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Investigation of variability among mouse aggregation chimaeras and X-chromosome inactivation mosaics

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SUMMARY

Mouse aggregation chimaeras were produced by aggregating C3H/HeH and C3H/HeHa–*Pgk-1*^{a}/Ws embryos. At mid-term the proportions of the two cell populations in these conceptuses and the X-inactivation mosaic female progeny of C3H/HeH ♀ × C3H/HeHa–*Pgk-1*^{a}/Ws ♂ matings were estimated using quantitative electrophoresis of phosphoglycerate kinase (PGK-1) allozymes. The percentage of PGK-1B was more variable in the foetus, amnion and yolk sac mesoderm of the chimaeras than in the corresponding tissues of the mosaic conceptuses. Positive correlations were found for the percentage of PGK-1B between these three primitive ectoderm tissues in both chimaeras and mosaics and between the two primitive endoderm tissues (yolk sac endoderm and parietal endoderm) of the chimaeras. There was no significant correlation between the primitive ectoderm and primitive endoderm tissues of the chimaeras. The results suggest that unequal allocation of cell populations to the primitive ectoderm and primitive endoderm considerably increases the variability among chimaeras but variation probably exists before this segregation occurs. The variation that arises before and at this allocation event is present before X-chromosome inactivation occurs in the primitive ectoderm lineage and explains why the proportions of the two cell populations are more variable among chimaeras than mosaics. Additional variation arises within the primitive ectoderm lineage, after X-inactivation. This variation may be greater in chimaeras than mosaics but the evidence is inconclusive. The results also have some bearing on the nature of the allocation of cells to the primitive ectoderm and primitive endoderm lineages and the timing of X-chromosome inactivation in the primitive ectoderm lineage.

INTRODUCTION

Mouse chimaeras, produced by aggregating two 8-cell embryos, and X-inactivation mosaics both comprise two populations of cells. Cellular heterogeneity in aggregation chimaeras is a consequence of experimental manipulation during early development. X-inactivation mosaics (henceforth referred to as mosaics), on the other hand, are produced by random X-chromosome inactivation in normal XX females. After X-chromosome inactivation some

Table 1. *Comparison of mouse aggregation chimaeras and X-inactivation mosaics.* (Modified after West, 1978)

	Aggregation chimaeras	X-inactivation mosaics
Number of zygotes	2(or more)	1
Time of 'marking' of different cell populations.	By aggregation. 8 + 8 cells (2½ days *p.c.*)	By X-chromosome inactivation (probably betwen 4½ and 5½–6½ days *p.c.* in the foetal lineage*).
Spatial relationship between cell populations at the time of 'cell marking'.	Non-random.	Probably random
Maximum genetic difference possible between cell populations.	Whole genome	X-chromosome
Difference in developmental age between cell populations.	Possible	None

p.c. = *post coitum*
*Gardner, 1974; Monk & Harper, 1979; Rastan, 1982.

cells express genes on the maternally derived X-chromosome (X^m) and other cells express genes on the homologous, paternally-derived X-chromosome (X^p). In both types of experimental mice the two cell populations can be visualized with appropriate genetic markers (see McLaren, 1976a).

In many respects chimaeras and mosaics are very similar: the proportions of the two cell populations in different organs of individuals are usually positively correlated (Nesbitt, 1971; Falconer, Gauld, Roberts & Williams, 1981) and the distribution of the two cell populations within a tissue often appears to be similar in chimaeras and mosaics. This is seen, for example, in patterns of coat melanocytes (Mintz, 1967; Cattanach, 1974), retinal pigment epithelium cells (West, 1976a) and hepatocytes (West, 1976b; Wareham, Howell, Williams & Williams, 1983).

One consistent difference between chimaeras and mosaics is that the proportions of the two cell populations are more variable among chimaeras than mosaics (Deol & Whitten, 1972; McLaren, 1976a; West, 1976a; Falconer & Avery, 1978). This is true even when a series of chimaeras is produced by aggregating embryos from two inbred strains.

Table 1 shows some differences between chimaeras and mosaics that could underlie this greater variability among chimaeras. Genetically based cell selection is unlikely to contribute to the extra variability of chimaeras when they are made by aggregating embryos of two inbred strains, unless the ocurrence of XX $\longleftrightarrow$ XX, XX $\longleftrightarrow$ XY and XY $\longleftrightarrow$ XY chimaeras is somehow significant in this respect. It seems more likely that the greater variability of chimaeras is a result of some embryological inequality between the two

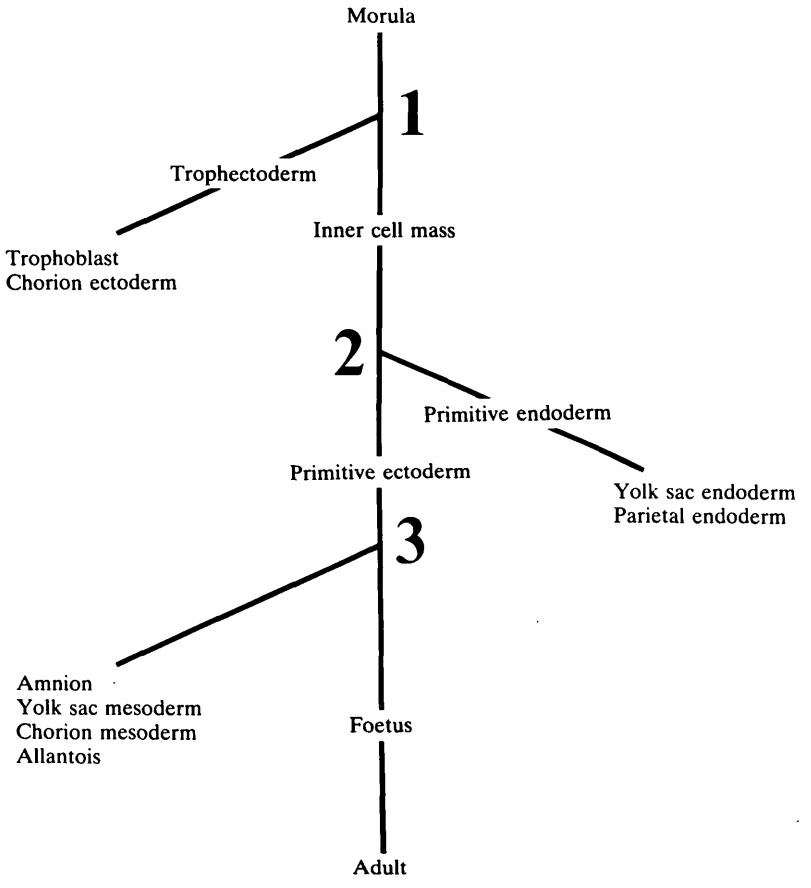

Fig. 1. Lineages established during mouse development. Allocation events are marked 1, 2 and 3. For simplicity the multiple allocation events that occur within the primitive ectoderm lineage are shown as a single event at 3. X-Chromosome inactivation in the foetal lineage occurs between allocation events 2 and 3 (see Discussion for references).

aggregated embryos or the allocation of unequal proportions of the two cell populations to the foetal lineage.

Falconer & Avery (1978) considered the theoretical aspects of unequal cell allocation in depth. They argued that the greater variability among chimaeras is a consequence of the first two allocation events. First, when cells are allocated to the inner cell mass (ICM) or trophectoderm and second, when ICM cells are allocated to the primitive ectoderm (epiblast) or primitive endoderm. They went on to conclude that 'most of the variation in chimaeras comes from the second sampling'. Their reasoning is based on a knowledge of 1) the allocation events in early mouse development (see Fig. 1), 2) the approximate timing of X-chromosome inactivation in the foetal lineage (be-

tween allocation events 2 and 3 in Fig. 1), and 3) the extent of cell mixing shortly after aggregation of the two 8-cell embryos.

Falconer & Avery (1978) reasoned that the first sampling event, where cells are allocated to either the trophectoderm or inner cell mass, will contribute some variation since small numbers of cells are involved but for geometrical reasons this variation will be limited. Their argument is based on two points. First, the cells allocated to the inner cell mass are believed to be located in the inside of the morula, surrounded by the presumptive trophectoderm cells (Tarkowski & Wroblewska, 1967; Hillman, Sherman & Graham, 1972). Second, little cell mixing occurs between the two aggregated embryos before the blastocyst is formed (Garner & McLaren, 1974). Thus, the plane of allocation divides the morula into two concentric spheres while the two aggregated cell populations remain approximately as two hemispheres. Falconer & Avery (1978) argued, therefore, that similar proportions of the two cell populations will be allocated to the trophectoderm and inner cell mass.

At the second sampling event, however, different proportions of the two cell populations may be allocated to the primitive ectoderm and primitive endoderm. This is illustrated in Fig. 2. Falconer & Avery (1978) proposed that on mathematical grounds this second allocation event was capable of generating most of the extra variability seen among chimaeras, if little cell mixing occurs within the inner cell mass. Their model makes the specific prediction that the cell proportions in the tissues derived from the primitive ectoderm should be

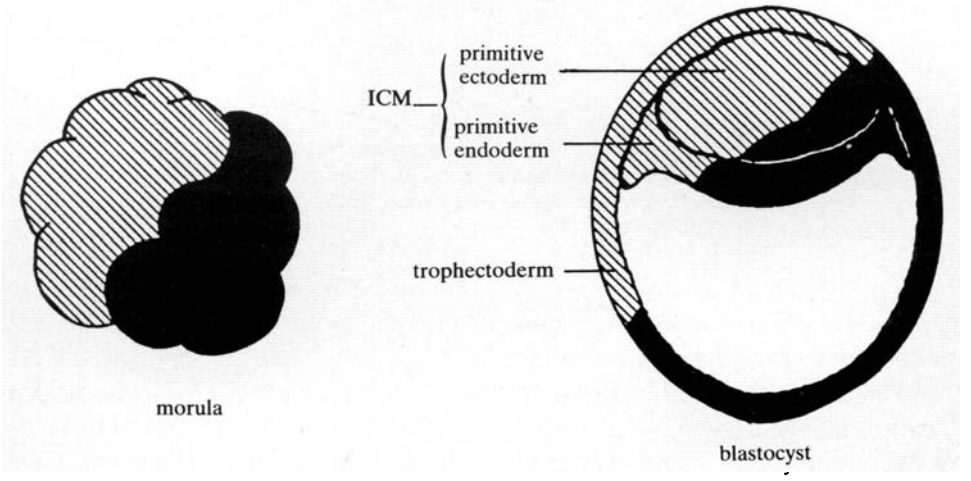

Fig. 2. Production of a chimaeric blastocyst (right) from a morula (left) composed of black and shaded cell populations. In the blastocyst shown, the trophectoderm and inner cell mass (ICM) have similar proportions of black cells. The primitive endoderm, however, has a higher proportion of black cells than the primitive ectoderm. As a result of this unequal cell allocation the foetus is likely to have a low proportion of 'black' cells.

negatively correlated with the proportions in the tissues of the primitive endoderm lineage, unless modified subsequently (Falconer & Avery, 1978, p.218).

The experiments reported here were designed to test Falconer and Avery's unequal allocation model. We have examined the proportions of the two cell populations in the foetus and various extraembryonic membranes of a series of chimaeras and mosaics constructed from partially congenic strains. This was done both to compare the variability of chimaeras and mosaics and to test whether, in chimaeras, the cell proportions in the primitive endoderm lineage are negatively correlated with those in the primitive ectoderm lineage. In addition, correlations of cell proportions in mosaic tissues have some bearing on the timing of X-chromosome inactivation in the foetal lineage.

MATERIALS AND METHODS

a) Production of chimaeras and mosaics

Chimaeras were made by aggregating preimplantation embryos from the C3H/HeH and C3H/HeHa–*Pgk-1*^{a}/Ws (abbreviated to C3H–*Pgk-1*^{a}) inbred strains. Embryos, at 2½ days *post coitum (p.c.),* were flushed from the ovidcuts of naturally mated females with the Hepes-buffered medium (M2) described by Quinn, Barros & Whittingham (1982). Normally the embryos were collected between 9·00 and 11·30 h, two days after finding the vaginal plug and the majority were at the 8-cell stage. The zonae pellucidae were removed in acid Tyrodes solution (Nicolson, Yanagimachi & Yanagimachi, 1975) and the embryos returned to M2 medium.

To facilitate aggregation small wells were made by melting areas in the bottom of a 5 cm diameter, plastic Petri dish with a heated, fine glass rod (A. McMahon and G. Porter-Goff, personal communication). The wells were filled with drops of Whittingham's embryo culture medium No. 16 (Whittingham, 1971), covered with paraffin oil and pairs of denuded embryos were placed in contact in each well. The culture dishes were placed in a plastic sandwich box. A humid, CO_2-enriched atmosphere was maintained by dissolving three soda tablets (468 mg sodium bicarbonate, 132 mg tartaric acid) in a Petri dish of water inside the sandwich box as described by Cattanach, Wolfe and Lyon (1972). The box was then sealed with adhesive tape and incubated at 37 °C for approximately 22 h.

After overnight culture nearly all of the pairs of embryos had aggregated and reached the late morula stage. Aggregated embryos were placed in M2 medium and surgically transferred to the uterus of a pseudopregnant recipient female on the third day of pseudopregnancy. Recipient females were (C3H/HeH × 101/H)F_1 hybrids that had been mated to genetically sterile males, heterozygous for the male sterile T145H translocation.

The transfer technique was similar to that reported by McLaren & Michie

(1956) and has been described elsewhere (West, Kirk, Goyder & Lyon, 1984). Most commonly six embryos were transferred, unilaterally to the right uterine horn.

Mosaic foetuses were the female progeny of natural matings between C3H/HeH females and C3H–*Pgk-1*^{a} males.

b) Analysis of chimaeras and mosaics

Conceptuses resulting from the transfer of aggregated embryos were removed from the uterus 12½ days after the recipient female mated (12½ days *p.c.*). Each normal conceptus was placed in M2 medium and the foetus, amnion, yolk sac mesoderm, yolk sac endoderm and parietal endoderm were separated as described by Papaioannou & West (1981). The endodoerm and mesoderm layers of the yolk sac were separated following treatment with a mixture of 0·5% trypsin and 2·5% pancreatin in either calcium- and magnesium-free Tyrode's solution or phosphate-buffered saline (Levak-Svajger, Svajger & Skreb, 1969). These samples were sonicated in a small volume of sample buffer (200 μl for the foetus and 50 μl for the other samples) using an MSE Soniprep 150. Sonicated samples were centrifuged and the supernatants were stored in 250 μl plastic tubes at −20 °C for up to three weeks before they were shipped to Munich, on dry ice, by air. The sample buffer was prepared as described by Papaioannou, West, Bücher & Linke (1981) according to the recipe of Bücher *et al.* (1980).

Conceptuses from matings between C3H/HeH females and C3H–*Pgk-1*^{a} males were dissected at 12½ days *p.c.* as described for the chimaeras. (In this case, however, the dissections were done in modified PB1 medium containing 10% foetal calf serum, as described by Papaioannou & West (1981), rather than M2 medium). The yolk sac endoderm and parietal endoderm samples were discarded from most of these conceptuses once preliminary analyses had confirmed that these tissues were not mosaic because of the preferential expression of X^m. Heterozygous *Pgk-1*^{b}/*Pgk-1*^{a} female conceptuses (mosaics) were distinguished from their *Pgk-1*^{b}/Y siblings by electrophoresis of phosphoglycerate kinase in the foetal sample (see below).

The propotions of the two cell populations in mosaic and chimaeric tissues were estimated by quantitative electrophoresis of the PGK-1A and PGK-1B allozymes of phosphoglycerate kinase. In somatic tissues PGK-1A and PGK-1B have equal specific activities (West & Chapman, 1978; Johnston & Cattanach, 1981; Mühlbacher, Kuntz, Haedenkampt & Krietsch, 1983). They are produced by the X-chromosome-linked gene *Pgk-1* and provide a genetic marker for the two cell populations in both mosaics and chimaeras.

The randomness of X-chromosome inactivation is influenced by the X-chromosome controlling elements (*Xce*) of the conceptus. The C3H/HeH inbred strain carries the *Xce*^{a} allele whereas the C3H–*Pgk-1*^{a} strain that we

used carries the *Xce*^{c} allele (Johnston & Cattanach, 1981). This C3H–*Pgk-1*^{a} strain was derived from a cross between a C3H/HeHa female and a single *Pgk-1*^{a}/Y male that was descended from a feral mouse trapped in Bjertrup, Denmark (Nielsen & Chapman, 1977). Heterozygous *Pgk-1*^{a}/*Pgk-1*^{b} females were crossed to C3H/HeHa males for seven more generations and then intercrossed and inbred to produce a homozygous *Pgk-1*^{a}/*Pgk-1*^{a} strain (West & Chapman, unpublished).*

Cellogel electrophoresis, staining and quantitation of the PGK-1 allozymes were done as described (Bücher *et al.*, 1980; Rabes *et al.*, 1982). The PGK-1A and PGK-1B allozymes were stained and visualized as fluorescent bands under long wavelength ultraviolet illumination. Their relative proportions were estimated by integrating the areas under the peaks that were produced by scanning the bands with a scanning fluorimeter fitted with a chart recorder. Statistical tests were done using a computer and a Hewlett Packard programmable calculator, programmed by Mr. D. G. Papworth of the MRC Radiobiology Unit.

RESULTS

a) Contamination controls

In order to determine whether maternal PGK-1 activity was present as a contaminant in the various samples that were analysed, 20 C3H–*Pgk-1*^{a} embryos were surgically transferred to three recipient *Pgk-1*^{b}/*Pgk-1*^{b} females. (The embryos were collected at 3½ days *p.c.* and transferred, without *in vitro* culture, to (C3H/HeH × 101/H)F_1 females during the third day of pseudopregnancy). All three recipients became pregnant and thirteen normal conceptuses and two small moles were recovered on the 13th day of pseudopregnancy. Ten normal conceptuses were dissected to provide separate samples of foetus, amnion, yolk sac mesoderm, yolk sac endoderm and parietal endoderm for analysis of PGK-1 allozymes.

Nine conceptuses showed no contaminating PGK-1B allozyme in any of the five samples and the remaining conceptus had no contamination in four of the five samples. One sample of parietal endoderm, that had been recorded as visibly contaminated, produced very low PGK-1 activity and contained about 7·5 % of the maternal PGK-1B allozyme. These results indicate that contamination with maternal PGK-1 is unlikely for the foetus, amnion, yolk sac mesoderm and yolk sac endoderm but may occur occasionally in the parietal endoderm.

**Footnote:* Although the *Xce*^{c} allele is associated with the closely linked *Pgk-1*^{a} allele in this partially congenic strain, feral mice carrying *Pgk-1*^{a} were trapped in several locations in Denmark by Nielsen & Chapman (1977) and at least one appeared to carry *Pgk-1*^{a} in association with a different *Xce* allele (West & Chapman, 1978).

b) Chimaeras

Of 190 pairs of embryos that were cultured overnight, 189 aggregated and were transferred to 27 recipient females. Twenty-one of these recipient females subsequently became pregnant, accounting for 149 of the embryos transferred. At 12½ days *p.c.* there were 56 implants comprising 34 small moles, 1 dead foetus and 21 live foetuses. These 21 normal conceptuses were dissected for analysis.

The percentage of PGK-1B in each of the five samples analysed from the 21 normal conceptuses is shown in Table 2. The conceptuses are grouped according to the distribution of the two cell populations among the five tissues examined. Those in which the foetus was not chimaeric are grouped according to the stage when one cell population was excluded from the foetal lineage. This occurs at allocation events 1, 2 and 3 (see Fig. 1) for groups 1, II and III respectively (Table 2).

One cell population was absent from all of the tissues examined in the nine conceptuses in group I. These represent either technical failures or chimaeras where one cell population was allocated exclusively to the trophectoderm lineage (allocation event 1 in Fig. 1).

The five conceptuses in group II (Table 2) were not chimaeric in any of the primitive ectoderm derivatives but analysis of the yolk sac endoderm and parietal endoderm revealed that the conceptuses were chimaeric. One cell

Table 2. *Percentage PGK-1B in twenty-one prospective chimaeric conceptuses*

Group*	Conceptus Number	Primitive ectoderm			Primitive endoderm	
		Foetus	Amnion	Yolk sac mesoderm	Yolk sac endoderm	Parietal endoderm
I	C1–C8	0	0	0	0	0
	C9	100	100	100	100	100
II	C10	0	0	0	9	0
	C11	0	0	0	46	0
	C12	0	0	0	59	62
	C13	0	0	0	52	17
	C14	100	100	100	0	0
III	C15	0	5	55	58	71
IV	C16	7	9	48	47	29
	C17	28	12	19	50	50
	C18	53	62	41	7	15
	C19	54	45	27	61	67
	C20	77	87	97	42	34
	C21	90	88	91	46	42

*The conceptuses are grouped according to the distribution of the two cell populations among the five tissues examined. Those in which the foetus was not chimaeric are grouped according to the stage when one cell population was excluded from the foetal lineage.

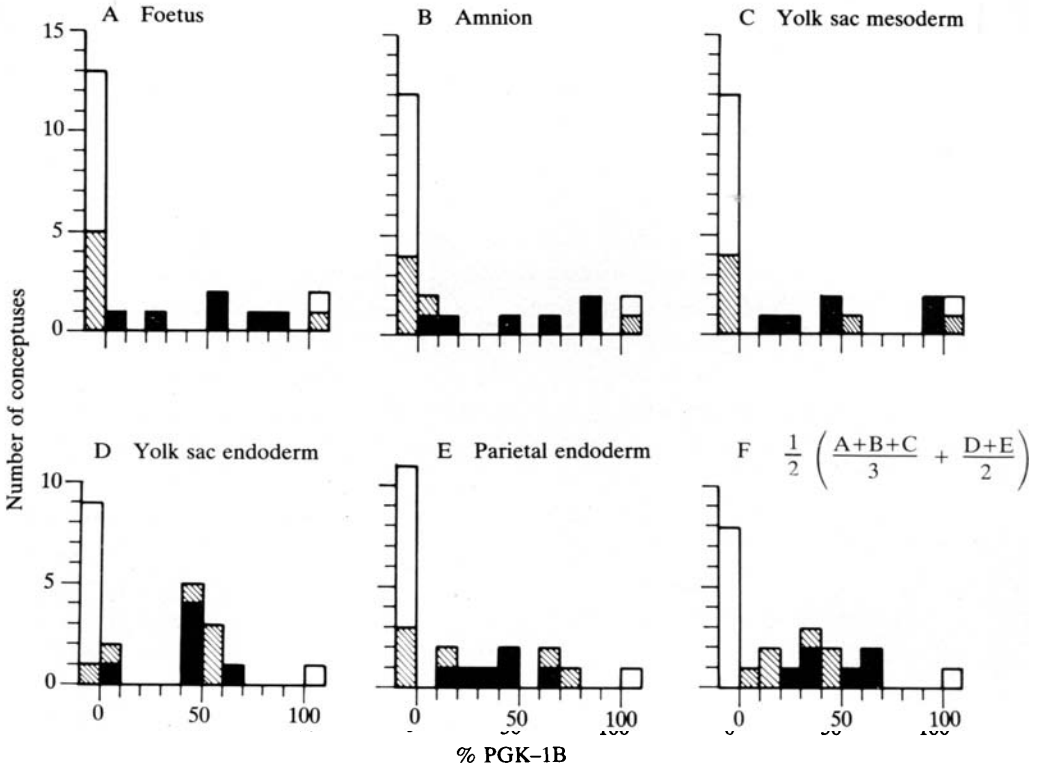

Fig. 3. A–E: Distributions of % PGK-1B in five tissues from chimaeric conceptuses. F: distribution of estimated % PGK-1B in the inner cell mass, calculated from the % PGK-1B in the five tissues analysed, as explained in the text. Open bars represent conceptuses C1–C9 (possible technical failures), the hatched bars represent C10–C15 (foetus not chimaeric but the conceptus is chimaeric) and the black bars represent C16–C21 (foetal chimaeras).

population was, therefore, lost from the foetal lineage when cells of the inner cell mass were allocated to the primitive ectoderm or primitive endoderm lineages. This illustrates that this allocation event can increase the variability among chimaeras. Conceptus C14 is an example of the presumably rare, extreme condition where the two cell populations segregate completely at cell allocation so that the primitive ectoderm comprises exclusively one cell population and the primitive endoderm is composed entirely of the other cell population.

In the one conceptus in group III (C15, in Table 2), one cell population was lost from the foetal lineage within the primitive ectoderm since the foetus was entirely PGK-1A but both PGK-1A and PGK-1B allozymes were present in the other four tissues. The remaining six conceptuses (group IV, in Table 2) were chimaeric in all five samples analysed.

Of the twelve known chimaeras (C10–C21), both cell populations were

present more frequently in the primitive endoderm lineage (11/12) than in the primitive ectoderm lineage (7/12). The PGK-1B (C3H/HeH) cell population was more frequently excluded than PGK-1A (C3H–*Pgk-1*^{a}) cells and it is possible that C3H ⟷ C3H–*Pgk-1*^{a} is an unbalanced strain combination, as defined by Mullen & Whitten, 1971.

The distributions of the % PGK-1B in the various individual chimaeric samples is shown in Figs. 3A–E. If the proportion of inner cell mass cells that are allocated to primitive ectoderm is reflected by the proportion that can be identified as such when they become overtly differentiated, it seems likely that about 50% of the inner cell mass cells are allocated to the primitive ectoderm and the remaining 50% are allocated to the primitive endoderm (see McLaren, 1976b). A crude estimate of the percentage of PGK-1B in the inner cell mass was calculated, on this basis as: ½ (unweighted ectoderm mean) + ½ (unweighted endoderm mean). The distribution of this estimate is shown in Fig. 3F.

The percentage PGK-1B in samples from the twelve known chimaeras did not fit a normal distribution (Fig. 3), so Spearman's rank correlation coefficient (r_s) was calculated to test Falconer and Avery's prediction that the cell proportions in the primitive ectoderm and primitive endoderm should be negatively correlated (Falconer & Avery, 1978). The results are shown in Table 3 and Fig. 4.

The three primitive ectoderm tissues were significantly positively correlated as were the two primitive endoderm tissues. Correlations between samples from the primitive endoderm and primitive ectoderm lineages however, were not statistically significant. Although there was no significant negative correlation between primitive endoderm and primitive ectoderm, the observation, that the correlations are lower between these two lineages than within them, supports Falconer and Avery's suggestion that allocation event 2 (see Fig. 1) is an important source of variation among chimaeras.

c) *Mosaics*

Thirty of the 68 (C3H/HeH × C3H–*Pgk-1*^{a})F_1 conceptuses that were examined produced both PGK-1A and PGK-1B allozymes in the foetus, amnion and yolk sac mesoderm. These were assumed to be mosaics (heterozygous *Pgk-1*^{b}/*Pgk-1*^{a} females). The remaining 38 conceptuses, which produced only PGK-1B in these samples, were assumed to be *Pgk-1*^{b}/Y males and were not considered further.

The percentage of PGK-1B in the individual samples is shown in Table 4 and the distributions shown in Fig. 5. The mean for all three tissues is lower than

Fig. 4. Correlations for % PGK-1B between five pairs of tissues from chimaeric conceptuses (A–E) and between the mean values for the primitive ectoderm and primitive endoderm (F).

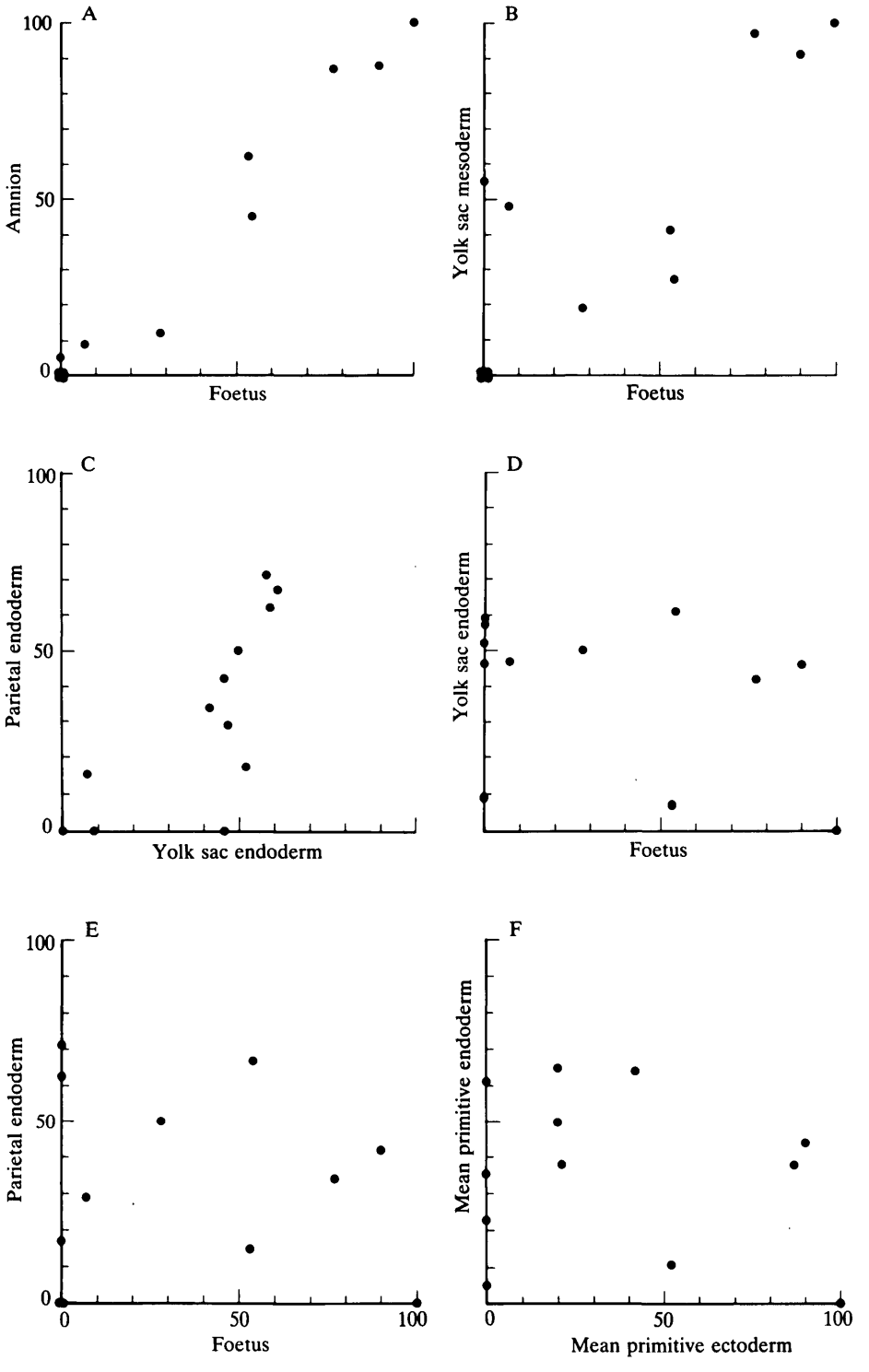
A
100
50
0
Amnion
Foetus
B
Yolk sac mesoderm
Foetus
C
Parietal endoderm
Yolk sac endoderm
D
Yolk sac endoderm
Foetus
E
Parietal endoderm
0
50
100
Foetus
F
Mean primitive endoderm
Mean primitive ectoderm

Table 3. *Spearman's rank correlation coefficients (r_s) for the percentage PGK-1B in different tissues from the twelve known chimaeric conceptuses.*

		Primitive ectoderm lineage			Primitive endoderm lineage
	Foetus	Amnion	Yolk sac mesoderm	Ectoderm mean	Yolk sac endoderm
Primitive ectoderm lineage					
Amnion	+0·974**	–	–	–	–
Yolk sac mesoderm	+0·790*	+0·877**	–	–	–
Primitive endoderm lineage					
Yolk sac endoderm	−0·414	−0·437	−0·376	–	–
Parietal endoderm	−0·015	+0·039	+0·118	–	+0·797*
Endoderm mean	–	–	–	−0·096	–

*0·001<P<0·01 **P<0·001

Table 4. *Percentage PGK-1B in mosaic conceptuses*

Conceptus number	Foetus	Amnion	Yolk sac mesoderm
M1	11	20	18
M2	16	14	17
M3	22	19	21
M4	23	11	23
M5	24	20	32
M6	24	23	26
M7	24	25	34
M8	25	18	28
M9	26	21	19
M10	27	23	33
M11	27	23	21
M12	27	22	31
M13	28	30	40
M14	29	24	32
M15	30	26	27
M16	31	24	29
M17	31	32	40
M18	33	25	29
M19	34	22	43
M20	34	22	25
M21	35	19	29
M22	35	32	35
M23	35	23	31
M24	36	34	39
M25	36	30	39
M26	36	28	30
M27	38	24	29
M28	40	29	31
M29	43	26	37
M30	50	33	47
Mean ± S.E.	30·3 ± 1·4	24·1 ± 1·0	30·5 ± 1·4

50% PGK-1B because the mosaic conceptuses are *Pgk-1*^{b}, *Xce*^{a}/*Pgk-1*^{a}, *Xce*^{c}. Heterozygosity for the X-chromosome controlling element, *Xce* results in non-random X-inactivation such that a majority of cells express the X-chromosome carrying *Xce*^{c} (Johnston & Cattanach, 1981).

The yolk sac endoderm and parietal endoderm are not normally mosaic for X-linked gene expression because the maternally derived X-chromosome is preferentially expressed in these tissues (West, Frels, Chapman & Papaioannou, 1977; Papaioannou & West, 1981). We tested these two tissues from eight (C3H × C3H–*Pgk-1*^{a})F$_1$ conceptuses to check whether this was true of *Xce*^{a}/*Xce*^{c} conceptuses, where the *Xce*^{c} allele was inherited from the father. Four of these conceptuses proved to be *Pgk-1*^{b}/*Pgk-1*^{a} females but none produced any PGK-1A allozyme in either the yolk sac endoderm or parietal endoderm. We concluded that preferential expression of the maternally derived *Pgk-1*^{b} allele occurred in the yolk sac endoderm and parietal endoderm of *Pgk-1*^{b}, *Xce*^{a}/*Pgk-1*^{a}, *Xce*^{c} females and so we excluded these tissues from further analysis of the mosaics. (Rastan & Cattanach (1983) have subsequently published cytogenetic evidence that suggests that X^p is active in some yolk sac endoderm cells of *Xce*^{a}/*Xce*^{c} conceptuses but this has yet to be confirmed.)

Table 5 shows that the percentage of PGK-1B in the three mosaic tissues analysed were positively correlated.

d) *Comparison of chimaeras and mosaics*

The distributions of the % PGK-1B in the foetus, amnion and yolk sac mesoderm samples from the chimaeras (Figs. 3A–C) was much broader than the corresponding distributions for mosaic samples (Fig. 5) even when the nine possible technical failures, C1–9 (shown as open bars in Fig. 3), are excluded.

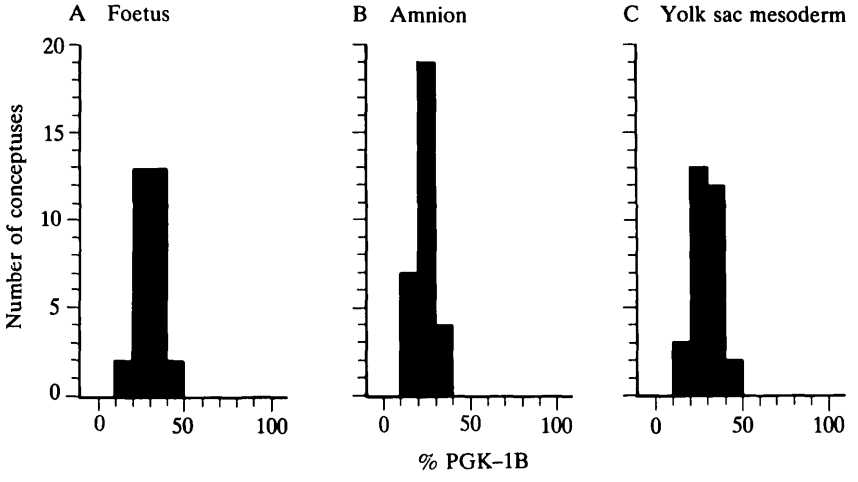

Fig. 5. Distribution of % PGK-1B in three tissues from mosaic conceptuses.

Table 5. *Correlations between the percentage PGK-1B in three tissues from thirty mosaic conceptuses*

	Foetus	Amnion
Correlation coefficients (r)		
Amnion	+0·643**	–
Yolk Sac Mesoderm	+0·680**	+0·716**
Spearman's rank correlation coefficients (r_s)		
Amnion	+0·678**	–
Yolk sac mesoderm	+0·568*	+0·693**

*P = 0·001 **P<0·001

Table 6. *Percentage PGK-1B in mosaic and known chimaeric conceptuses*

	% PGK-1B			
Sample	Mean ± S.E.	Number	Variance	Coefficient of Variation (%)*
CHIMAERAS (C3H/HeH ⟷ C3H/HeHa – *Pgk-1^a*)				
Foetus	34·1 ± 11·2	12	1513·4	114·1
Amnion	34·0 ± 11·5	12	1596·4	117·5
Yolk sac mesoderm	39·8 ± 11·3	12	1522·7	98·0
Yolk sac endoderm	39·8 ± 6·2	12	467·7	54·4
Parietal endoderm	32·3 ± 7·6	12	697.1	81·9
Mean primitive ectoderm (ect.)	36·0 ± 10·9	12	1435·9	105·3
Mean primitive endoderm (end.)	36·0 ± 6·5	12	504·5	62·4
½ (ect. + end.)**	36·0 ± 5·8	12	396·5	55·3
MOSAICS (C3H/HeH × C3H/HeHa – *Pgk-1^a*)F_1♀♀				
Foetus	30·3 ± 1·4	30	62·3	26·0
Amnion	24·1 ± 1·0	30	29·1	22·4
Yolk sac mesoderm	30·5 ± 1·4	30	56·1	24·6
Yolk sac endoderm	100	4	–	
Parietal endoderm	100	4	–	

*Coefficient of Variation (%) = (Standard Deviation/Mean) × 100 %.
**This represents a crude estimate of the % PGK-1B in the inner cell mass (see text).

This is also reflected by the higher variances and coefficients of variation found for the twelve known chimaeras than for the mosaics (Table 6). The crude estimates of the variability among the inner cell masses of the twelve known chimaeras, before cells were allocated to primitive ectoderm or primitive endoderm, (Fig. 3F and Table 6) is lower than for the individual chimaera samples but still higher than the variation seen among individual mosaic samples.

DISCUSSION

Previous reports have shown that the proportions of the two cell populations contributing to various adult tissues in mouse aggregation chimaeras are more variable than in the corresponding tissues of X-inactivation mosaics. Our present results show that this greater variability is not confined to the foetal lineage but is also true of the amnion and the yolk sac mesoderm.

Our observation that one cell population may be excluded from a particular developmental lineage yet contribute to others in chimaeric conceptuses (Table 2) may have wider implications. Unequal allocation may also occur, for example, in those chromosome mosaics that arise by non-disjunction during early cleavage, and result in a restricted pattern of mosaicism. Kalousek & Dill (1983) reported two cases of restricted human chromosome mosaicism and pointed out that restricted mosaicism could cause difficulties when prenatal diagnosis of foetal mosaicism is based on samples of extraembryonic tissues.

The quantitative results for the chimaeric conceptuses did not show a statistically significant negative correlation between the percentage of PGK-1B in the primitive ectoderm and primitive endoderm. Nevertheless, Falconer and Avery's argument, that unequal allocation of cells to the primitive ectoderm and primitive endoderm is an important source of variation in chimaeras, is supported by the results shown in Tables 2 and 3. One cell population was excluded from the foetal lineage at this allocation event in five of the twelve known chimaeras (group II in Table 2). Also, the correlations between primitive ectoderm and primitive endoderm were considerably lower than those within these lineages (Table 3).

However, the absence of a significant negative correlation between the primitive ectoderm and primitive endoderm strongly suggests that variation between inner cell masses was higher than predicted by Falconer and Avery. This variation would tend to cause a positive correlation between all inner cell mass derivities and reduce the significance of any negative correlation subsequently produced by unequal allocation of inner cell mass cells to the primitive ectoderm and primitive endoderm. The variability seen among chimaeras would then be a result of two superimposed sources of variation.

Variability between inner cell masses is perhaps to be expected *a priori*. Cell death, chance exclusion of cells from the aggregate or differences in developmental stage between the aggregants would all contribute to variation between aggregates. Unequal allocation of cells to the inner cell mass (event 1 in Fig. 1) could also occur by chance, because of the small numbers of cells involved, or perhaps because of developmental asynchrony between the aggregants. (Kelly, Mulnard & Graham (1978) showed that cells that divide early are more likely to contribute to the inner cell mass and Spindle (1982) showed that 8-cell embryos contributed disproportionately more cells to the inner cell masses of blastocysts formed from the aggregates of various numbers of 4-cell and 8-cell embryos.)

The distribution of the crude estimate of the percentage of PGK-1B in the inner cell masses of the present series of chimaeras (Fig. 3F) shows considerable variation (see also Table 6). Although much of this variation may be attributable to experimental error, since the estimate is very indirect, inspection of Table 2 confirms that variation must exist among inner cell masses. Chimaeras C10–C13 and C16 almost certainly developed from inner cell masses where C3H–*Pgk-1*^{a} cells (PGK-1A) predominated, whereas chimaeras C20 and C21 probably developed from inner cell masses in which C3H cells (PGK-1B) formed the majority.

Some direct evidence of the variation that exists between chimaeric inner cell masses comes from the chimaeric blastocysts that were produced by Garner & McLaren (1974) by aggregating radiolabelled and unlabelled 8–cell embryos. The mean percentage of labelled cells in the inner cell masses of six blastocysts was 45·2 %. The range was 27·3 %–56·6 %, the variance was 161·0 and the coefficient of variation was 28·1 %.

Falconer & Avery (1978) used the predicted negative correlation between the endoderm and ectoderm and the observed positive correlation, between the proportions of the two cell populations represented in the gametic output and coat pigmentation of adult chimaeras, to argue that primordial germ cells arise from the primitive ectoderm rather than the primitive endoderm. Although we have not confirmed the predicted negative correlation between the primitive ectoderm and the primitive endoderm our observations do support Falconer & Avery's argument. Tissues that are derived from the pimitive ectoderm were positively correlated with each other (Table 3 and also Falconer *et al.*, 1981) but not with those derived from the primitive endoderm.

Our results also have some bearing on the nature of allocation of inner cell mass cells to the primitive ectoderm and primitive endoderm. The lack of a positive correlation between the primitive ectoderm and primitive endoderm lineages in chimaeras rules out the rather unlikely possibility that one daughter cell of each inner cell mass cell is allocated to each of these two lineages. Also, the positive correlation between the yolk sac endoderm and the parietal endoderm, and the lack of correlations between these two tissues and any of the tissues derived from the primitive ectoderm suggest that the two endoderm tissues share a common pool of cells that is separate from the primitive ectoderm lineage. This supports the traditional lineage relationship (Fig. 1) rather than the alternative model, proposed by Dziadek (1979). Dziadek suggested that cells in the inner cell mass were allocated to the parietal endoderm and visceral (yolk sac) endoderm in two successive allocation events such that the two endoderm tissues did not share a common pool of cells other than the inner cell mass. (See also Gardner, 1982 for discussion.)

The significance of the lower variance for primitive endoderm tissues in chimaeras, compared to primitive ectoderm derivitives (Table 6) is unclear. It could be interpreted to suggest that fewer inner cell mass cells are allocated to

the primitive ectoderm than the primitive endoderm. However, according to McLaren (1976b) direct cell counts revealed approximately equal numbers of primitive ectoderm and primitive endoderm cells by the time they were overtly differentiated.

The positive correlations for the percentage of PGK-1B in the three primitive ectoderm derivatives analysed in the mosaic conceptuses (Table 5) are relevant to our understanding of the timing of X-chromosome inactivation in the primitive ectoderm. Individual (C3H × C3H–*Pgk-1*^{a})F_1 female conceptuses should be genetically almost identical, so the probability of say X^m being inactivated in a particular cell should be the same in all individuals. The positive correlations, therefore, indicate that the proportions of the two cell populations were influenced by an event that caused some variation between individuals and occurred before cells were allocated to the three separate lineages (foetus, amnion and yolk sac mesoderm). In principle this variation could arise by an earlier allocation event or by X-chromosome inactivation itself.

The previous allocation event, where inner cell mass cells are allocated to primitive ectoderm or endoderm, occurs *before* X-inactivation in the primitive ectoderm lineage (Gardner, 1974) and so cannot contribute to this variation. Therefore, the variation is attributable either to random X-inactivation in a limited number of cells or to the death of some cells after X-inactivation has occurred. It seems likely, therefore, that X-inactivation occurs sufficiently early to allow enough cell mixing between different regions of the primitive ectoderm, allocated to the foetus, amnion and yolk sac mesoderm, to produce similar proportions of cells that express *Pgk-1*^{b} among these three tissues within an individual. (Extensive cell mixing may also explain why local amplification of the subset of cells in the proliferative zone (Snow, 1977) does not destroy the positive correlations between foetus, amnion and yolk sac mesoderm in either the mosaics or chimaeras.)

Unless there is interflow of cells between these three tissues once they have begun to differentiate, cell allocation is likely to occur at or before about 7 days *post coitum*. (For origin of these tissues see Snell & Stevens, 1966; Theiler, 1983.) X-chromosome inactivation in the primitive ectoderm presumably occurs at least several cell generations earlier which is consistent with the current idea that it occurs between 4½ and 5½–6½ days *p.c.* (Gardner, 1974; Monk & Harper, 1979; Rastan, 1982).

A similar conclusion about the time of X-inactivation in the primitive ectoderm was reached by McMahon, Fosten & Monk (1983) who found positive correlations for the percentage of PGK-1A between the yolk sac mesoderm and three foetal samples (neural ectoderm, heart and liver) among three *Pgk-1*^{b}/*Pgk-1*^{a} and five *Pgk-1*^{a}/*Pgk-1*^{b} mosaic conceptuses. In this case, however, the use of outbred mice as one of the parents complicates the interpretation of the correlations and of the more sophisticated covariance

analysis, that these authors used, because the *Xce* genotypes were unknown and several *Xce* alleles could be present in the outbred stock.

Finally, the late allocation events that distribute cells to different lineages within the primitive ectoderm (event 3 in Fig. 1) could also be a source of variation. Since these events occur after X-inactivation, they would only generate more variability among chimaeras than mosaics if the two cell populations were less completely mixed in the chimaeras than in the mosaics. The evidence, discussed above, suggests that quite extensive cell mixing occurs within the primitive ectoderm of mosaics between the time of X-inactivation and allocation of cells to the foetus, amnion and yolk sac mesoderm. More variation, between these three tissues, was seen in two chimaeras (C15 and C16 in Table 2) than in any of the mosaics (Table 4) so it is possible that less cell mixing occurred in the chimaeras. In our experiments, genetic differences between the two cell populations of the chimaeras were minimized by using partially congenic strains but it is still possible that genetic differences existed that inhibited cell mixing between cells of different genotypes (see West, 1976a). Although we cannot be certain whether these late allocation events contribute to the greater variability among chimaeras they are probably less significant than the first two allocation events since the variation in the individual primitive ectoderm tissues is not significantly larger than for the mean (Table 6).

In conclusion, we have shown that cells can be allocated unequally to the primitive ectoderm and primitive endoderm. (This is particularly obvious in group II in Table 2.) It seems very likely that variation exists among chimaeric inner cell masses before this allocation event. We, therefore, propose that the effects of these two sources of variation, that arise at sequential stages of development, are superimposed and thereby generate significant variability among chimaeras before X-chromosome inactivation occurs.

This conclusion supports the general arguments of the model proposed by Falconer & Avery (1978). However, our experiments did not reveal the inverse correlation between the primitive ectoderm and primitive endoderm that was predicted (Falconer & Avery, 1978, p.213). The variability present among inner cell masses may, therefore, be more extensive than Falconer and Avery envisaged. Falconer and Avery's assumption, that the cells of the inner cell mass are still relatively unmixed when they are allocated to the primitive ectoderm or primitive endoderm, may be unnecessary to account for the greater variability among chimaeras if this arises from two superimposed sources of variability. With recent improvements in cell markers for chimaeras it should now be possible to test this assumption directly.

CONCLUSIONS

1. The proportions of the two cell populations in the foetus, amnion and yolk sac mesoderm are more variable among mouse aggregation chimaeras than among X-chromosome inactivation mosaics.
2. Much of this extra variability among chimaeras arises when cells of the inner cell mass are allocated to either the primitive ectoderm lineage or the primitive endoderm lineage.
3. Variation probably already exists among chimaeric inner cell masses before cells are allocated to the primitive ectoderm and endoderm lineages.
4. The greater variability among chimaeras can probably be attributed to two superimposed sources of variation. One that exists among inner cell masses and the other that arises, subsequently, as cells are allocated to the primitive ectoderm lineage. Both sources of variation exist before X-chromosome inactivation occurs in the primitive ectoderm lineage.
5. Variation that arises within the primitive ectoderm lineage (after X-chromosome inactivation has occurred) may be greater in chimaeras than mosaics but the evidence on this point is inconclusive.

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REFERENCES

BÜCHER, TH., BENDER, W., FUNDELE, R., HOFNER, H., & LINKE, I. (1980). Quantitative evaluation of electrophoretic allo- and isozyme patterns for developmental genetics. *FEBS* Lett. **115**, 319–324.

CATTANACH, B. M. (1974). Position effect variegation in the mouse. *Genet. Res.* **23**, 291–306.

CATTANACH, B. M., WOLFE, H. G. & LYON, M. F. (1972). A comparative study of the coats of chimaeric mice and those of heterozygotes for X-linked genes. *Genet. Res. Camb.* **19**, 213–228.

DEOL & WHITTEN (1972). Time of X-chromosome inactivation in retinal melanocytes of the mouse. *Nature New Biol.* **238**, 159–160.

DZIADEK, M. (1979). Cell differentiation in isolated inner cell masses of mouse blastocysts *in vitro*: onset of specific gene expression. *J. Embryol. exp. Morph.* **53**, 367–379.

FALCONER, D. S. & AVERY, P. J. (1978). Variability of chimaeras and mosaics. *J. Embrol. exp. Morph.* **43**, 195–219.

FALCONER, D. S., GAULD, I. K., ROBERTS, R. C. & WILLIAMS, D. A. (1981). The control of body size in mouse chimaeras. *Genet. Res.* **38**, 25–46.

GARDNER, R. L. (1974). Microsurgical approaches to the study of early mammalian

development. In *Birth Defects and Fetal Development: Endocrine and Metabolic Factors,* (ed.) K. S. Moghissi, pp. 212–233. Illinois. C. C. Thomas, Springfield.

GARDNER, R. L. (1982). Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J. Embryol. exp. Morph.* **68**, 175–198.

GARDNER, W. & MCLAREN, A. (1974). Cell distribution in chimaeric mouse embryos before implantation. *J. Embryol. exp. Morph.* **32**, 495–503.

HILLMAN, N., SHERMAN, M. I. & GRAHAM, C. (1972). The effect of spatial arrangements on cell determination during mouse development. *J. Embryol. exp. Morph.* **28**, 263–278.

JOHNSTON, P. G. & CATTANACH, B. M. (1981). Controlling elements in the mouse. IV. Evidence of non-random X-inactivation. *Genet. Res. Camb.* **37**, 151–160.

KALOUSEK, D. K. & DILL, F. J. (1983). Chromosomal mosaicism confined to the placenta in human conceptions. *Science* **221**, 665–667.

KELLY, S. J., MULNARD, J. G. & GRAHAM, C. F. (1978). Cell division and cell allocation in early mouse development. *J. Embryol. exp. Morph.* **48**, 37–51.

LEVAK-SVAJGER, B., SVAJGER, A. & SKREB, N. (1969). Separation of germ layers in presomite rat embryos. *Experientia* **25**, 1311–1312.

MCLAREN, A. (1976a). *Mammalian Chimaeras.* Cambridge: Cambridge University Press.

MCLAREN, A. (1976b). Growth from fertilization to birth in the mouse. In *Embryogenesis in Mammals, Ciba Foundation Symposium* **40** *(new series)*, (A. McLaren, ed.), pp. 47–51. Amsterdam: Elsevier/Excerpta Medica/North Holland).

MCLAREN, A. & MICHIE, D. (1956). Studies on the transfer of fertilized mouse eggs to uterine foster mothers. I. Factors affecting the implantation and survival of native and transferred eggs. *J. exp. Biol.* **33**, 394–416.

MCMAHON, A., FOSTEN, M. & MONK, M. (1983). X-chromosome inactivation mosaicism in the three germ layers and the germ line of the mouse embryo. *J. Embryol. exp. Morph.* **74**, 207–220.

MINTZ, B. (1967). Gene control of mammalian pigmentary differentiation. I. Clonal origin of melanocytes. *Proc. natn. Acad. Sci., U.S.A.* **58**, 344–351.

MONK, M. & HARPER, M. (1979). Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature* **281**, 311–313.

MÜLBACHER, C., KUNTZ, G. W. K., HAEDENKAMPT, G. A. & KRIETSCH, W. K. G. (1983). Comparison of the two purified allozymes (1B and 1A) of X-linked phosphoglycerate kinase in the mouse. *Biochemical Genetics* **21**, 487–496.

MULLEN & WHITTEN (1971). Relationship of genotype and degree of chimaerism in coat colour to sex ratios and gametogenesis in chimaeric mice. *J. Exp. Zool.* **178**, 165–176.

NESBITT, M. N. (1971). X-chromosome inactivation mosaicism in the mouse. *Devl Biol.* **26**, 252–263.

NICOLSON, G. L., YANAGIMACHI, R. & YANAGIMACHI, H. (1975). Ultrastructural localization of lectin-binding sites of the zonae pellucidae and plasma membranes of mammalian eggs. *J. Cell Biol.* **66**, 263–274.

NIELSEN, J. T. & CHAPMAN, V. M. (1977). Electrophoretic variation for X-chromosome linked phosphoglycerate kinase (PGK-1) in the mouse. *Genetics* **87**, 319–325.

PAPAIOANNOU, V. E. & WEST, J. D. (1981). Relationship between the parental origin of the X-chromosomes, embryonic cell lineage and X-chromosome expression in mice. *Genet. Res. Camb.* **37**, 183–197.

PAPAIOANNOU, V. E., WEST, J. D., BÜCHER, TH. & LINKE, I. M. (1981). Non-random X-chromosome expression early in mouse development. *Devl Genet.* **2**, 305–315.

QUINN, P., BARROS, C. & WHITTINGHAM, D. G. (1982). Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fert.* **66**, 161–168.

RABES, H. M., BÜCHER, TH., HARTMANN, A., LINKE, I. & DÜNNWALD, M. (1982). Clonal growth of carcinogen-induced enzyme-deficient preneoplastic cell populations in mouse liver. *Cancer Research* **42**, 3220–3227.

RASTAN, S. (1982). Timing of X-chromosome inactivation in postimplantation mouse embryos. *J. Embryol. exp. Morph.* **71**, 11–24.

RASTAN, S. & CATTANACH, B. M. (1983). Interaction between the *Xce* locus and imprinting of the paternal X chromosome in mouse yolk-sac endoderm. *Nature* **303**, 635–637.

SNELL, C. D. & STEVENS. L. C. (1966). Early Embryology. In *Biology of the Laboratory Mouse,* (Second Edition), (ed. E. L. Green). pp. 205–245. New York: McGraw Hill/ Dover Publications.

SNOW, M. H. L. (1977). Gastrulation in the mouse: growth and regionalization of the epiblast. *J. Embryol. exp. Morph.* **42**, 293–303.

SPINDLE, A. (1982). Cell allocation in preimplantation mouse chimaeras. *J. exp. Zool.* **219**, 361–367.

TARKOWSKI, A. K. & WROBLEWSKA, J. (1967). Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *J. Embryol. exp. Morph.* **18**, 155–180.

THEILER, K. (1983). Embryology. In *The Mouse in Biomedial Research. Vol. III. Normative Biology, Immunology and Husbandry* (eds. H. L. Foster, J. D. Small and J. G. Fox) New York, London: Academic Press.

WAREHAM, K. A., HOWELL, S., WILLIAMS, D. & WILLIAMS, E. D. (1983). Studies of X-chromosome inactivation with an improved histochemical technique for ornithine carbomoyltransferase. *Histochemical J.* **15**, 363–371.

WEST, J. D. (1976a). Clonal development of the retinal epithelium in mouse chimaeras and X-inactivation mosaics. *J. Embryol. exp. Morph.* **35**, 445–461.

WEST, J. D. (1976b). Patches in the livers of chimaeric mice. *J. Embryol. exp. Morph.* **36**, 151–161.

WEST, J. D. (1978). Analysis of clonal growth using chimaeras and mosaics. In *Development in Mammals* **3**, (ed. M. H. Johnson), pp. 413–460. Amsterdam: Elsevier/North Holland Inc.

WEST, J. D. & CHAPMAN, V. M. (1978). Variation for X-chromosome expression in mice detected by electrophoresis of phosphoglycerate kinase. *Genet. Res.* **32**, 91–102.

WEST, J. D., FRELS, W. I., CHAPMAN, V. M. & PAPAIOANNOU, V. E. (1977). Preferential expression of the maternally derived X chromosome in the mouse yolk sac. *Cell* **12**, 873–882.

WEST, J. D., KIRK, K. M., GOYDER, Y. & LYON, M. F. (1984). Discrimination between the effects of X-ray irradiation of the mouse oocyte and uterus on the induction of dominant lethals and congenital anomalies. I. Embryo transfer experiments. *Mutation Research.* (In press).

WHITTINGHAM, D. G. (1971). Culture of mouse ova. *J. Reprod. Fert. Suppl.* **14**, 7–21.

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