

Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo

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SUMMARY

Cloning of cells from peri-implantation embryos by blastocyst injection was used to investigate the time of X-chromosome inactivation in that part of the ectoderm lineage giving rise to foetal tissues of the mouse. Matings were arranged so that the two X-chromosomes of female donor cells controlled two distinct coat colours and host blastocysts were of a third colour genotype. No coat chimaeras were obtained in experiments using donor cells from the primitive ectoderm of 6th or 7th day embryos or from lactationally delayed implanting or reactivated blastocysts. In contrast, a minimum of 80 unequivocal coat chimaeras were obtained in experiments in which primitive ectoderm cells from 5th day implanting blastocysts were used for injection. The majority of these chimaeras that had received a female cell exhibited both donor colours in addition to host colour in their coats, suggesting that the donor cell had not undergone X-inactivation until one or more cycles after transplantation. The remainder of such chimaeras exhibited only one or other donor coat colour. Determination of the parental origin of the allocyclic X-chromosome in donor metaphase preparations in internal tissues of several chimaeras revealed that the coat pattern did not always reflect the X-activity status of the donor cell clone as a whole. Nevertheless, the findings suggest that X-inactivation takes place shortly after implantation in the primitive ectoderm cell population from which the foetus is derived.

Of the 68 chimaeras in which the sex of both the donor and host component was established 62 proved to be fertile. Furthermore, 21 of the 37 fertile chimaeras whose sex corresponded with that of the donor cell yielded functional gametes of donor origin. Injection of cells from a single donor blastocyst into a series of host blastocysts established that at least 2 cells in 5th day primitive ectoderm can give rise to both somatic cells and functional germ cells among their mitotic descendants.

INTRODUCTION

It is now well established that most gene loci on one of the two X-chromosomes are genetically inactive in somatic cells of female eutherian mammals (Gartler & Riggs, 1983). This mechanism for equalizing the dosage of such genes in the two sexes comes into operation during embryogenesis in both the soma and germ line of females, but is reversed around the time of onset of meiosis in germ cells (Johnston, 1981; McLaren, 1981). Much effort has been devoted in recent years to establishing the stage in development at which X-chromosome inactivation takes place (reviewed by West, 1982). This work has revealed marked differences in the

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pattern of inactivation between different cell lineages in the early rodent embryo. Thus, while the process appears to be essentially random with respect to parental origin of the X-chromosomes in primitive ectoderm-derived tissues, preferential inactivation of the paternally-inherited X-chromosome has been demonstrated unequivocally in derivatives of the trophoctoderm and primitive endoderm (Takagi & Sasaki, 1975; West, Papaioannou, Frels & Chapman, 1978; Papaioannou & West, 1981; Papaioannou, West, Bucher & Linke, 1981). Furthermore, DNA from the inactive X-chromosome in primitive ectoderm derivatives is ineffective in cellular transformation, whereas that from primitive endoderm-derived visceral yolk sac endoderm can be effective, implying that inactivation also differs with respect to DNA modification in these two lineages (Kratzer *et al.* 1983).

An attractive explanation for the difference between ectoderm derivatives and trophoctoderm and primitive endoderm derivatives in random *versus* paternal X-inactivation is based on the notion of differential imprinting of the two X-chromosomes (Lyon, 1977; Monk, 1978). According to this hypothesis, some imprint is conferred on one or other X during gametogenesis which renders either the paternal X more susceptible or, more probably, the maternal X more resistant to inactivation (Lyon & Rastan, 1984). This imprint is erased during early development, and inactivation occurs before erasure in trophoctoderm and primitive endoderm and after in primitive ectoderm. However, while available evidence suggests that trophoctoderm and primitive endoderm may indeed undergo somewhat earlier X-inactivation than primitive ectoderm, this evidence is not compelling because conflicting results have been obtained for the latter tissue. Thus, from measurements of the ratio of activity of an X-*versus* an autosomally coded enzyme in individual embryos, Kozak & Quinn (1975) concluded that inactivation had taken place by 6 days *postcoitum* (*p.c.*) in the primitive ectoderm. Using basically the same approach with a different pair of enzymes, Monk & Harper (1979) argued that the process does not occur until 6.5 days *p.c.* However, on the basis of examination of metaphase spreads for the presence of a cytogenetically differentiated X-chromosome, Rastan (1982) claimed that X-inactivation had already occurred by 5.5 days *p.c.*

There are problems in using either of the above approaches to obtain an accurate estimate of the time of X-inactivation. Enzyme activity measurements will inevitably err towards giving a late estimate unless the rates of turnover of the enzyme and degradation of its mRNA is high. Furthermore, if the stability of an enzyme or its mRNA was markedly lower in cells undergoing overt differentiation than in those that were not, differences in the timing of X-inactivation based on activity measurements might be apparent rather than real. An obvious shortcoming in the use of chromosome morphology is that while there are strong grounds for believing that an allocyclic X-chromosome is inactive (Cohen & Rattazzi, 1971; Rattazzi & Cohen, 1972; Hamerton *et al.* 1971), the temporal relationship between genetic inactivation and onset of visible chromosome differentiation has yet to be established.

In the present investigation use has been made of a third approach to attempt to estimate the time of X-inactivation in the primitive ectoderm. This entails cloning early embryonic cells by injecting them individually into genetically dissimilar host blastocysts which are then returned to the uterus to continue their development *in vivo* (Gardner, 1978a; Gardner & Lyon, 1971). Matings are arranged so that female donor embryos have different coat colour genes controlled by their two X-chromosomes and host embryos are of a third distinct coat colour genotype. The status of the donor cell with regard to X-chromosome activity can be determined from the coat colours of the resulting chimaeric offspring, as illustrated in Fig. 1. Using this approach, Gardner & Lyon (1971) obtained evidence that X-inactivation had not taken place in foetal precursor cells of the 3.5-day *p.c.* inner cell mass (ICM). The results of cloning primitive ectoderm cells from later embryos are presented here, and have a bearing on the origin of the definitive germ cell lineage as well as the timing of X-inactivation. A preliminary report of part of this work has been published earlier (Gardner, 1974, 1978a).

MATERIALS AND METHODS

Mice

Donor embryos were obtained from albino ($X^N X^N cc$) females mated to, cc, cc^e or $c^e c^e$ males that also carried a c^+ allele inserted in their X-chromosome (X^T , Cattanaach, 1961). All female ($X^T X^N$) offspring from this cross should therefore have a flecked coat which is white (cc^e or cc) in areas populated by melanocytes in which the paternally inherited X^T is inactive and wild type in areas populated by melanocytes in which it is the active X-chromosome. Male offspring should be white only ($X^N Y cc^e$ or cc ; see Fig. 1). When available, males and/or females that were also homozygous for the Rb(6.15)1Ald metacentric marker chromosome were used in the above cross to enable donor and host metaphase preparations to be distinguished in chimaeras. The host blastocysts were from PT (pc^{ch}/pc^{ch}) or CBA-*p* ($p+/p+$) females mated to $p+/p+$ males so that all offspring should be sandy coloured with pink eyes ($p+/pc^{ch}$ or $p+/p+$). Female mice from several different random-bred stocks were mated with either vasectomized or genetically sterile males (carrying the male-sterile translocation T145H) to provide pseudopregnant recipients for the injected blastocysts (preferentially, when available parous females that had successfully raised a litter of pups were used as recipients). The mice were normally kept under conventional lighting conditions. However, for certain experiments mice used to provide donor or host embryos were placed on an altered lighting regime in which the dark period was from 14.00–23.00h. Embryos obtained in these conditions are assumed to be approximately 6 h ahead of those from mice on a standard lighting regime. Delayed implanting donor blastocysts were obtained by allowing mating to take place during *postcoitum* oestrus and enhancing the suckling stimulus by fostering additional new born pups onto the mated females (Mantalenakis & Ketchel, 1966). Reactivation of delayed blastocysts was produced by removal of the suckling young (McLaren, 1968).

Recovery, manipulation and culture of embryos

In earlier experiments medium M199 plus 10% (v/v) heat-inactivated foetal calf serum was used for recovery and manipulation of donor and host embryos and for postoperative culture of injected blastocysts. Subsequently, PB1 medium (Whittingham & Wales, 1969) modified as described elsewhere (Gardner, 1984) was adopted. Donor embryos were recovered in the late morning or afternoon of the 5th, 6th or 7th day of pregnancy from non-delayed females and in the afternoon of the 7th to 11th day from those in lactationally delayed implantation. Reactivated delayed implanting blastocysts were recovered around noon on the 8th to 10th day

after mating, approximately 20 h after removal of the suckling young. Donor blastocysts were invariably removed by flushing after inflating the uterine horns by introducing medium via the cervix while keeping the oviductal end closed with forceps. The yield of blastocysts was typically lower than that of postimplantation embryos, especially from 5th day non-delayed females on the altered lighting regime, presumably because of failure to dislodge those that were more advanced in implantation. The postimplantation donor embryos were recovered by dissection from their investing decidual swellings. Host blastocysts were recovered on the afternoon of the 4th day of pregnancy.

Postimplantation embryos were dissected with siliconized (Repelcote, Hopkin & Williams, U.K.) watchmaker's forceps or glass microneedles to release the egg cylinder from investing parietal yolk sac tissue. Embryonic regions were then isolated by cutting the cylinder in two across the embryonic-extraembryonic junction. ICMs were isolated microsurgically from donor blastocysts either directly or, in later experiments, after the embryos had been torn open murally and exposed to a mixture of trypsin and pancreatin at 4°C (Gardner, 1982). Finally, the embryonic fragments or ICMs were incubated in a solution of 0.6% sodium chloride plus 0.296% sodium citrate containing 0.25% (w/v) trypsin (Difco 1/250) at 37°C for up to 30 min prior to return to medium for dissociation by pipetting. In more recent experiments, pronase treatment followed by incubation in calcium-free medium (Gardner, 1982, 1984) was used to assist dissociation of ICMs.

Primitive ectoderm cells were distinguished from those of the endoderm by their relatively smooth appearance following dissociation (Gardner & Rossant, 1979), and injected singly into host blastocysts as described elsewhere (Gardner, 1968, 1978*b*). In some experiments groups of blastocysts were injected with cells from the same donor embryo and each such group transferred separately to pseudopregnant recipients. In the remainder, cells for injection were selected from dissociated ICMs or embryonic fragments pooled from several embryos. Injected blastocysts were transferred to one or both uterine horns of recipients during the afternoon of the third day of pseudopregnancy, either directly, or following postoperative culture for 1–2 h.

Analysis of offspring

Recipients were allowed to deliver their young which were briefly examined shortly after birth for evidence of pigmentation and to ensure that they were being looked after. Pups were fostered onto females that had recently given birth in cases where they were clearly being neglected or failed to gain weight. All offspring obtained from injected blastocysts were inspected closely at 2–3 weeks postpartum and those lacking any external signs of chimaerism were killed. Overt chimaeras were assigned code numbers at this stage and a visual estimate made of the proportions of donor and host colour in their coats. A coloured photographic record of each chimaera was normally obtained prior to maturity and, in most animals additional photographs were taken thereafter. Mature chimaeras were paired with pc^{ch}/pc^{ch} , $p+/p+$ or, in some cases, with $+c/+c$ mice to investigate their fertility and germ-line constitution. Chromosome preparations from cornea (Fredga, 1964) were used to confirm the origin of offspring from the donor component in chimaeras in which this component carried the Rb1Ald chromosome.

Chimaeras obtained in experiments in which donor cells carried the Rb1Ald marker chromosome were bled from the tail so that chromosome preparations could be obtained from Concanavalin A stimulated cultures of peripheral blood for establishing the sex of the donor and host components. The cultures were grown for 3–4 days and then exposed to colcemid ($0.05 \mu\text{g ml}^{-1}$) for up to 2 h before harvest. The sex of the mitotic cells was determined by the presence or absence of a Y chromosome by virtue of either, or both, its unique morphological features after conventional staining (Ford, 1966*a*) or its minimal C-band (Nesbitt & Francke, 1973) after, for example, hydrolysis in 5 M-hydrochloric acid, treatment in 2xSSC (0.3 M-sodium chloride, 0.03 M-tri-sodium citrate) at 60°C and Giemsa staining (modified from McKenzie & Lubs, 1973).

Chimaeras that were used for more extensive chromosomal analysis were injected intraperitoneally with colcemid (0.25 ml of 0.04% wt/vol.) one hour before they were killed, and chromosome preparations made from lymphomyeloid tissues by air drying (Ford, 1966*b*) after hypotonic treatment in 0.56% KCl solution. Cellular sex was determined on some of these slides

by the method described earlier. In cases where the donor cells were female, their inactive X was scored using either, or both, the technique of Kanda (1973) or by prolongment of the combined trypsin/ASG G-banding technique of Gallimore & Richardson (1973). With Kanda's technique the inactive X is retained as a uniformly dark-staining chromosome amongst the remaining denatured and pale-staining chromosomes. After modified G-banding this chromosome displays diffuse bands which contrast with the more sharply defined bands of the active X and the autosomes. Preparations of metaphase spreads from corneas were made by air drying from 60 % acetic acid (Ford, Evans & Gardner, 1975) and, where appropriate, the inactive X was scored after treating slides for 15 mins in 2xSSC at 60 °C and staining them in Giemsa.

The following criteria were used to establish the sex of the donor and host component of chimaeras in cases where this could not be done chromosomally. Any chimaera exhibiting wild-type pigmentation in the coat, eye or skin was assumed to have a female donor component. If the donor and/or host component yielded functional gametes its sex was assumed to correspond with that of the chimaera.

RESULTS

Rates of development and chimaerism

The results are summarized in Table 1. A total of 566 live young were obtained from 988 injected blastocysts, representing a rate of development to term of 57 %. If only those blastocysts that were transferred to recipients that became pregnant are considered, the success rate is 72 % (566/783). No overtly chimaeric offspring were found in experiments in which the donor cells were from 6th or 7th day postimplantation embryos, delayed implanting blastocysts, or blastocysts that had been reactivated after a period of 3 days or more in delay. In view of these negative results, efforts were concentrated on obtaining a larger series of chimaeras using donor cells from 5th day implanting blastocysts. A minimum of 80 coat colour chimaeras were found among the 434 offspring produced in these experiments, representing an overall cloning efficiency of at least 18 % for early primitive ectoderm cells (Table 1). The rate of chimaerism was lower using donor cells from advanced (11/99 = 11 %) than standard (69/335 = 21 %) 5th day blastocysts. As indicated in Table 2 the proportion of donor colour(s) in the coats of these chimaeras was very variable, ranging from approximately 1 % to more than 95 %. The donor contribution was present as patches or flecks that were widely distributed along the body axis in all except five cases. The exceptions, in which donor melanocytes appeared to be restricted to a single region, all exhibited a donor contribution that was estimated to be less than 15 % (Table 2).

X-chromosome inactivation

The sex of the donor component was not established in 10 of the 80 chimaeras, and in 32 of the remainder it was classified as male. The donor coat colour in each of the 38 chimaeras that had evidently developed from blastocysts injected with a female donor cell is indicated in Table 3. The majority of these chimaeras, including seven of the ten in which the donor contribution was estimated to be less than 15 % (Table 2), exhibited both wild-type and white patches. The relative proportions of the two colours were very variable in such animals, although wild type was more frequently the major donor component than white (Table 4).

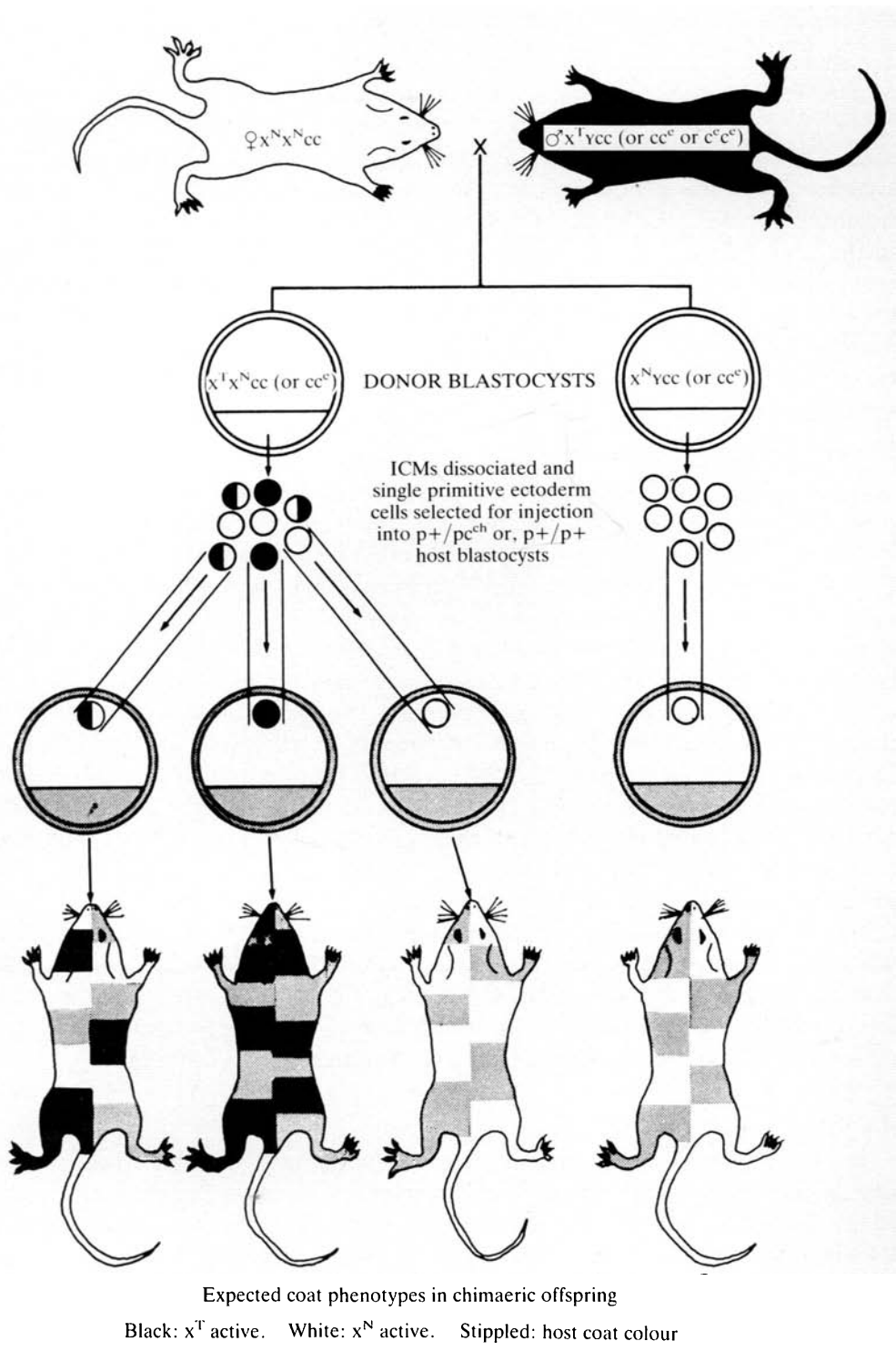


Fig. 1. Diagram of procedure for clonal analysis of X-chromosome inactivation.

Wild-type colour only was present in the coats of seven additional chimaeras with female donor cells, and white only in the remaining two (Table 3).

The proportion of donor cells and relative frequency of inactivated X^N versus X^T in such cells was estimated for various tissues of seven of the chimaeras listed in Table 3 by karyotypic analysis at autopsy (Table 5). Three points of interest emerged from this analysis. First, the donor contributions to these additional tissues could vary quite widely and did not necessarily correspond closely with that in the coat. Second, in all samples except one, X^N appeared to be the inactive chromosome more often than X^T . Finally, the one animal in which X^T appeared to be active in all donor melanocytes of the coat seemed nevertheless to have a minor population of cells with X^N active in all tissues that were examined chromosomally (C*41(♀), Table 5). Although this chimaera exhibited a modest donor component in the coat, it was not spatially restricted (Fig. 2).

Sexual differentiation and frequency of donor contribution to the germ line

49 of the 80 chimaeras were phenotypically male, 30 were phenotypically female, and 1 was a lateral hermaphrodite. 12 of the chimaeras proved uninformative in this part of the investigation because of failure to establish the sex of the host (two cases), the donor (seven cases), or both components (three cases). In 27 of the 68 for which the sex of both components could be established, the donor and host were of different sexes. In all except three of these, the sexual phenotype of the chimaera corresponded with that of the host. One of the exceptions was the lateral hermaphrodite mentioned above, and the remaining two were fertile males with male donor and female host components. No breeding data were obtained for 6 of the 68 chimaeras. Three of these animals died prior to maturity and the remainder, including the hermaphrodite, failed to breed. The

Table 1. *Offspring obtained following transfer to pseudopregnant mice of blastocysts injected with single primitive ectoderm cells*

| Source of donor cells | No. of injected blastocysts | | No. of offspring | No. of coat chimaeras |
|---|-----------------------------|---|------------------|-----------------------|
| | Total transplanted | No. transplanted to ♀♀ that became pregnant | | |
| 5th day implanting blastocyst | 736 | 583 | 434* | 80† |
| Delayed implanting blastocyst | 96 | 64 | 41 | 0 |
| Reactivated delayed implanting blastocyst | 42 | 36 | 20 | 0 |
| 6th day embryo | 65 | 51 | 32 | 0 |
| 7th day embryo | 49 | 49 | 39* | 0 |

* Excluding remains of new born in one litter.

† Five additional offspring that died before the coat developed showed unequivocal donor pigmentation.

Table 2. *Classification of chimaeras according to estimated percentage of donor coat colour*

| Sex of donor cell | Donor colour(s) in coat | % Donor colour (No. with spatially restricted as opposed to more generalized distribution of donor cells) | | | | | | | | | | | |
|----------------------|----------------------------|--|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|--|
| | | <5 | 5-14 | 15-24 | 25-34 | 35-44 | 45-54 | 55-64 | 65-74 | 75-84 | 85-94 | 95 or + | |
| male | white only | 1(1) | 9(3) | 7 | 7 | 3 | 2 | 1 | — | 1 | — | 1 | |
| female | wild type and white | 2(1) | 5 | 6 | 2 | 2 | 2 | 4 | 3 | 1 | 1 | 1 | |
| female | wild type only | 1 | 1 | 2 | 2 | — | — | 1 | — | — | — | — | |
| female | white only | — | 1 | — | — | — | 1 | — | — | — | — | — | |
| not established | white only | 1 | 3 | 4 | 1 | — | 1 | — | — | — | — | — | |
| Total | | 5(2) | 19(3) | 19 | 12 | 5 | 6 | 6 | 3 | 2 | 1 | 2 | |



Fig. 2. Chimaera C*41(0) exhibiting mainly host-type coat colour with wild-type only donor colour due to activity of X^T on head (particularly the left side), back, and rump.

composition of the germ line relative to genotypic constitution in the 62 fertile chimaeras is shown in Table 6. It will be seen that 21 of the 37 animals whose phenotypic sex corresponded with the genetic sex of the donor cell produced functional gametes of donor origin, and that the proportion of male \rightarrow male chimaeras with a donor contribution to the germ line was higher than female \rightarrow female chimaeras.

Table 3. *Coat phenotypes of chimaeras in which donor cell was female*

| Sex of chimaera | Donor colour present* | | |
|-----------------|-----------------------|---------------------|------------|
| | Wild type only | Wild type and white | White only |
| Female | 5 | 11(2)† | 0 |
| Male | 2(2)† | 18(2)† | 2 |
| Total | 7 | 29 | 2 |

* All animals showed some host (sandy) colour.

† Numbers in brackets refer to chimaeras whose donor cells were derived from advanced as opposed to standard 5th day blastocysts.

Table 4. *Relative proportions of wild type and white in coats of chimaeras exhibiting both donor colours*

| No. of chimaeras with | | | | |
|------------------------|---------------------|---------------------------|---------------------|------------------------|
| wild type \geq white | wild type $>$ white | wild type \approx white | white $>$ wild type | white \geq wild type |
| 5 | 6 | 12 | 3 | 3 |

Table 5. Coat and karyotypic analysis of selected chimaeras with female donor cell

| Chimaera Code No. | Proportion and type of donor coat colour | % Donor metaphases (total scored) | | % Donor metaphases with X ^N inactive (total scored) | | | | | | |
|----------------------|--|--------------------------------------|-------------------|--|-------------------|--------------------|------------------|------------------|--------------|---|
| | | Highest | | Lowest | | | | | | |
| | | | | | | | | | | |
| C* 15(♂) | ca. 15-20 % + and c (+ ≥ c) | 78(50) LC | 29(100) LN | 84(50) BM(i) | 75(75) BM(ii) | 82(71) LN | 58(50) LC | — | — | — |
| C* 22(♂) | ca. 85 % + and c (c > +) | 98(100) BM | 0(15) LC | 73(100) BM(i) | 79(100) BM(ii) | 74(100) BM(iii) | 79(100) Sp(i) | 70(50) Sp(ii) | 81(32) LN | — |
| C* 26(♂) | ca. 55 % + and c (+ > c) | 48(86) PP | 17(18) LC + RC | 71(100) BM(i) | 73(100) BM(ii) | 83(35) LN | 76(41) PP | — | — | — |
| C* 32(♂) | ca. 20 % + and c | 55(100) BM(i) | 40(50) PP | 46(100) BM(i) | 68(100) BM(ii) | 65(20) PP | — | — | — | — |
| C* 41(♀) | ca. 15 % + only | 60(100) BM(ii) | 0(20) LC + RC | 89(100) BM(i) | 73(78) BM(ii) | 80(54) Sp | 84(50) LN | 82(50) PP | — | — |
| C* 42(♀) | ca. 15 % + and c (+ ≥ c) | 26(100)* BM | 26(100)* BM | 55(27)* BM | — | — | — | — | — | — |
| C* 48(♂) | ca. 15-20 % + and c (+ ≥ c) | 4(100) BM | 2(100) PP | 59(70) BM | — | — | — | — | — | — |

Key: BM, bone marrow; (), sample no.; LC, left cornea; LN, pooled lymph nodes; PP, Peyer's patches; RC, right cornea; Sp, spleen.
* Only samples scored.

Observations on chimaeras with the same donor embryo

One quartet, two triplets and two pairs of such chimaeras were produced, the relevant features of which are presented in Table 7. It is evident that both the extent and, in the case of female donor cells, type of coat colour can vary between members of such groups of chimaeras. In addition, 5th day ICMs can contain at least two primitive ectoderm cells that are capable of giving rise to both germ and somatic cells among their progeny.

DISCUSSION

Results of analysis of midgestation conceptuses suggest that the lack of overt chimaeras among offspring in experiments using 6th or 7th day donor embryos was

Table 6. *Breeding data on 62 fertile chimaeras in which sex of donor and host components were established*

| Genotypic composition | | Sex of chimaera | Origin of germ line | | |
|-----------------------|------|-----------------|---------------------|-------|-------|
| Donor | Host | | Host | Mixed | Donor |
| ♂ | ♀ | {♀ | 5 | — | — |
| ♀ | ♂ | ♂ | — | — | 2 |
| ♀ | ♀ | ♀ | 20 | — | — |
| ♂ | ♀ | ♀ | 9 | 5 | — |
| ♂ | ♂ | ♂ | 7 | 14 | — |

Table 7. *Coat and germ line in groups of chimaeras whose donor cells originated from the same embryo*

| Sex of donor embryo | Code No. and phenotypic sex of chimaera | | Donor coat colour | | Composition of germ line |
|---------------------|---|---|-------------------|----------------------|--------------------------|
| | | | Type | Estimated percentage | |
| ♂ | C* 17 | ♂ | c | 25 | mixed |
| | C* 18 | ♀ | c | 10 | host |
| | C* 19 | ♀ | c | 5 | host |
| | C* 20 | ♂ | c | 5(R)† | mixed |
| | C* 37 | ♂ | c | 50 | mixed |
| ♂ | C* 38 | ♂ | c | 35 | mixed |
| | C* 39 | ♀ | c | 5–10(R)† | host |
| | C* 5 | ♀ | + only | 30 | host |
| ♀ | C* 6 | ♂ | + and c | 10 | host |
| | C* 7 | ♀ | + only | 7 | host |
| ♀ | C* 15 | ♂ | + and c | 25 | host |
| | C* 16 | ♂ | + and c | 20 | host |
| ♀ | C* 21 | ♂ | + and c(+ > c) | 75 | host |
| | C* 22 | ♂ | + and c(c ≧ +) | 85 | host |

† R, Spatially restricted donor contribution

+, wild type; c, white

probably due to failure of the transplanted cells to form clones rather than to their colonizing tissues other than neural crest (R. L. Gardner, unpublished data; Rosa Beddington, personal communication; Janet Rossant, personal communication). It is likely that this explanation also accounts for the negative results with cells from delayed and reactivated blastocysts. Even with 5th day implanting blastocysts the cloning efficiency of primitive ectoderm cells is much lower than that of corresponding endoderm cells (Gardner & Rossant, 1979; Gardner, 1982, 1984), and was found to decline significantly over the 6h or so by which advanced embryos were ahead of those produced under the standard lighting regime in the present study. It is conceivable, therefore, that only very early primitive ectoderm cells can be cloned by blastocyst injection.

Concerning the timing of X-inactivation there are two main relevant findings from this work. First, most but not all chimaeras with a female donor component displayed both wild-type and white colour in their coats. Second, in those chimaeras which displayed both colours the proportions of the two types in some cases differed very markedly from equality, in either direction. Possible explanations for the few animals with only white or wild-type are (i) X-inactivation had already occurred before the cell was transferred, (ii) inactivation occurred one or a few divisions after transfer and by chance all descendant cells had the same X active, (iii) inactivation occurred after transfer, some cells had X^T and others X^N active, but by chance those contributing to the melanocytes were all of one type.

Explanation (iii) is a possibility where the contribution of donor type melanocytes is limited, as in chimaera C*41 (Table 5) which had wild-type colour only in its coat but some cells with X^T inactive in other tissues. It is also a possibility if the proportions of donor cells with X^T and X^N active at the time of inactivation were highly unequal. Such an inequality could arise by chance if only few descendant cells were present at the time of inactivation. Thus, if inactivation occurred within a very few divisions after transfer it could result both in animals with donor melanocytes all of one colour, and in those with marked inequality of the two colours. It would also explain the findings in the second triple and second twin pair in Table 7, where the phenotypes resulting from individual donor cells from the same embryos differed. Therefore, the evidence points to inactivation having occurred at about one to four divisions after transfer. After four divisions $2 \times (1/2)^4$ or $1/8$ of embryos would be expected to have all descendant donor cells with the same X active. A proportion of $2 \times 4 \times (1/2)^4$ or $1/2$ would have three descendant cells of one type and one of the other and hence an equal contribution of wild-type and white pigment.

In general, more donor cells appeared to have an inactive X^N than X^T , judging by the relative amounts of wild-type and white pigmentation and the features of the two X-chromosomes in metaphase spreads (Tables 3, 4 and 5). The possibility that this reflects a bias in inactivation rather than a proliferative advantage of cells in which X^T was active is suggested by the fact that in all except two of the nine chimaeras with a female donor component that exhibited one donor colour this was wild type. However, there may have been additional cases in which X^N only

was active in melanocytes among the ten chimaeras for which the donor sex could not be established.

A possibility has to be considered that manipulation of donor cells during isolation and transplantation had, in fact, reversed a prior state of X-inactivation (Gardner & Lyon, 1971). Having failed by the present approach to define a stage in development at which all donor cells form clones with only X^T or X^N active, we cannot formally exclude this possibility. However, the simplest interpretation of the data is that both X-chromosomes are active in primitive ectoderm cells at approximately $4\frac{1}{2}$ days *p.c.*, and that inactivation of one or other occurs in cells of this lineage within the next 24 h, as suggested by Rastan (1982).

The size of the donor cell clones was very variable judging both from the coat and from chromosomal analysis of additional tissues in the chimaeras. Furthermore, as will be discussed elsewhere, the extent of the donor contribution to different tissues is perhaps more variable in these and other single cell injection chimaeras than in those produced by embryo aggregation. Although there was a marked excess of phenotypic males in the present series, the pattern of sexual differentiation was dictated by the sex of the host component in all except three of the chimaeras that were classified as XX/XY. This suggests that single cell injection chimaeras may provide more suitable material for confirming whether XY germ cells can form functional oocytes than conventional aggregation chimaeras (Evans, Ford & Lyon, 1977).

A particularly interesting finding was that 57% of donor clones whose sex corresponded with that of the chimaera formed both somatic cells and functional gametes. This provides the first direct evidence that the germ cell lineage originates within the primitive ectoderm. A mean of 23.1 (s.d. = 7.2; N = 17) primitive ectoderm cells was found in 5th day blastocysts of a random-bred mouse stock (Gardner, 1985). Attempts to estimate the proportion of such cells that possess the potential to give rise to germ cells is complicated by the relatively low efficiency with which such cells can be cloned by blastocyst injection, although it is clear that at least two cells from a single ICM can do so (Table 7). If one assumes that the cloning procedure is not enriching for a subpopulation of germ-line antecedents, the present results suggest that the majority of early primitive ectoderm cells possess this property. The lower frequency of donor germ-line contributions found among chimaeras in which donor and host components were female rather than male probably reflects failure to detect all cases because fewer offspring were normally obtained from the former than the latter. Hence, a substantial majority, if not all, early primitive ectoderm cells may possess the capacity to give rise to germ cells. How many of them so do normally is a matter on which the present findings have no bearing.

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