the most likely explanation of their poor contribution to chimaeras, Dvorak *et al*. (1995) reached a different conclusion. They noted a high frequency of postimplantation losses in their series of chimaeras and proposed that both embryo survival and changes in the composition of chimaeras depended on immune interactions between the foster mothers and chimaeric fetuses. Evidence that cell interactions affect the chimaeric phenotype includes a report of delayed protein synthesis when cells from 8-cell stage embryos were aggregated to 2-cell stage embryos (Prather and First, 1988) and several cases of vegetative heterosis, where the measured value exceeds the quantitative range defined by the two parental genotypes (Falconer *et al*., 1981; Mikami and Onishi 1985; Crusio et al, 1990). Nevertheless, the poor contribution of BALB/c cells to chimaeras seems more likely to be a result of cell-autonomous differences between genotypes rather than cell interactions.

In conclusion, the present study shows that BALB/c cells made a poor contribution to BALB/c↔[(C57BL × CBA/Ca)F_1 × TGB] chimaeras even when two half-sized embryos were aggregated, to avoid the effects of size regulation. In each case the chimaeras were made by aggregating an equal number of cells yet, by E12.5, BALB/c cells were under-represented. Further studies are needed to establish whether this depletion occurs gradually or at a specific stage and to test whether BALB/c cells are also depleted from the mural trophectoderm lineage.

Materials and Methods

Mouse strains

Mice referred to here as "CF_1" are F_1 hybrids of two congenic strains that are both homozygous for albino and *Gpi1^c* (West and Flockhart, 1994). The TGB stock was derived from a cross between (C57BL × CBA/Ca)F_1 and strain 83, which carries a homozygous reiterated ß-globin transgenic sequence TgN(Hbb-b1)83Clo (Lo, 1983; 1986; Lo *et al* 1987). Offspring were backcrossed to (C57BL × CBA/Ca)F_1 then intercrossed until homozygous for the transgene. CBA/Ca males were obtained from the Institute of Cell, Animal and Population Biology, University of Edinburgh; BALB/c/Eumm and some (C57BL × CBA/Ca)F_1 mice were purchased from the Department of Medical

Microbiology, University of Edinburgh and A/J/Ola/Hsd mice were purchased from Harlan Olac Ltd (Bicester, UK). All other animals were bred and maintained, under conventional conditions of 14 hours light (05:00-19:00h) and 10 hours dark. Chimaeras were made either by combining inbred BALB/c embryos with [(C57BL × CBA/Ca)F_1 × TGB] embryos (unbalanced series) or by combining (BALB/c × A/J)F_2 embryos with [(C57BL × CBA/Ca)F_1 × TGB] embryos (balanced series).

Superovulation and embryo collection

Female mice (5-7 weeks old) were superovulated by intraperitoneal injections of 5 IU pregnant mare's serum gonadotrophin (PMSG) at 12:00h, followed by 5 IU human chorionic gonadotrophin (hCG) 48h later. After hCG injection, the females were caged individually with the appropriate males. Mating was verified by the presence of a vaginal plug the following morning, which was designated 0.5 day *post coitum* (*p.c.*) or E0.5. On the same day when the plugs were checked, a group of CF_1 females was examined and those in oestrus were mated to vasectomised CF_1 males to produce pseudopregnant females.

Eight-cell stage embryos were flushed from the reproductive tracts of pregnant females at E2.5 with HEPES-buffered M2 medium (Quinn *et al.*, 1982) and their zonae pellucidae removed by brief exposure to warm acidic Tyrode's solution (Nicolson *et al.*, 1975).

Production of chimaeras

Four series of chimaeras were produced and are listed in Table 1. The ($^8/_8 \leftrightarrow ^8/_8$) chimaeras were made by pushing pairs of zona-free 8-cell stage embryos together in drops of M2 medium containing phyotohaemagglutinin (PHA, M form, Gibco 10576-015; diluted 1:19 v/v in M2 medium) for two minutes (Mintz *et al.*, 1973; Pratt, 1987). For production of ($^4/_8 \leftrightarrow ^4/_8$) chimaeras, the blastomeres were dissociated in M2 medium by drawing zona-free 8-cell stage embryos through a fine, flame-polished pipette (Kelly, 1977). Four cells from each of the two strains involved in the combination were then aggregated in M2 medium containing PHA. All the aggregates were rinsed in drops of M2 medium and transferred to drops of pre-equilibrated M16 medium (Whittingham, 1971) under paraffin oil (Boots) and cultured at 37ºC in an atmosphere of 5% CO_2 in air

Embryos which had formed well-developed morulae or early blastocysts by the morning after aggregation were transferred surgically to the uterine horns of CF_1 females at 2.5 days of pseudopregnancy. The females were anaesthetised with Hypnorm / Hypnovel as previously described (West *et al,* 1995b) and pregnancies were timed according to the pseudopregnant females.

Analysis of chimaeras

CF_1 females were killed at 12.5 days of gestation and the conceptuses were dissected to provide five samples: fetus, amnion, yolk sac mesoderm, yolk sac endoderm and placenta. The mesoderm and endoderm layers of the visceral yolk sac were separated as previously described (West and Flockhart, 1994). Briefly, the visceral yolk sacs were put into separate wells, of 16-well plates, containing trypsin/pancreatin solution (0.5 g trypsin and 2.5 g pancreatin in 100 ml phosphate buffered saline), at 4°C for approximately 3.5 hours (Levak-Svajger *et al.*, 1969). They were then transferred to fresh M2 medium for at least 30 min. at 4°C and finally transferred to another dish of fresh M2 medium and dissected with watchmaker's forceps. The whole conceptus was weighed, the fetus and placenta were each weighed separately, the crown/rump length was measured and the morphological index, based on an assessment of hind limb development (McLaren and Buehr, 1990; Palmer and Burgoyne, 1991), was recorded.

All the tissues were stored at -20°C in 50% glycerol in water, 200µl each for fetus and placenta in 1.5 ml eppendorf microfuge tubes, 20µl each for the others in 96-well plates. Samples were lysed by three cycles of freeze/thawing with mechanical disruption. Each chimaera was a mixture of homozygous *Gpi1*$^{a/a}$ and *Gpi1*$^{b/b}$ cells, and the recipient CF_1 females were homozygous *Gpi1*$^{c/c}$. The proportions of the two cell populations in the chimaeric tissues were estimated from the proportions of GPI1-A and GPI1-B allozymes, after electrophoresis, staining for GPI1 activity and densitometry with a Helena Process-24 gel scanner (West *et al.*, 1986). Any maternal contamination appeared as a GPI1-C band which was excluded from the calculations.

Control groups

Some non-chimaeric 8-cell stage embryos from each mating were used as controls. After removing the zonae pellucidae, they were cultured overnight and transferred to CF_1 pseudopregnant females, as described for the chimaeras. They were dissected at E12.5 and their physical development assessed as described above.

Statistical analysis

Statistical tests were performed on an Apple Macintosh computer using the statistical packages 'StatView 4.1' (Abacus Concepts Inc., Berkeley, USA) and MultiStat (Biosoft, Cambridge).

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