An *in situ* cell marker for clonal analysis of development of the extraembryonic endoderm in the mouse*

By R. L. GARDNER

Sir William Dunn School of Pathology, South Parks Road, Oxford, OX1 3RE, U.K.

SUMMARY

Conditions were found for staining whole mid-gestation capsular parietal endoderms and visceral yolk sacs for malic enzyme activity that gave excellent discrimination between wild-type ($Mod-1^+/Mod-1^+$) cells and mutant ($Mod-1^n/Mod-1^n$) cells that lack the cytoplasmic form of the enzyme. Reciprocal blastocyst injection experiments were undertaken in which single primitive endoderm cells of one genotype were transplanted into embryos of the other genotype. In addition, $Mod-1^+/Mod-1^+$ early inner cell mass (ICM) cells were injected into $Mod-1^n/Mod-1^n$ blastocysts, either in groups of two or three singletons or as daughter cell pairs. A substantial proportion of the resulting conceptuses showed mosaic histochemical staining in the parietal endoderm, visceral yolk sac, or in both these membranes. Stained cells were invariably intimately intermixed with unstained cells in the mosaic parietal endoderms. In contrast, one or both of two distinct patterns of staining could be discerned in mosaic visceral yolk sacs. The first, a conspicuously 'coherent' pattern, was found to be due to endodermal chimaerism; the second, a more diffuse pattern, was attributable to chimaerism in the mesodermal layer of this membrane. The overall distribution of cells with donor staining characteristics resulting from primitive endoderm versus early ICM cell injections was consistent with findings in earlier experiments in which allozymes of glucosephosphate isomerase were used as markers.

The conspicuous lack of phenotypically intermediate cells in predominantly stained areas of mosaic membranes suggested that the histochemical difference between $Mod-1^+/Mod-1^+$ and $Mod-1^n/Mod-1^n$ genotypes was cell-autonomous. This conclusion was strengthened by the results of staining mixed *in vitro* cultures of parietal endoderm in which presence or absence of phagocytosed melanin granules was used as an independent means of distinguishing wild type from null cells.

By substituting tetranitro blue tetrazolium for nitro blue tetrazolium in the incubation medium, satisfactory differential staining was obtained for both the extraembryonic endoderm and other tissues of earlier postimplantation wild type versus null embryos.

Finally, absence of cytoplasmic malic enzyme activity does not appear to have a significant effect on the viability or behaviour of mutant cells.

INTRODUCTION

The extraembryonic endoderm of the mouse conceptus consists of two types of cell, parietal and visceral, that are spatially, morphologically and biochemically

---

* This paper is dedicated to Dr Sydney Smith who kindled my interest in embryological research.

distinct (see Gardner, 1983, for review). Two different lineage schemes have been proposed to account for the origin of these cell populations. According to the first, parietal and visceral endoderm cells share a common origin from the primitive endoderm that is formed on the free surface of the inner cell mass (ICM) in the mature blastocyst (Gardner & Papaioannou, 1975; Gardner, 1978a). The second scheme equates primitive with early parietal endoderm, visceral endoderm being assigned a separate and somewhat later origin from the underlying primitive ectoderm (Dziadek, 1979). Support for this second scheme comes from observations on isolated ICMs maintained *in vitro* (Pedersen, Spindle & Wiley, 1977; Atienza-Samols & Sherman, 1979; Dziadek, 1979).

Experiments in which the technique of blastocyst injection (Gardner, 1968, 1978b) was used to assay the developmental potential of ICM-derived cells in intact embryos *in vivo* have provided results that support the first of the above schemes rather than the second. Thus, ability to colonize the extraembryonic endoderm of host embryos was found to be restricted to primitive endoderm cells from the mature blastocyst, and to parietal and visceral endoderm cells from early postimplantation embryos (Rossant, Gardner & Alexandre, 1978; Gardner & Rossant, 1979; Gardner, 1982). Furthermore, such experiments demonstrated unequivocally that clones formed by transplanting single primitive endoderm cells could span both the visceral and parietal endoderm of the same conceptus (Gardner, 1982). Nevertheless, the majority of such primitive endo-dermal clones were confined to the parietal endoderm. Indeed, even within the parietal endoderm, distal colonization seemed to be preferred over proximal. More surprising, perhaps, was the finding that established visceral endoderm cells exhibited as marked a preference for parietal rather than visceral coloniza-tion as their primitive endodermal progenitors (Gardner, 1982). Unfortunately, it was necessary to resort to electrophoresis of tissue homogenates in order to separate the allozymes of glucosephosphate isomerase (GPI, E.C. 5.3.1.9.) that were used to distinguish donor from host cells in these blastocyst injection ex-periments. Hence, spatial resolution was too poor both to enable more detailed analysis of the distribution of donor cells within the extraembryonic endoderm and to determine whether visceral cells that had colonized the parietal endoderm displayed a phenotype appropriate for this layer.

Clearly, much more effective use could be made of clonal analysis to study both the growth of parietal and visceral endoderm and the relationship between them if individual donor cells could be distinguished from host cells *in situ*. Several genetic markers are currently available that enable mosaicism to be visualized at the cellular level in certain adult tissues (reviewed by McLaren, 1976; West, 1984), although not all of these have been critically evaluated (Gard-ner, 1984).

The most promising attempts to exploit genetic polymorphism as the basis of a more general *in situ* cell marker that might also be employed prenatally have depended on three different approaches. The first involves the production of

interspecific chimaeras in which species-specific antisera or DNA probes can be used to visualize mosaicism in all tissues (Gardner & Johnson, 1973, 1975; Siracusa *et al* 1983; Rossant, Vijh, Siracusa & Chapman, 1983). One cannot neglect the possibility that adoption of this particular solution to the marker problem might entail substantial perturbation of the developmental phenomena under investigation. Such concern would seem to be fully justified with regard to rat–mouse chimaeras which typically show runting at birth and have so far failed to survive thereafter (Gardner & Johnson, 1973, 1975). It is perhaps less warranted in the case of chimaeras that have been produced more recently between *Mus musculus* and *Mus caroli* because these organisms can develop into healthy, fertile adults (Rossant & Chapman, 1983). The second approach has been to obtain allozyme-specific antisera against GPI as a means of harnessing this ubiquitous enzyme as an *in situ* marker in intraspecific chimaeras in the mouse (Gearhart & Oster-Granite, 1978, 1980; Oster-Granite & Gearhart, 1981). Finally, haplotype-specific monoclonal antibodies have recently enabled H-2 antigens to be used as markers in a variety of tissues of post-natal mouse chimaeras (Ponder, Wilkinson & Wood, 1983). However, the task of assigning individual cells to one or other H-2 type may be difficult at the interfaces between patches in certain epithelia and impossible throughout other tissues that show very fine grained mosaicism (Ponder *et al*. 1983). In addition, it is not clear how useful H-2 antigens will be prenatally as markers since they do not appear to be expressed in many tissues until a relatively late stage of development (Kirkwood & Billington, 1981).

An important limitation of all three of these approaches to *in situ* cell marking is that they utilize reagents that are applicable to sectioned material rather than whole-mount preparations. They are therefore ill-suited for studying patterns of chimaerism in extraembryonic membranes (Gardner, 1984).

The present investigation was prompted by the recent discovery of a null mutation at the *Mod-1* locus which codes for cytoplasmic malic enzyme in the mouse (Lee *et al*. 1980a; Lee, Lee, Lewis & Johnson, 1980b; Johnson *et al*. 1981). Its aim was to explore the possibility of using the presence versus absence of a functional gene at this locus as an *in situ* marker with which to undertake clonal analysis of development of the extraembryonic endoderm. Initially cytoplasmic malic enzyme did not appear to be a very promising candidate for such a marker for several reasons. First, it evidently belongs to the class of soluble cytoplasmic enzymes which tend to be particularly difficult to localize histochemically (Pearse, 1972). Second, virtually nothing is known about either the temporal or spatial patterns of expression of the *Mod-1* locus during prenatal development. Engel & Wolfe (1971) could not detect the presence of the enzyme prior to the 10th day of gestation using an electrophoretic assay. However, homogenates of entire conceptuses were used in this study and no indication of the sensitivity of the assay was provided. Finally, as illustrated in Table 1, cytoplasmic malic enzyme is but one of five different mammalian cell enzymes

that can utilize L-malate as a substrate. However, the fact that there are differences in intracellular location, co-enzyme requirements, and in certain physico-chemical properties between the various enzymes (see Tables 1 and 2) suggested that conditions might nevertheless be found in which wild-type cells could be distinguished unequivocally from homozygous mutant cells in chimaeric membranes. All reagents that are needed for the histochemical demonstration of malic enzyme activity are relatively small molecules (Pearse, 1972). This meant that staining could be attempted using intact cells, a strategy that has been employed very successfully in similar studies in *Drosophila* (Janning, 1972; Kuhn & Cunningham, 1978; Szabad, Schupbach & Wieschaus, 1979; Lawrence, 1981).

MATERIALS AND METHODS

Mice

A stock of mice that was homozygous for the lack of cytoplasmic malic enzyme activity ($Mod-1^n/Mod-1^n$ mice) was obtained by intercrossing heterozygous progeny from the fifth generation of backcrossing to the C57BL/6J inbred strain in which the null mutation had originated. Corresponding wild-type mice belonged to the random-bred PO (Pathology, Oxford) albino strain and are designated $Mod-1^+/Mod-1^+$ mice because no attempt was made to determine whether they were $Mod-1^a/Mod-1^a$, $Mod-1^b/Mod-1^b$, or heterozygous for these two functional alleles. Females were paired overnight (dark period 19.00–07.00 h) with fertile males of the same strain and checked for the presence of vaginal plugs the following morning. Additional $Mod-1^+/Mod-1^+$ females were mated to vasectomized males to serve as recipients of transplanted blastocysts. The day on which the plug was found was designated the first day of pregnancy or pseudo-pregnancy.

Media

PB1 medium (Whittingham & Wales, 1969) containing glucose (1 g/l) in place of lactate and foetal calf serum (10 % v/v) in place of bovine serum albumin, was used for the recovery, storage at room temperature and manipulation of blastocysts. It was also used for the recovery and dissection of conceptuses whose visceral yolk sacs and or capsular parietal endoderms were intended for *in vitro* culture. Culture of blastocysts, isolated inner cell mass (ICM) cells, and cells or tissues for postimplantation embryos was done in modified α-medium (Stanners, Eliceiri & Green, 1971), which was also supplemented with 10 % (v/v) foetal calf serum, at 37 °C in an atmosphere of 5 % CO_2 in air.

Production of chimaeric conceptuses

Chimaeras were produced by injecting cells into blastocysts (Gardner, 1968) using a technique that has been described fully elsewhere (Gardner, 1978b). All

micromanipulative procedures were done in hanging drops maintained at approximately 11 °C by means of a thermocirculator (Model 02/CTCV/H.P., Churchill, U.K.) connected to the microscope stage (Leitz, heating and cooling stage 80). Donor $Mod-1^+/Mod-1^+$ blastocysts were recovered at approximately 11.00 h on the 4th or 14.30 h on the 5th day of pregnancy. Host $Mod-1^n/Mod-1^n$ blastocysts were invariably recovered before 10.00 h on the 4th day because they usually showed such a marked degree of developmental precocity as to suggest that ovulation was being induced by coitus in this stock. In reciprocal cell injection experiments, donor $Mod-1^n/Mod-1^n$ blastocysts were recovered at roughly 10.00 h on the 5th day and host $Mod-1^+/Mod-1^+$ blastocysts at 15.00 h on the 4th day of pregnancy.

Primitive endoderm cells were isolated from 5th day blastocysts as described recently (Gardner, 1982), and one such cell injected into each host blastocyst. Fourth day $Mod-1^+/Mod-1^+$ blastocysts were incubated for up to 1 h to compensate for any adverse effects of recovery and removal of the zona pellucida (Nichols & Gardner, 1984) on their state of expansion. Those whose blastocoele did not exceed the ICM in volume following culture were selected as early ICM cell donors. The trophectoderm of these blastocysts was destroyed immunosurgically (Solter & Knowles, 1975; Nichols & Gardner, 1984) using normal rat rather than guinea pig serum as the source of complement (see Spielmann, Jacob-Muller & Beckord, 1980). Following elimination of trophectodermal debris by pipetting, the ICMs were incubated in 0·25 % pronase (Calbiochem. Grade B) in Dulbecco 'A' phosphate-buffered saline (PBS, Oxoid, U.K.) for 12 min at room temperature. Thereafter, they were rinsed and then incubated in OC medium (table 6.5 of Biggers, Whitten & Whittingham, 1971) without calcium at 37 °C for the 15–35 min required to decompact them. Once decompacted, the ICMs were transferred to drops of PB1 for mechanical dissociation. The dissociated single cells were then transferred in groups of 10–30 into approximately 2 mm diameter drops of α-medium that had been placed on sterile siliconized coverslips and covered with paraffin oil (Boots, Pure Drug Co., U.K.). The cells were deposited well apart from each other, and the precise number in each drop recorded before culture. They were incubated for up to $2\frac{1}{2}$ h at 37 °C and examined at roughly half hourly intervals in orders to identify any that had divided. Daughter cell pairs were removed to a manipulation chamber in which they were kept at 11 °C until transplantation. Finally, a daughter cell pair was injected into each of one series of blastocysts, and a group of either two or three single cells that had not divided during culture into each of another.

Production of mixed parietal endoderm cell cultures

Conceptuses were removed from the uteri of females killed either on the morning of the 11th or 12th day of pregnancy and placed in PB1. Capsular Reichert's membrane was detached from the placental region of each conceptus with iris scissors, dissected free of adherent trophoblast and capsular decidual

tissue, and rinsed in fresh PB1 to eliminate maternal blood cells. Five or six isolated Reichert's membranes of the same genotype were then transferred to a 35 × 10 mm tissue culture dish (Falcon, Beckton-Dickinson, U.S.A.) containing α-medium. Each membrane was stretched out flat with its parietal endoderm layer uppermost and held thus by focally embedding it in the floor of the dish with the tips of watchmaker's forceps at intervals round its periphery. A dense suspension of melanin granules was added either to the dish containing the wild-type or mutant Reichert's membranes or, in controls for assessing the effect of labelling with melanin granules on staining for malic enzyme activity, to one of a pair of dishes containing wild type membranes.

The melanin granule suspension was prepared by repeatedly teasing the freshly recovered pigmented iris epithelia from three mice in α-medium. After standing for 5–10 min to allow debris to sediment, the suspension was transferred to a sterile siliconized glass centrifuge tube and made up to approximately 8 ml with additional α-medium. The granules deposited by centrifugation at 4300 r.p.m. for 25 min were resuspended in 0·25–0·5 ml of fresh α-medium and added to the appropriate dish of Reichert's membranes to make a final volume of approximately 2 ml.

Pairs of dishes were incubated for 3 h, the one containing the melanin granule suspension being pipetted briefly at hourly intervals so as to ensure that granules continued to sediment on the parietal endoderm cells throughout. Following culture, the central flattened part of each Reichert's membrane was cut out with iris scissors so as to avoid the peripheral folds in which cells were shielded from melanin granules and ensure that equivalent regions were obtained from both labelled and unlabelled specimens. All excised fragments from a given dish were pooled and transferred to a mixture of 0·05 % w/v trypsin (Difco, 1/250) and 0·02 % w/v sodium diaminoethanetetra-acetate (E.D.T.A., B.D.H. Poole, U.K.) in PBS for 12 min at room temperature. Thereafter, they were transferred via two rinses of PB1 to a cavity cell containing approximately 1 ml of this medium and stripped of cells by being raised repeatedly through the medium-air interface. The resulting suspension of the cells that had not been exposed to melanin granules was refrigerated while single unequivocally labelled cells were selected at ca. 11 °C with a micropipette held in a micromanipulator from the suspension prepared from membranes that had been exposed to these particles. When sufficient labelled cells had been collected they were mixed with an excess of those from the unlabelled suspension and the mixture deposited at as high a density as possible on a piece of glass coverslip placed in the bottom of a 35 × 10 mm tissue culture dish containing α-medium (see Fig. 1). Dispersal of the cells by convection was reduced by refrigerating each dish prior to seeding. The dishes were incubated either for $2\frac{1}{4}$–$2\frac{1}{2}$ h or for approximately 16 h. Thereafter, the medium was replaced by PBS and each dish swirled vigorously to detach all loosely adhering cells before the cultures were fixed with glutaraldehyde. Each dish was rinsed three times with PBS following removal of the fixative. After a further change of PBS, the dishes were refrigerated for 12–36 h.
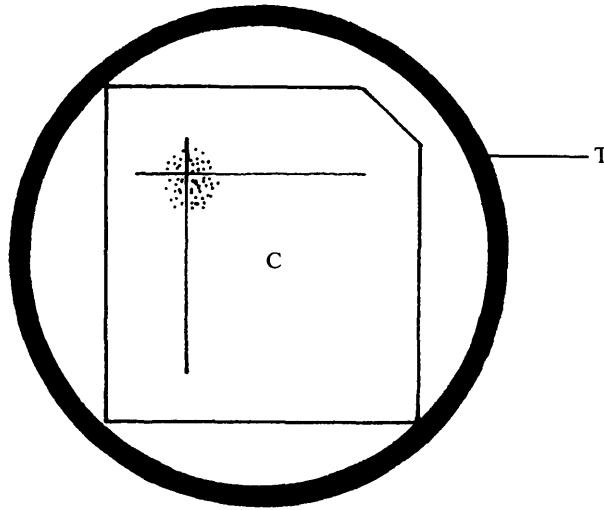
Fig. 1. Diagram of a mixed parietal endoderm cell culture. A roughly square piece of glass coverslip (C) was prepared which occupied most of the floor of a 35 mm diameter plastic tissue culture dish (T). Perpendicular lines were etched in the coverslip and one corner removed to enable identification of its upper surface during processing. The cells, indicated by dots, were deposited in the region of intersection of the etched lines to facilitate subsequent photography of the culture.

Recovery and handling of parietal and visceral yolk sacs

Conceptuses of $Mod-1^+/Mod-1^+$, $Mod-1^n/Mod-1^n$ and of presumptively chimaeric constitution were usually recovered from females killed on the 10th to 12th day of pregnancy. Capsular parietal endoderms were isolated as described earlier, except that a single proximodistal cut was made in those of presumptive chimaeras prior to their final separation from the placenta. A corresponding incision was made in the rim of the placenta so that a circumferentially coincident proximodistal cut could be made later in the visceral yolk sac during its fixation. Such cuts enabled mutual alignment of pairs of membranes following histo-chemical staining so that the pattern of distribution of wild-type and mutant cells in one could be compared with that in the other in cases in which both were chimaeric. Capsular parietal endoderms were usually pinned out on the floor of plastic culture dishes for fixation to avoid the wrinkling and folding that tended to occur if they were unattached. In order to retain the shape of visceral yolk sacs during fixation, conceptuses from which the capsular parietal yolk sac only had been removed were placed in several ml of fixative. After approximately $\frac{1}{2}$ min, each visceral yolk sac was cut open proximodistally and the foetus plus amnion removed as quickly as possible through this aperture so that the fixative had access to the mesodermal as well as the endodermal surface of the membrane.

The parietal endoderm was fixed *in situ* in 7th to 9th day embryos which had been cut in two just below the base of the ectoplacental cone or developing

placenta, after removal of as much decidual and trophoblast tissue as possible from Reichert's membrane. It retained its natural shape if removed carefully thereafter. Additional embryos from which both the placental region and Reichert's membrane had been removed were fixed after opening the amnion to ensure ready access of the fixative to the internal as well as the external surface.

Fixation

Following initial trials with various reagents, the principle of minimal fixation with dilute glutaraldehyde (Flitney, 1966) was adopted for all material that was to be stained for malic enzyme activity. Two different stocks of aqueous glutaraldehyde were tested. One was a standard (approximately 25 %) solution (B.D.H., Poole, U.K.) and the other a vacuum-distilled 8 % solution stored under nitrogen (Polysciences, U.S.A.). The stocks were diluted in 0·2 M-phosphate buffer which, after addition of L-malate at a final concentration of 10 mM, was adjusted to a pH of 7·2. Fixation was invariably done at room temperature and, as described in the Results, a range of concentrations and durations of exposure to fixative were explored. It was followed by three rinses in PBS during which the 10th to 12th day visceral yolk sacs were separated from their placentae. Initially, corresponding parietal endoderms that were pinned out for fixation were detached at this stage. In later experiments they were not released until after staining. Finally, the PBS was changed once again and the fixed specimens either refrigerated or incubated at 37 °C for various periods in order to deplete the cells of endogenous substrates and or co-enzymes which might impair the specificity of the subsequent histochemical reaction.

All specimens were refrigerated in Carnoy's fixative after histochemical staining. Capsular parietal endoderms were then spread on slides and mounted in DPX, either directly or via cedar wood oil (B.D.H., Poole, U.K.). Some visceral yolk sacs were also made into flat whole-mount preparations. Others were kept in cedar wood oil for inspection and photography in order to conserve their natural shape. Pieces of some of these yolk sacs, and the remainder in their entirety, were embedded in paraffin wax for sectioning.

Additional visceral yolk sacs which were to be used for morphometric analysis were placed directly in Carnoy's fixative on recovery for subsequent wax embedding, sectioning and staining with haematoxylin and eosin. All embedded material was sectioned at 5–7 μm.

Histochemistry

The following reagents were purchased from Sigma: amo barbital (amytal), dicoumarol, β-glycerophosphate, iodoacetic acid, D-L-lactic acid, L-malic acid, malonic acid, β-nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide phosphate (NADP), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), sodium azide and tetranitro blue tetrazolium (TNBT). Additional reagents were cupric acetate, manganous chloride and zinc

sulphate (B.D.H., Poole, U.K.); mercuric chloride and oxide (Fisons', U.K.); meldola-blue (Boehringer, Mannheim, West Germany), and 5,5'-dithiobis-(2-nitrobenzoic acid) (D.T.N.B., Emanuel, U.S.A.).

Staining for lactate dehydrogenase activity was done as described by Pearse (1972). The recipe of Pearse (1972), with inclusion of PMS and sodium azide, was also used initially for histochemical staining for malic enzyme activity. Except where stated otherwise, the tetrazolium used throughout this investigation was NBT. The various modifications to the incubation medium that were found to be necessary to obtain satisfactory discrimination between mutant and wild-type parietal and visceral endoderm cells are discussed in the Results. Whenever possible, wild-type and null mutant control specimens were processed and stained simultaneously with those from presumptive chimaeras.

Appraisal of histochemical staining of parietal and visceral endoderm cells in situ

The frequency of stained versus unstained cells in capsular parietal endoderm was assessed by scoring square fields of membrane of approximately $225 \times 225\,\mu m$ with the aid of camera-lucida drawings made with an R.A. microscope plus drawing tube (Zeiss, West Germany). In wild-type and null controls fields were chosen at low magnification with essentially dark-ground illumination so as to avoid folded areas without risk of bias with respect to possible regional variation in staining. Two such fields, one proximal and the other distal, were scored for each of the chimaeric specimens analysed in this way. The proximal field was selected from the part of the proximal extremity deemed to contain the highest proportion of donor cells. Obviously, because the capsular parietal endoderm is essentially dome shaped, there is much less flexibility in choice of the corresponding distal field. Hence, this procedure will tend to overestimate the proximal relative to distal contribution of donor cells.

Chimaeric visceral endoderms could usually be analysed satisfactorily as intact specimens after staining. Control visceral yolk sacs were sectioned before examination because mesodermal staining precluded scoring of the endoderm in whole mounts of wild-type specimens, and histochemical preparations of enzymatically isolated endoderm were unsatisfactory. However, although the number of inappropriately stained cells could readily be determined, the total number scored could not because it was often impossible to discern cell boundaries, particularly in sections of wild-type controls. Hence, total cell number was estimated as follows. The length (L) of the endodermal surface of haematoxylin- and eosin-stained sections of 12th day wild-type visceral yolk sac, in which cell counts could be made, was estimated from camera-lucida drawings by McIntyre's method (see Aherne & Dunnill, 1982). Dividing L by the number of cells counted yielded a mean width of $5.72\,\mu m$ (s.d. $= \pm 0.35$) for endoderm cells in $5\,\mu m$ thick sections. (Similar values were obtained on applying this procedure to the parts of histochemically stained sections of null controls in which cell boundaries could be resolved.) Three sections taken at four-section

intervals from a serial sequence were scored for each control specimen. The mean length (L) of the central of the three sections was estimated by McIntyre's method, and the total number of cells scored (N) calculated as follows:

$$N = 3 \times \frac{L}{5 \cdot 72}$$

Cell viability in parietal endoderm tissue

Freshly isolated capsular parietal endoderms were exposed to a 0·36 mg/ml solution of erythrosin B (Sigma) in PBS for 10 min at room temperature immediately after they had been pinned out. They were then rinsed in PBS and kept moist by addition of a coverslip while two or three fields per specimen were selected and scored for stained cells as described earlier for the histochemical control preparations of this tissue.

Analysis of mixed parietal endoderm cell cultures

Prior to staining for malic enzyme activity, a photographic record was obtained of the seeded area of each coverslip at a magnification of ×32 or ×64 with a Leica MDa camera attached to a Leitz Diavert microscope. The coverslips were transferred to Carnoy's fixative after histochemical staining and then mounted individually on glass slides in DPX (B.D.H., Poole, U.K.). Finally, the seeded area of each preparation was reproduced on layout paper under ×40 objective and ×10 ocular magnification by means of the drawing tube. As each cell was outlined it was marked as positive or negative for malic enzyme activity and assigned a consecutive number. An independent observer inspected the photographs that had been taken of the preparations before staining in order to identify cells which could not be scored either as unequivocally labelled or unlabelled with melanin granules. (Such equivocally labelled cells were rare, and attributable principally to irregularities in the coverslips which resulted in local departures from focus rather than inadequate uptake of the granules.) Finally, the photographic and camera-lucida records of each culture were compared so that the presence or absence of histochemical staining could be recorded for the first 1000 of the consecutively numbered cells classified as unequivocally labelled or unlabelled with melanin granules.

RESULTS

Histochemical staining of wild-type versus mutant membranes

Capsular parietal endoderm was used in initial investigations because this membrane consists of a monolayer of separate or very tenuously associated cells attached to a tough but relatively porous basement membrane (Jollie, 1968). Hence, optimal conditions for fixation, rinsing and staining were likely to be defined more readily for cells of this tissue than those of the substantially thicker

and much more densely populated visceral yolk sac. Specimens were exposed to concentrations of glutaraldehyde ranging from 0·06 % to 1·5 % for periods of between 1 and 20 min. The most marked differential staining between wild-type and null 11th or 12th day parietal endoderm cells was observed consistently after fixation of the membranes in 0·6 % standard glutaraldehyde for 2 min. Results were inferior with vacuum-distilled glutaraldehyde, even if the concentration was as low as 0·06 %.

It was necessary to refrigerate the membranes in PBS for $3\frac{1}{2}$–4 days after fixation before an acceptably low level of background staining was obtained when either L-malate or, more particularly NADP, was omitted from the incubation medium. When the staining of $Mod-1^+/Mod-1^+$ tissue was compared with that of $Mod-1^n/Mod-1^n$ tissue in complete incubation medium of Pearse (1972) after such prolonged storage in PBS, the majority of cells in wild-type specimens stained more strongly than those in their null counterparts. However, the results were not very satisfactory because there was clearly substantial overlap in staining intensity of cells of the two genotypes in specimens that had been treated identically throughout. It was considered that such differential staining would be unacceptable for discriminating between cells of the two genotypes in chimaeric membranes.

Dependence of the histochemical reaction on exogenous L-malate and NADP suggested that staining in null cells was most probably due to activity of the mitochondrial form of malic enzyme and or to the more recently described NAD(P)-dependent malic enzyme which also appears to reside in mitochondria (see Table 1). However, malate dehydrogenase(s) might also contribute to staining if significant conversion of the exogenous NADP to NAD was occurring as a result of endogenous alkaline phosphatase activity (Leeflang-de Pijper & Hulsmann, 1974; Meijer & de Vries, 1975). This possibility was examined by comparing the staining of parietal endoderm tissue for lactate dehydrogenase (LDH) activity with NAD versus NADP as co-enzyme. Since LDH appears to have an absolute requirement for NAD (Dixon & Webb, 1964), NADP can only substitute for this co-enzyme in the reaction if it is converted to it. Specimens of parietal endoderm that had received exactly the same pretreatment as those used for malic enzyme histochemistry were incubated in aliquots of stain in which L-malate was replaced by the same concentration of DL-lactate (Pearse, 1972) and which contained either NAD or NADP, or neither co-enzyme. Whereas the presence of NAD ensured very intense staining, cells incubated in NADP-containing medium for up to 6 h exhibited the same low background level of staining as those incubated in the absence of exogenous co-enzyme. These results argued against NAD-dependent malate dehydrogenase activity playing a significant role in the staining of null cells, as did those obtained after addition of 16 mM β-glycerophosphate to the malic enzyme incubation medium as a competitive inhibitor of alkaline phosphatase (Leeflang-de Pijper & Hulsmann, 1974). Therefore, further attempts to enhance the differential staining between

Table 1. *Enzymes in the mouse which utilize L-malate as substrate*

Malic enzyme (E.C. 1.1.1.40)

$$\text{L-malate} + \text{NADP} \rightleftharpoons \text{pyruvate} + \text{CO}_2 + \text{NADPH}_2 \,[1]$$
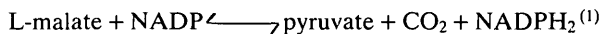
Cytoplasmic enzyme – coded by *Mod-1* locus on chromosome 9.[2,3]
Alleles – *Mod-1*^{a}, *Mod-1*^{b} and *Mod-1*^{n}.[3,4,5]
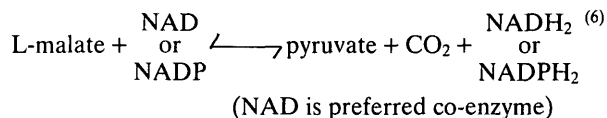**Mitochondrial enzyme** – coded by *Mod-2* locus on chromosome 7.[2,3]
Alleles – *Mod-2*^{a} and *Mod-2*^{b}.[3]

Malate dehydrogenase (E.C. 1.1.1.37)

$$\text{L-malate} + \text{NAD} \rightleftharpoons \text{oxaloacetate} + \text{NADH}_2 \,[1]$$
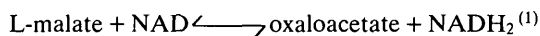
Cytoplasmic enzyme – attributed to *Mor-2* locus for which neither the location nor existence of alleles seems to be known.[3]
Mitochondrial enzyme – coded by *Mor-1* locus on chromosome 5.[2,3]
Alleles – *Mor-1*^{a} and *Mor-1*^{b}.[2,3]

NAD(P)-dependent malic enzyme (E.C. ?)[6]

$$\text{L-malate} + \genfrac{}{}{0pt}{}{\text{NAD}}{\text{or}}{\text{NADP}} \rightleftharpoons \text{pyruvate} + \text{CO}_2 + \genfrac{}{}{0pt}{}{\text{NADH}_2}{\text{or}}{\text{NADPH}_2} \,[6]$$

(NAD is preferred co-enzyme)

This is a mitochondrial enzyme which does not seem as yet to have been investigated genetically.

References (1) = Dixon & Webb (1964); (2) = Green (1981); (3) = Shows, Chapman & Ruddle (1970); (4) = Lee *et al.* (1980*a,b*); (5) = Johnson *et al.* (1981); (6) = Sauer & Dauchy (1978).

---

null and wild-type cells were directed towards obtaining selective inhibition of mitochondrial malic enzyme activity.

There is evidence from studies on various types of mammalian cells that mitochondrial malic enzyme is more sensitive to inhibition by a number of reagents than its cytoplasmic counterpart (Table 2). The effect of several such inhibitors on the staining of wild-type versus null parietal endoderm cells was examined using the concentrations and conditions of exposure to each reagent shown in Table 3. No consistent improvement in differential staining was observed following any of the experimental treatments listed in this table. However, a striking difference in the degree of staining of wild-type and null cells was evident in malonate controls in which the concentration of L-malate had been reduced to 10 mM or less in the absence of the inhibitor (Table 3). Strong staining of wild-type cells was obtained consistently at this low substrate concentration, providing the period of incubation was increased to approximately 48 h (Fig. 2). Null cells, in contrast, remained essentially unstained under these conditions, typically exhibiting at most a dozen or so small grains of diformazan against an otherwise straw-coloured background (Fig. 3). Furthermore, the period for which the fixed specimens had to be refrigerated in PBS prior to incubation could

Table 2. *Some differences between cytoplasmic and mitochondrial malic enzyme in mammals*

1. Conventional antisera can be raised against the cytoplasmic enzyme which do not cross-react with the mitochondrial enzyme and vice versa.[1,2]

2. The cytoplasmic enzyme has a lower K_m for L-malate, pyruvate, NADP and $NADPH_2$ than the mitochondrial enzyme.[3,4]

3. The ratio of V_{max} forward/V_{max} reverse reaction is lower for the cytoplasmic than mitochondrial enzyme.[3]

4. The mitochondrial enzyme is more sensitive to inhibition by a variety of reagents than the cytoplasmic enzyme. Included among such reagents are the following: Hg^{2+}, Cu^{2+}, Zn^{2+}, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), iodoacetate, dicoumarol, and malonate (at low concentrations of L-malate).[5,6]

References (1) = Lee *et al.* (1980*a,b*); (2) = Bernstine (1979); (3) = Simpson & Estabrook (1969); (4) = Bartholomé, Brdiczka & Pette (1972); (5) = Frenkel & Cobo-Frenkel (1973); (6) = Lee, Yuan & Moser (1978).

Table 3. *Selective inhibitors of mitochondrial malic enzyme (MOD-2) tested for effects on differential staining of wild type versus null parietal endoderm*

Inhibitor	Range of concentrations tested	Stage at which tissue exposed to reagent
Hg^{2+}	10^{-4}–10^{-7} M	throughout staining in standard medium*
Cu^{2+}	10^{-4}–10^{-7} M	throughout staining in standard medium
Zn^{2+}	10^{-4}–10^{-7} M	throughout staining in standard medium
Dicoumarol	1.5×10^{-4}–1.5×10^{-5} M	throughout staining in standard medium
Iodoacetate	10^{-1}–10^{-3} M	throughout staining in standard medium
Iodoacetate	10^{-3} M	in PBS for 20 min at ca. 18 °C prior to staining
DTNB	10^{-4}–10^{-6} M	throughout staining in standard medium
DTNB	10^{-6} M	in PBS for 30–60 min at ca. 18 °C prior to staining
Malonate	10^{-1}–2.5×10^{-2} M	throughout staining in standard medium
Malonate	2.5×10^{-2} M	throughout staining in medium containing 10^{-2} or 10^{-3} M L-malate

*Standard medium is the incubation medium described by Pearse (1972) which contains 10^{-1} M L-malate.

be reduced to 24 h without adversely affecting differential staining. Additional trials indicated that 10 mM was the optimal concentration of substrate for obtaining satisfactory staining of wild-type parietal endoderm cells with minimal background in the nulls. Conspicuous staining of null cells occurred once the concentration of L-malate reached 20 mM. Further modifications to the basic
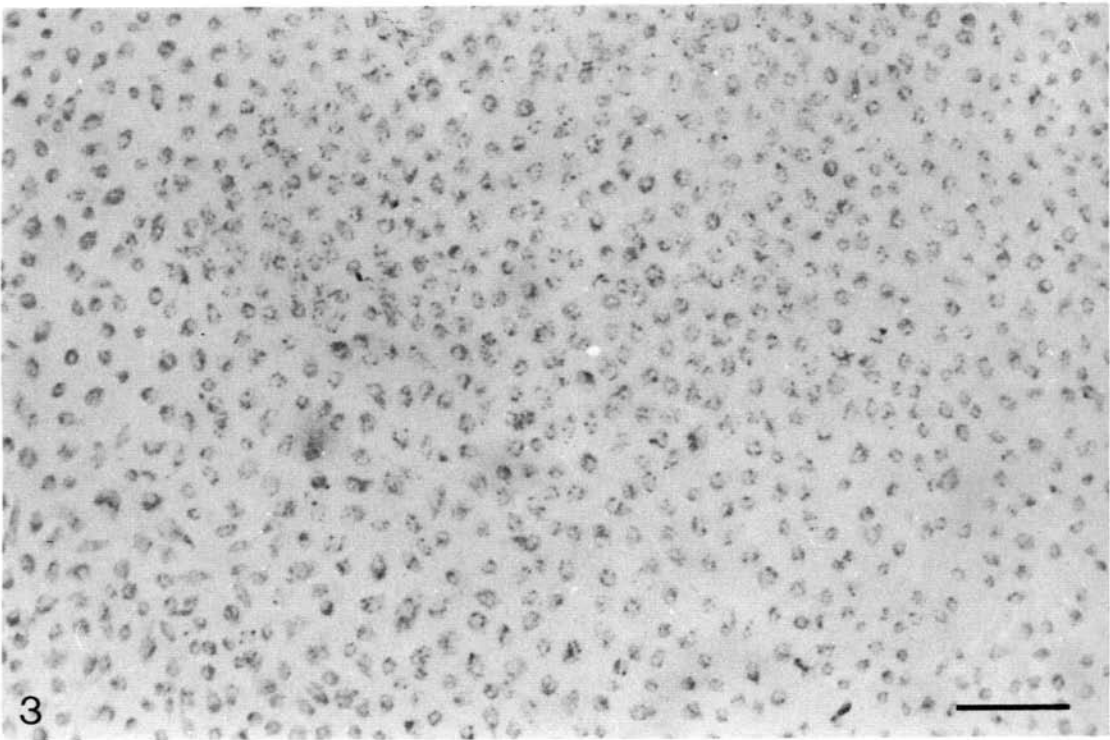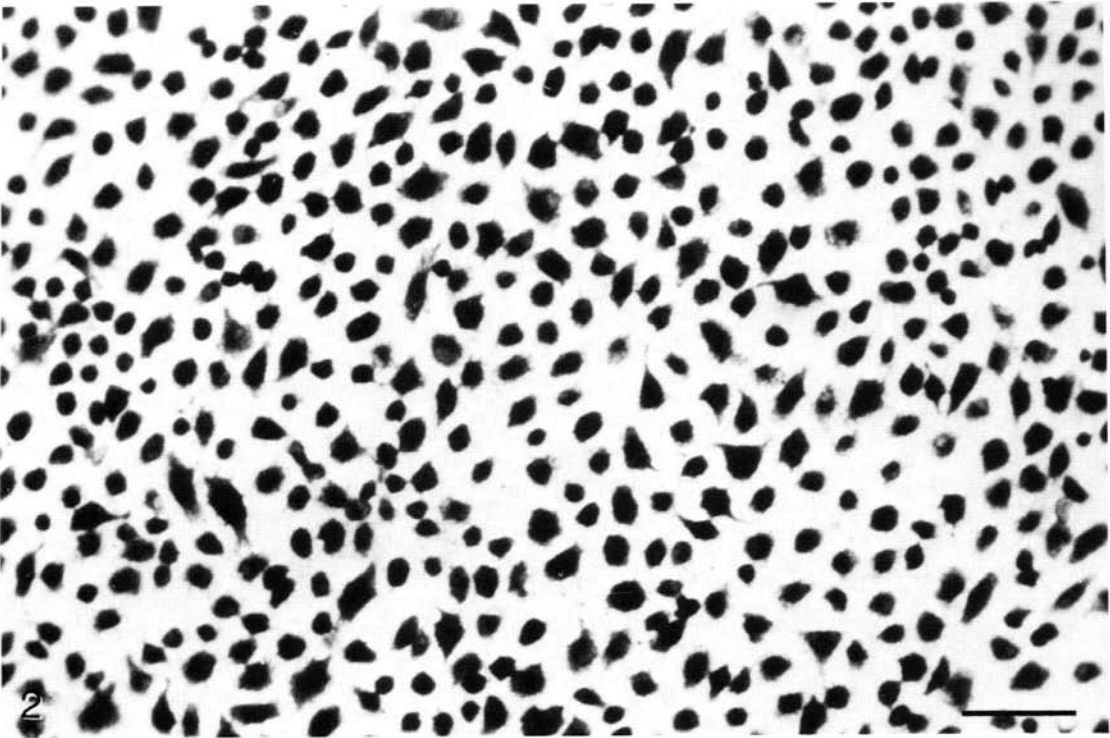
Figs 2 & 3

Table 4. *Modified incubation medium for enhancing differential staining of* Mod-1$^+$/Mod-1$^+$ *versus* Mod-1^n/Mod-1^n *cells for malic enzyme activity*

Component	Conditions of storage	Quantity used per unit of incubation medium
NADP	powder — at −20 °C — weighed out immediately prior to use	10 mg
NBT (or TNBT)*	1·0 mg/ml in distilled water at 4 °C	2·5 ml
Tris-HCl	0·2 M in distilled water at 4 °C	2·5 ml
Manganous chloride	0·9 mg/ml in distilled water at 4 °C	1·0 ml
Sodium azide	6·5 mg/ml in distilled water at 4 °C	1·0 ml
Amytal**	125 mg/ml in absolute ethanol at 4 °C	40 μl
L-malate	1 M in distilled water$^+$ — 100 μl aliquots at −20°C	100 μl
Distilled water	not stored	3·0 ml

pH of solution adjusted to 7·6 with 0·2 M Tris or 0·2 M HCl at this stage, prior to addition of one or other intermediate electron acceptor as follows:

either^{++} { PMS   30·6 mg/ml in distilled water at 4 °C   5 μl
or   { Meldola blue   0·5 mg/ml   5 μl

* Specimens stained in NBT medium were usually incubated for approximately 48 h during which the medium was changed 1–3 times. Satisfactory differential staining could often be obtained in 14–24 h using TNBT without changing the medium unless a somewhat longer period of incubation proved necessary. TNBT gives better differential staining in extra-embryonic endoderm at earlier stages of development than NBT, though it produces a higher background in null cells.

** This reagent was omitted routinely from TNBT incubation medium.

$^+$ L-malate was neutralized as described by Pearse (1972).

$^{++}$ PMS is used in conjunction with NBT incubation medium for staining visceral yolk sacs. Meldola blue is used invariably with TNBT medium and with NBT medium for staining parietal endoderm.

incubation medium described by Pearse (1972) were tested for their effects on differential staining. These included varying the concentration of NBT and NADP, incorporating amytal in addition to sodium azide (Meijer & de Vries, 1975) and replacing Mg^{2+} by Mn^{2+} (Lee, Yuan & Moser, 1978) as well as PMS by meldola blue (Turner & Hopkinson, 1979). The basic composition of the incubation medium which was finally adopted for all subsequent work is given in Table 4,

Fig. 2. Part of a 12th day wild-type preparation of capsular parietal endoderm after staining for malic enzyme activity in the modified incubation medium described in Table 4. Bar = 50 μm.

Fig. 3. Part of a 12th day null preparation of capsular parietal endoderm that had been treated identically to the wild-type specimen shown in Fig. 2. Bar = 50 μm.

Table 5. *Incidence of inappropriately stained cells in wild-type and null parietal and visceral endoderm stained for malic enzyme activity (NBT stain)*

Tissue	Genotype	Age post-coitum	No. specimens	Total No. of fields/sections scored+	Total No. of cells scored+	No. (and %) inappropriately stained
Parietal endoderm	Wild-type	11th day	2	8	3346*	59(1·76 %)
	Null	11th day	2	8	4293*	10(0·23 %)
	Wild-type	12th day	15	21	7974*	120(1·50 %)***
	Null	12th day	15	21	9886*	13(0·13 %)
Visceral endoderm	Wild-type	12th day	4	12	18850**	33(0·18 %)
	Null	12th day	4	12	12977**	2(0·02 %)

+ Fields were scored for the parietal and sections for the visceral endoderm. The total number of cells scored was counted directly for the parietal endoderm and estimated as described in Materials and Methods for the visceral endoderm.

* Note that the total number of cells is approximately 20 % less for wild-type than null parietal endoderm on both the 11th and 12th day, despite the fact that counts were based on comparable areas of membrane in specimens of the two genotype at each stage. The mean cell densities ($\pm$ S.D.) per square field of $225 \times 225\ \mu m$ are as follows:
11th day: Null = 537 $\pm$ 132, wild-type = 418 $\pm$ 65; 0·05<P>0·01.
12th day: Null = 471 $\pm$ 95, wild-type = 380 $\pm$ 91; 0·01<P>0·001.

** There was no obvious difference in size of null versus wild-type visceral yolk sacs on the 12th day (see Fig. 4). The substantial difference in estimated total cell number between wild-type and null specimens has a trivial explanation. Pairs of specimens of each genotype were embedded and sectioned together. Sections for examination were taken from the part of the block which appeared to be central for the wild-type specimen because lack of staining in the null specimen made its outline difficult to discern.

*** Three fields in each of five fresh-mounted 12th day wild-type parietal endoderms that had been exposed to erythrosin B were scored for the presence of cells that failed to exclude the dye. Unequivocal staining was observed in 114/4283 cells (= 2·66 %).

together with the specific modifications that were found to be necessary for different tissues or stages of development.

The proportion of cells staining in specimens of mid-gestation null parietal endoderm exposed to the incubation conditions specified in Table 4 was only a fraction of 1 % (Table 5). Conversely, less than 2 % of cells failed to stain in corresponding wild-type specimens (Table 5). The proportion of non-viable cells in 12th day parietal endoderm was somewhat over 2 %, as judged by staining of freshly isolated wild-type tissue with erythrosin B (see footnote 4 of Table 5). Hence, the frequency of overlap in histochemical phenotype between cells in wild-type and mutant parietal endoderm was very low using these modified incubation conditions and, particularly in the case of wild-type cells, was unlikely to be further improved. Even if pairs of null and wild-type membranes were pinned out one on top of the other so that part of their endodermal surfaces were in contact throughout incubation, no elevation of the null background was discernible in the region of apposition. This suggested that blurring of the distinction between wild-type and null cells due to diffusion of $NADPH_2$ or reduced intermediate electron acceptor from the site of co-enzyme reduction (Leeflang-de Pijper & Hulsmann, 1974) was not likely to be a significant complication in chimaeric tissue.

Following initial trials, mid-gestation visceral yolk sacs were fixed routinely for $2\frac{1}{2}$ min in 0·6 % standard glutaraldehyde. Further improvement in histochemical differentiation between wild-type and null cells was obtained in later experiments by exposing the yolk sacs to 0·1 % standard glutaraldehyde for 4 min. At first, the incubation conditions described in Table 4 yielded relatively poor differential staining of wild-type versus mutant yolk sacs, particularly in the proximal folded or villous region of the organ that adjoins the placenta *in situ*. Conspicuous proximal staining was also seen in both null and wild-type specimens incubated in control stain without NADP, even if they had been refrigerated in PBS for up to 5 days between fixation and staining. Enzyme activity decreased markedly in wild-type cells if storage in PBS was extended further in an attempt to eliminate such non-specific staining. This fairly high background in null specimens complicated the task of identifying visceral yolk-sac mosaicism in the earliest series of presumptive chimaeras. However, it was discovered somewhat later that reactivity could be eliminated altogether in null specimens without its being obviously diminished in wild-type ones if the yolk sacs were incubated in PBS at 37 °C for approximately 24 h prior to staining (Fig. 4). Under these conditions histochemical discrimination between visceral endoderm cells of the two genotypes was found to be even more reliable than between parietal endoderm cells (Table 5).

Fig. 4. Three wild-type and three null 12th day visceral yolk sacs that were incubated in PBS at 37 °C for approximately 24 h prior to staining for malic enzyme activity. Note the almost complete opacity of the wild-type specimens and absence of staining throughout the nulls. Scale bar = 2 mm.
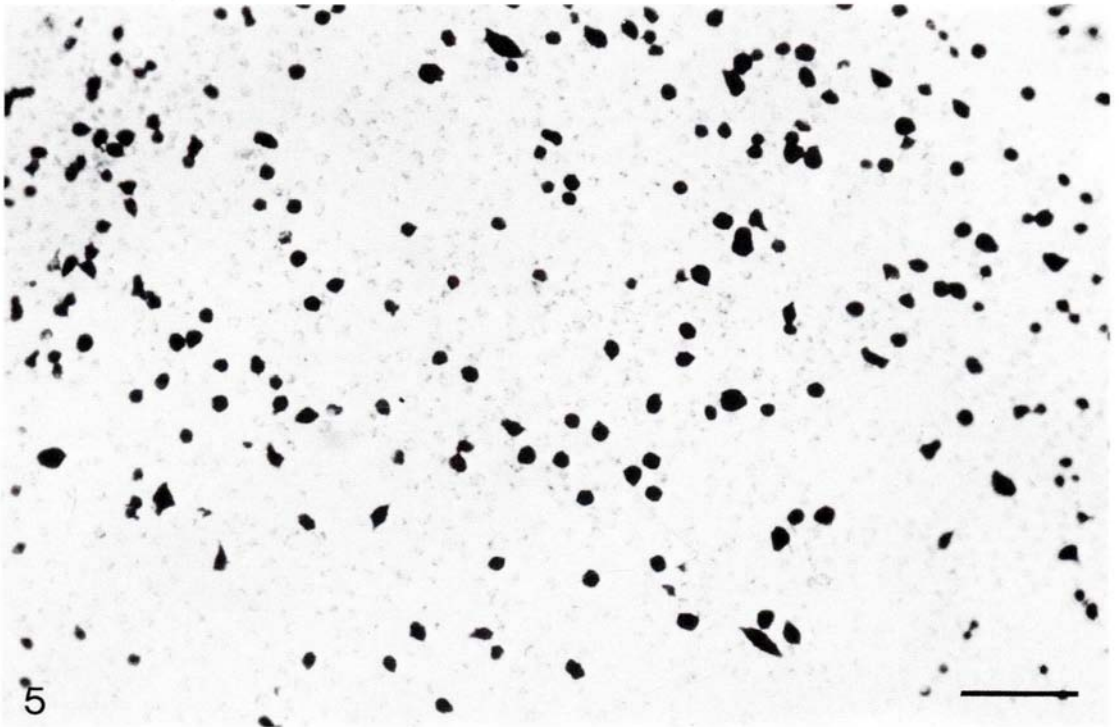
Fig. 5. Part of the histochemically mosaic parietal endoderm from a 12th day null conceptus (5RM36) that received a single wild-type primitive endoderm cell at the blastocyst stage. Note the extensive intermingling of stained and unstained cells. Lack of focus in parts of the periphery of the field is due to unavoidable slight undulations in the preparation. Bar = 50 μm.

Studies on presumptive extraembryonic endodermal chimaeras

Initially, a series of injections of single primitive endoderm cells from 5th day $Mod\text{-}1^{+}/Mod\text{-}1^{+}$ blastocysts into 4th day $Mod\text{-}1^{n}/Mod\text{-}1^{n}$ blastocysts was undertaken. The histochemical staining of the capsular parietal endoderms and visceral yolk sacs of all viable conceptuses obtained in these experiments was compared with that of corresponding membranes from conceptuses developing from uninjected $Mod\text{-}1^{n}/Mod\text{-}1^{n}$ control blastocysts.

Fig. 6. Flat-mount of the entire visceral yolk sac from an 11th day null conceptus (5RM17) that received a single wild-type primitive endoderm cell at the blastocyst stage. This specimen was stained before the practice of incubating yolk sacs in PBS at 37 °C for ca. 24 h had been introduced. Several 'coherent' patches of stained cells are nevertheless clearly discernible, though an unacceptable level of background staining is present in the proximal (upper) region of the preparation. The round dark mass towards the lower left is a remnant of the amnion. Bar = 1 mm.

Fig. 7. Part of the proximal region of the histochemically mosaic visceral yolk sac from a 12th day null conceptus (4IDCP8) that received a daughter pair of wild-type early ICM cells at the blastocyst stage. The distinction between stained and unstained cells is as marked at the boundary between patches as elsewhere. Bar = 100 μm.

6

7
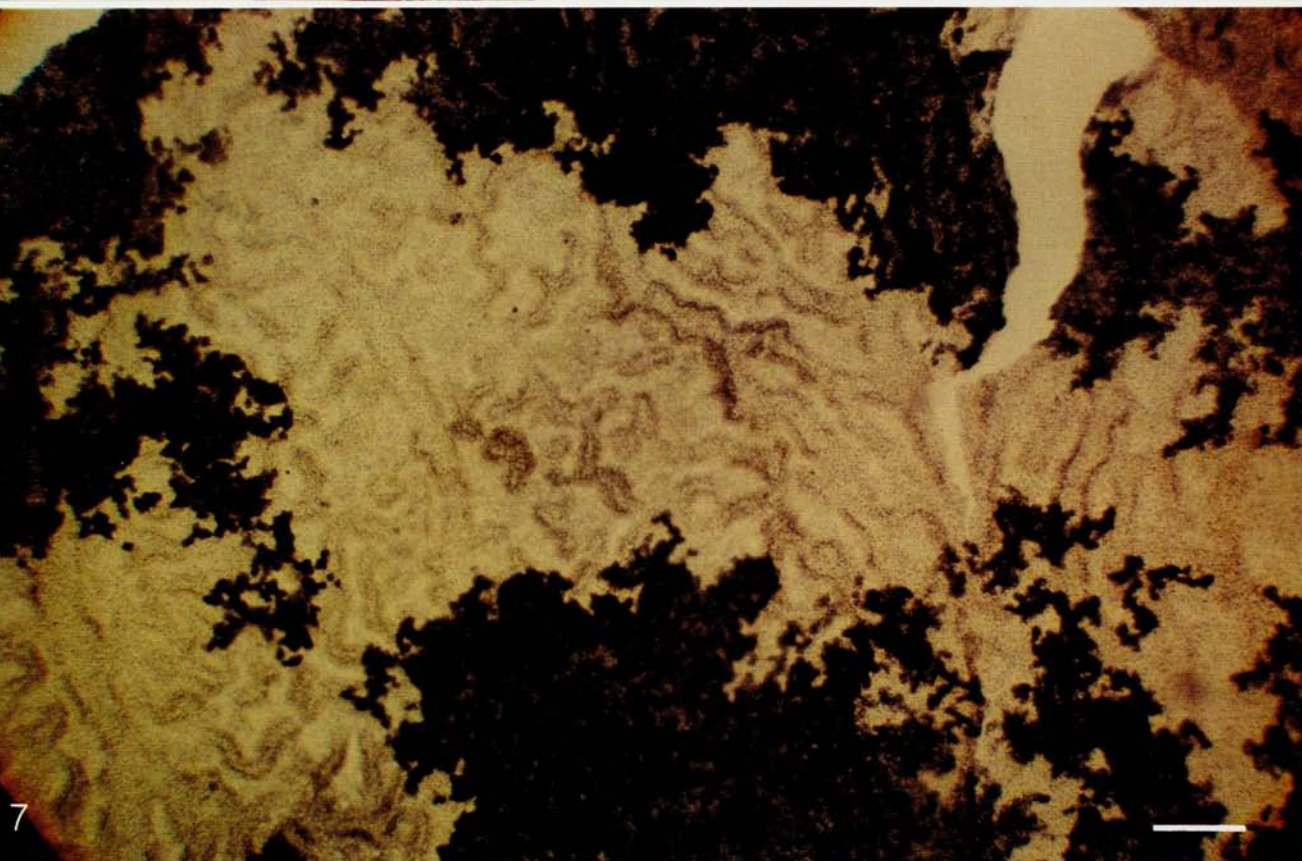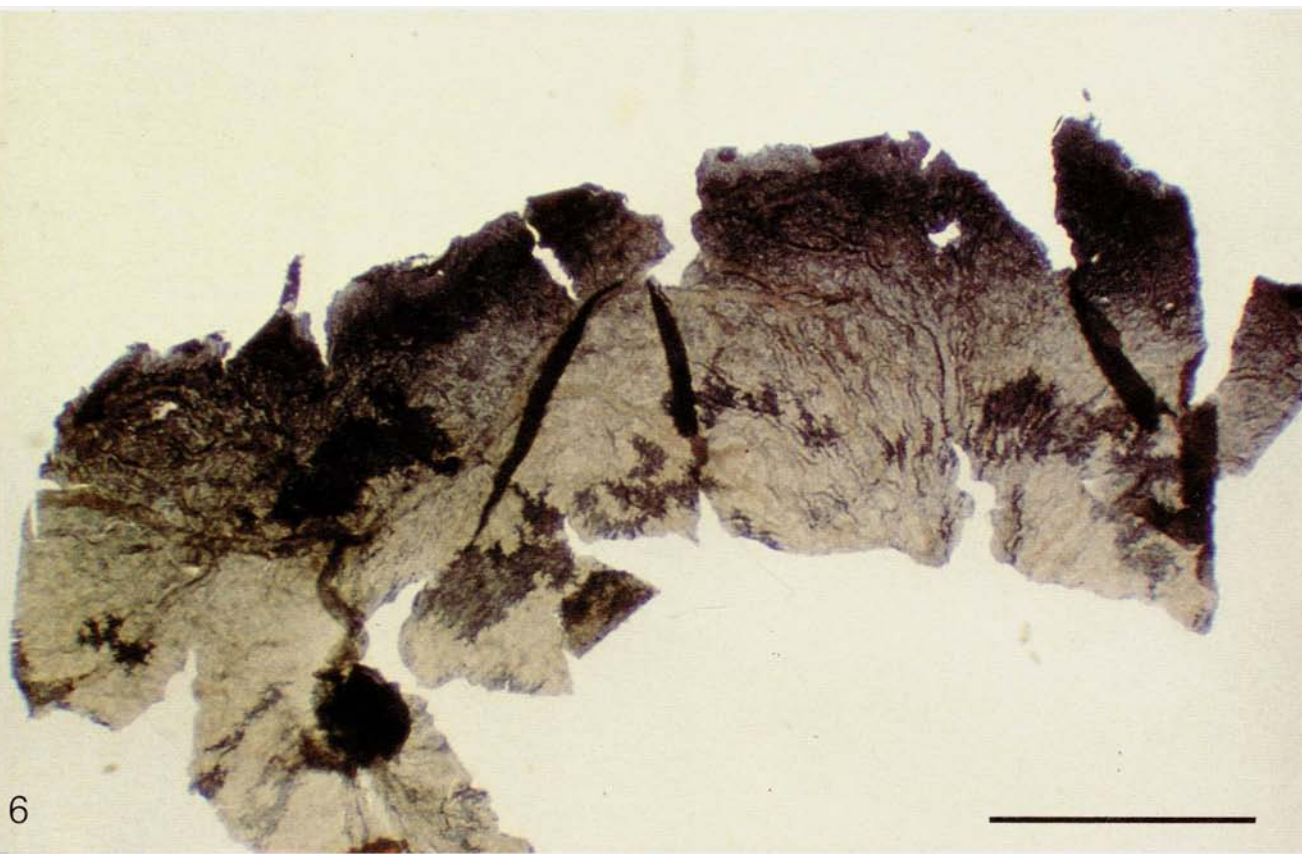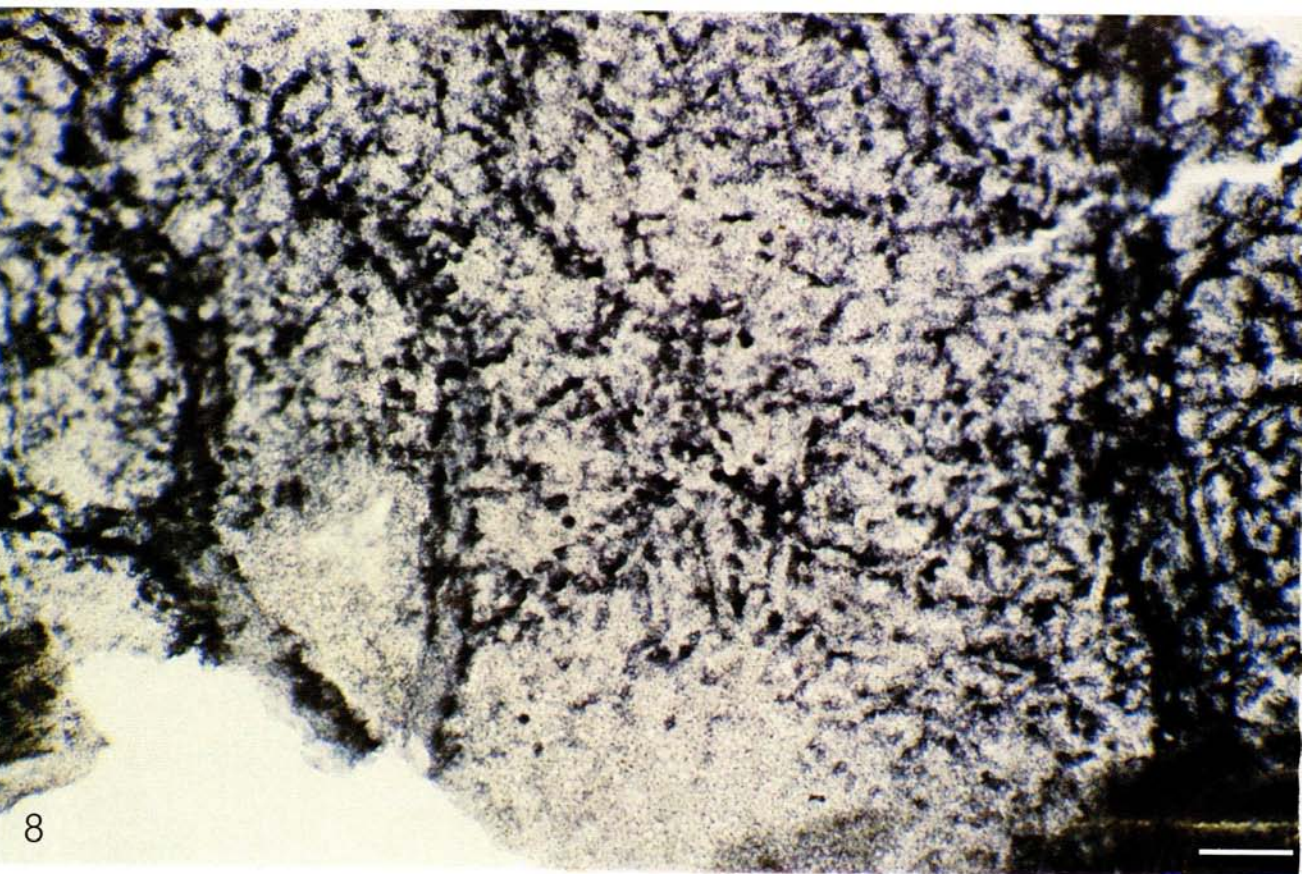
The membranes of conceptuses derived from injected blastocysts could be assigned to one of two distinct categories after staining. They were either a very conspicuous mosaic of stained and unstained cells or indistinguishable from those of null controls in appearance. The proportion of these experimental conceptuses showing histochemical mosaicism in the visceral yolk sac and/or parietal endoderm was similar to that of extraembryonic endodermal chimaeras found in previous single primitive endoderm cell injections using GPI allozymes as markers (Table 6 and Gardner, 1982). Most interestingly, the spatial arrangement of the stained cells was strikingly different in the two membranes. In all 36 cases in which such cells occurred in the capsular parietal endoderm they were intimately intermixed with unstained cells regardless of their location within the tissue (Fig. 5). Furthermore, as indicated in Tables 7 and 8, the stained cells were more abundant distally than proximally in most of the mosaic specimens irrespective of whether they were disseminated throughout the membrane or confined to a limited part of it. In contrast, unequivocally stained cells were arranged in one or more irregularly shaped patches in the three cases in which they were detected in the visceral yolk sac in addition to the parietal endoderm (Table 6 and Fig. 6). Except in parts of the proximal villous region, the distinction between stained and unstained cells was extremely sharp wherever they confronted one another in these visceral yolk sac preparations. Likewise, the differential staining of cells in mosaic parietal endoderms was as marked in areas where one class of cell predominated as in those where the other did so.

The proportion of stained cells in the three positive visceral yolk sacs was estimated visually to be 15 % or less. No attempt was made to obtain corresponding visual estimates for parietal endoderm because, as noted earlier, stained cells were not arranged in patches in this tissue and they typically showed considerable regional variation in frequency. Nevertheless, the contribution of stained cells to this membrane was clearly very variable, ranging from a sparse smattering on the one hand to cases in which they rivalled unstained cells in abundance on the other (Table 7).

A smaller series of reciprocal injection experiments was also carried out in which single 5th day $Mod-1^n/Mod-1^n$ primitive endoderm cells were introduced into $Mod-1^+/Mod-1^+$ blastocysts. Histochemical staining of parietal endoderms and visceral yolk sacs of the resulting conceptuses was compared with that of corresponding $Mod-1^+/Mod-1^+$ controls. (Incubation of the visceral yolk sacs in

---

Fig. 8. Part of the histochemically mosaic visceral yolk sac from an 11th day null conceptus (4IDCP7) that received a daughter pair of wild-type early ICM cells at the blastocyst stage. Note the diffuseness of staining in this specimen compared to that shown in Fig. 7. Bar = $100 \mu m$.

Fig. 9. Entire histochemically mosaic visceral yolk sac from a 12th day null conceptus (4IDCP10) that received a daughter pair of wild-type early ICM cells at the blastocyst stage. Both 'diffuse' and 'coherent' patches of staining are clearly discernible in this specimen. Bar = 1 mm.

Table 6. *Summary of results of cloning single primitive endoderm cells from 5th day blastocysts in 4th day blastocysts using MOD-1 as a cell marker*

Genotype of donor blastocysts	Genotype of host blastocysts	No. of injected blastocysts transferred to recipients that became pregnant*	Total No. of implantations (%)	No. of normal conceptuses (% of total implantations)	No. of chimaeras among normal conceptuses (% of normal conceptuses)	Classification of chimaeras according to distribution of mosaic staining for malic enzyme activity		
						No. with chimaerism in parietal endoderm only (% total chimaeras)	No. with chimaerism in both parietal and visceral endoderm (% total chimaeras)	No. with chimaerism in visceral endoderm only (% total chimaeras)
$Mod-1^+/Mod-1^+$	$Mod-1^n/Mod-1^n$	79**	60(76)+	59(92)	36(65)	33(92)	3(8)	0(0)
$Mod-1^n/Mod-1^n$	$Mod-1^+/Mod-1^+$	15	13(87)	10(77)	9(90)	9(100)	0(0)++	0(0)

* Six $Mod-1^n/Mod-1^n$ host blastocysts were transferred to a further recipient that did not become pregnant.
** Six of these 79 blastocysts were transferred to one oviduct of a female on the first day of pseudopregnancy because of a temporary shortage of day 3 recipients. The resulting conceptuses were recovered 10 rather than 8 or 9 days later to compensate for the delay in implantation that blastocysts suffer in such circumstances.
+ Four of these implantation sites were classified as resorptions. A fifth contained a conceptus which, despite exhibiting some stained cells in the parietal endoderm, was discounted because it was clearly non-viable.
++ Scoring of whole-mount preparations of $Mod-1^+/Mod-1^+$ visceral yolk sacs for the presence of unstained cells in the endodermal layer is complicated by the presence of staining throughout the adjacent mesoderm. Hence, while no unequivocal cases of chimaerism were encountered in this series of visceral yolk sacs, one cannot altogether exclude the possibility of a minor donor cell contribution.

Table 7. *Contribution of donor cells to proximal versus distal fields in capsular parietal endoderms* ⁺ *colonized by primitive endoderm or early ICM cell clones*

Clone No.	Donor → host genotype*	Donor cell type**	Proximal field count			Distal field count			Relative donor contribution⁺
			No. of donor cells	Total no. of cells	% donor cells	No. of donor cells	Total no. of cells	% donor cells	
5RM1	+ → n	R(s)	19	721	2·6	157	657	23·9	d > p
5RM2	+ → n	R(s)	28	554	5·1	160	545	29·4	d > p
5RM3	+ → n	R(s)	8	258	3·1	93	383	24·3	d > p
5RM4	+ → n	R(s)	23	704	3·3	79	488	16·2	d > p
5RM5	+ → n	R(s)	12	375	3·2	48	605	7·9	d > p
5RM6	+ → n	R(s)	112	813	13·8	202	725	27·9	d > p
5RM8	+ → n	R(s)	5	325	1·5	77	324	23·8	d > p
5RM9	+ → n	R(s)	49	490	10·0	237	586	40·4	d > p
5RM11	+ → n	R(s)	180	491	36·7	459	743	61·8	d > p
5RM14	+ → n	R(s)	119	536	22·2	95	432	22·0	d ⩽ p
5RM26	+ → n	R(s)	203	481	42·2	228	457	49·9	d > p
5RM36	+ → n	R(s)	117	409	28·6	84	415	20·2	p > d
5RM37	− → +	R(s)	28	273	10·3	290	477	60·8	d > p
5RM39	− → +	R(s)	71	202	35·1	156	232	67·2	d > p
5RM40	− → +	R(s)	60	129	46·5	31	204	15·2	p > d
5RM41	− → +	R(s)	21	153	13·7	58	275	21·1	d > p
5RM42	− → +	R(s)	263	380	69·2	65	151	43·0	p > d
5RM44	− → +	R(s)	102	245	41·6	141	281	50·2	d > p
5RM45	− → +	R(s)	130	216	60·2	123	190	64·7	d > p
4IDCP8	+ → −	I(dcp)	210	365	57·5	11	247	4·5	p > d
4IDCP10	+ → −	I(dcp)	310	381	81·4	90	288	31·3	p > d

⁺These represent the specimens recorded in rows 1–3 of Table 8 which were technically suitable for proximal versus distal field counts.
* + = wild-type; n = null.
** R(s) = single primitive endoderm cell
I(dcp) = daughter pair of early ICM cells.

Table 8. *Proximal (p) versus distal (d) distribution of donor cells in chimaeric capsular parietal endoderms*

Series of experiments	No. d > p	No. d ≂ p	No. p > d	No. p only	No. unscorable[+]
Single wild-type primitive endoderm cells into null blastocysts	24	4	2	0	6
Single null primitive endoderm cells into wild-type blastocysts	6	0	2	0	1
Single daughter pairs of wild-type early ICM cells into null blastocysts	0	0	3	2	0
2–3 singleton wild-type early ICM cells into null blastocysts	0	2	3	0	0

* Specimens excessively folded or torn so that distinction between proximal and distal region not clear.

PBS at 37 °C following fixation, which resulted in selective elimination of staining throughout null specimens (Fig. 4), was introduced at this stage in the investigation.) The proportion of conceptuses developing from injected blastocysts that exhibited unequivocal mosaicism in the parietal endoderm was even higher than in the initial series of experiments (Table 6). Furthermore, both the pattern of distribution and range of contributions of unstained cells in this tissue was comparable with that of stained cells in the reciprocal injection experiments (Tables 7 and 8). However, in no case did progeny of the transplanted null cells appear to have colonized the visceral yolk sac (Table 6). It is obviously more difficult to discern null cells in the endodermal layer of whole-mounted wild-type host visceral yolk sacs than *vice versa* because of the presence of staining throughout the adjacent mesoderm. Nevertheless, had patches of null cells been present they should have been detectable, especially in view of the fact that the yolk sacs were incubated in PBS at 37 °C for approximately 24 h prior to staining.

It was clearly necessary to obtain further cases of mosaicism in the visceral yolk sac in order to establish whether the pattern of distribution of stained versus unstained cells in this membrane differed consistently from that in the parietal endoderm. However, transplanting primitive endoderm cells between blastocysts is evidently a very inefficient way of obtaining visceral as opposed to parietal colonization (Table 6; also see Gardner, 1982). Hence, in the final series of injection experiments, donor cells were taken from the ICMs of 4th day $Mod-1^+/Mod-1^+$ blastocysts and injected into $Mod-1^n/Mod-1^n$ blastocysts of similar post-coital age in an attempt to increase the frequency of visceral yolk sac mosaicism (Gardner, 1981). This strategy was successful, as shown in Table 9, regardless of whether host blastocysts each received two or three single cells or a daughter cell pair.

Table 9. *Summary of results of injecting ICM cells from 4th day* Mod-1+/Mod-1+ *blastocysts into 4th day* Mod-1ⁿ/Mod-1ⁿ *blastocysts*

No. of cells injected/ blastocyst	No. injected blastocysts trans- planted*	No. of implanta- tions (%)	No. of normal conceptuses (% of total implanta- tions)	No. of chimaeras among normal conceptuses (% of normal conceptuses)	Classification of chimaeras according to distribution of mosaic staining for malic enzyme activity. (As verified histologically endodermal chimaerism could be recognized by 'coherent' and mesodermal chimaerism by 'diffuse' staining in whole-mounted visceral yolk sacs.)				
					No. (%) with chimaerism in parietal endoderm only	No. (%) with chimaerism in visceral endoderm only	No. (%) with chimaerism in parietal and visceral endoderm only	No. (%) with chimaerism in parietal endoderm, visceral endoderm and visceral mesoderm++	No. (%) with chimaerism in visceral mesoderm only++
1 daughter cell pair	24	22(92)+	14(64)	11(79)	0(0)	0(0)	2(18)	3(27)	6(55)
2 singleton cells	6	6(100)	6(100)	2(33)	0(0)	0(0)	1(50)	0(0)	1(50)
3 singleton cells	8	8(100)	6(75)	6(100)	0(0)	0(0)	1(17)	3(50)	2(33)

* All recipient mice became pregnant.
+ Four implantation sites were classified as resorptions. A further four contained very retarded conceptuses, one of which clearly exhibited stained cells in the parietal endoderm. These conceptuses, three of which were recovered from one recipient, were therefore classified as abnormal.
++ The amnion was also studied in those conceptuses in which part or all of it remained attached to the visceral yolk sac. A precise correspondence was noted between the visceral mesoderm and amnion regarding presence versus absence of unequivocally stained cells in all cases in which both were examined.

Two distinct patterns of staining could clearly be resolved in histochemically mosaic yolk sacs with these younger donor cells. The first pattern consisted of irregular coherent patches of staining (= 'coherent' pattern) closely resembling that seen earlier in the three cases of colonization of null yolk sacs by wild-type primitive endoderm cells (e.g. compare Figs 6 and 7). The second pattern was a more granular and diffuse one (= 'diffuse' pattern) also occurring in irregular patches, through which vitelline blood vessels could be readily discerned (Fig. 8). Some yolk sacs exhibited only one or other pattern of staining. However, both patterns were evident in other specimens, including three obtained following transplantation of daughter cell pairs (Table 9). When they occurred in the same yolk sac the two patterns bore no consistent spatial relationship to each other (Fig. 9). Presence of the 'coherent' pattern of staining in the visceral yolk sac was invariably accompanied by the occurrence of stained cells in the corresponding parietal endoderm (Table 9). In contrast, stained cells were absent from the capsular parietal endoderms of all conceptuses exhibiting only the 'diffuse' pattern of staining in their visceral yolk sacs (Table 9). Fragments of amnion often remained attached to visceral yolk sacs following their isolation. Histochemical mosaicism was apparent in such fragments in all cases in which 'diffuse' staining was present in the corresponding visceral yolk sac (see footnotes to Table 9). In conjunction with the primitive endoderm cell injection data (Table 6), these findings suggested that the 'coherent' pattern was due to staining in the endoderm and the 'diffuse' pattern to staining in the mesodermal layer of this organ. This was indeed confirmed by comparing the distribution of staining between the two layers in sections of pieces of yolk sac excised from regions exhibiting one or other pattern relative to that in corresponding wild-type and null controls (see Figs 10–13).

All specimens of parietal endoderm that displayed mosaicism in the early ICM cell injection series showed a similar degree of intermixing of stained and unstained cells to that encountered in the experiments with primitive endoderm cells (Fig. 14). However, whereas the progeny of donor primitive endoderm cells were typically more abundant distally than proximally, those of early ICM cells were located primarily or exclusively in the proximal region of the tissue in most cases (Tables 7 and 8).

Mixed parietal endoderm cell cultures

Dissociated null and wild-type cells were mixed thoroughly and then seeded

---

Figs 10–13. Sections through stained 12th day visceral yolk sacs with the endodermal layer uppermost. Bar = 75 μm. Fig. 10. Wild-type control showing staining in both the endoderm and mesoderm. Fig. 11. Null control showing absence of staining in both layers. Fig. 12. Histochemically mosaic yolk sac with 'coherent' pattern of staining in which stained cells are confined to the endoderm layer. Fig. 13. Histochemically mosaic yolk sac with 'diffuse' pattern of staining in which stained cells are confined to the mesoderm layer.
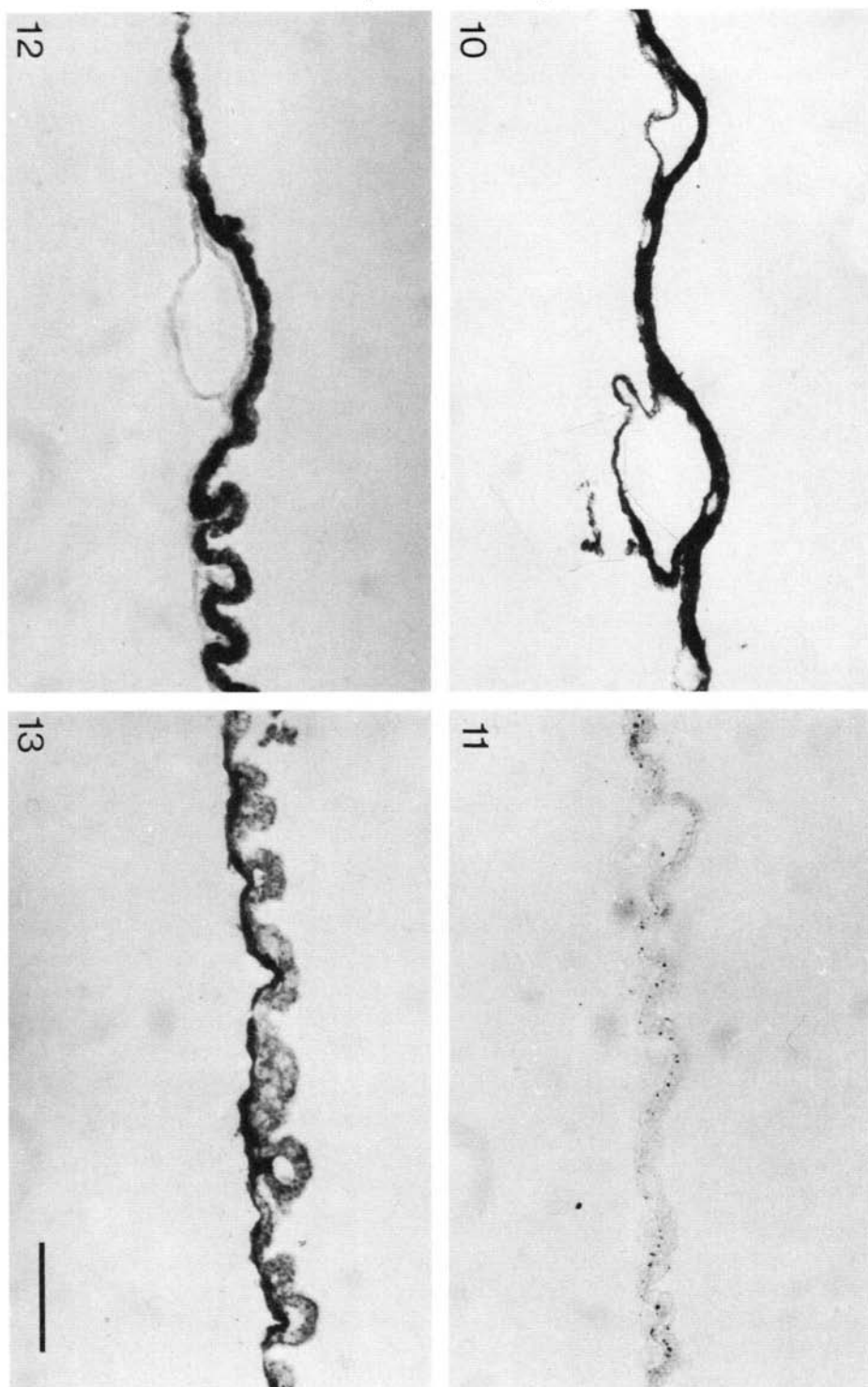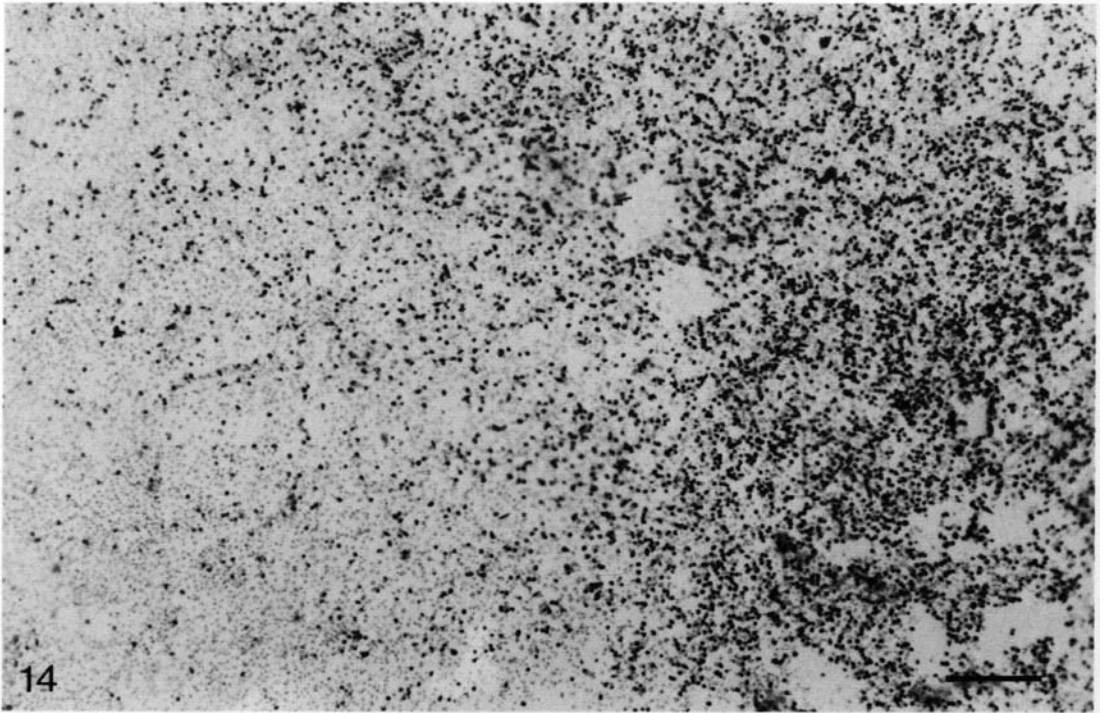
Figs 10–13

Fig. 14. Part of the parietal endoderm from the conceptus (4IDCP8) whose visceral yolk sac is illustrated in Fig. 9. The proximal region of the membrane is to the right and the distal region to the left. Although stained cells are much more abundant proximally than distally, mosaicism is clearly very fine-grained throughout the field. Bar = 200 μm.

locally on a marked fragment of coverslip (Fig. 1) at high concentration in order to mimic as closely as possible their density on Reichert's membrane. By ensuring that all members of one or other population in the mixture contained melanin granules, it was possible to determine the fidelity with which the phenotype of cells following staining for malic enzyme activity reflected their genotype at the *Mod-1* locus (Figs 15–18). The results of this comparison are presented in Table 10. Approximately 1 % or less of null cells were misclassified as wild-type cells on the basis of their histochemical phenotype in short-term

Fig. 15. Part of a mixed parietal endoderm cell culture with melanin granule labelled wild-type cells.

Fig. 16. Same field as Fig. 15 after staining. Note the correspondence between labelling and histochemical staining of cells.

Fig. 17. Part of a mixed parietal endoderm cell culture with melanin granule labelled null cells.

Fig. 18. Same field as Fig. 17 after staining. Note the inverse relationship between labelling and histochemical staining of cells. Bar in Figs 15–17 = 100 μm.
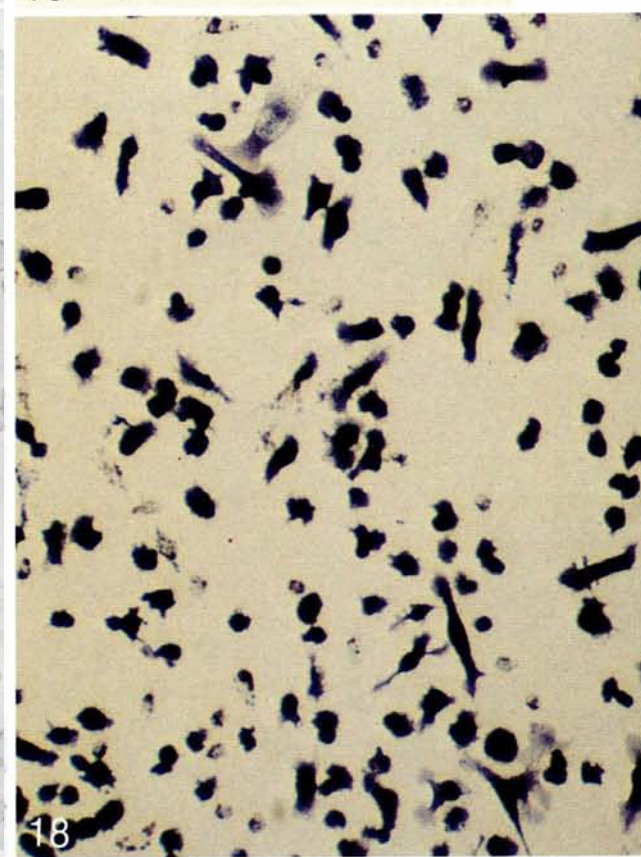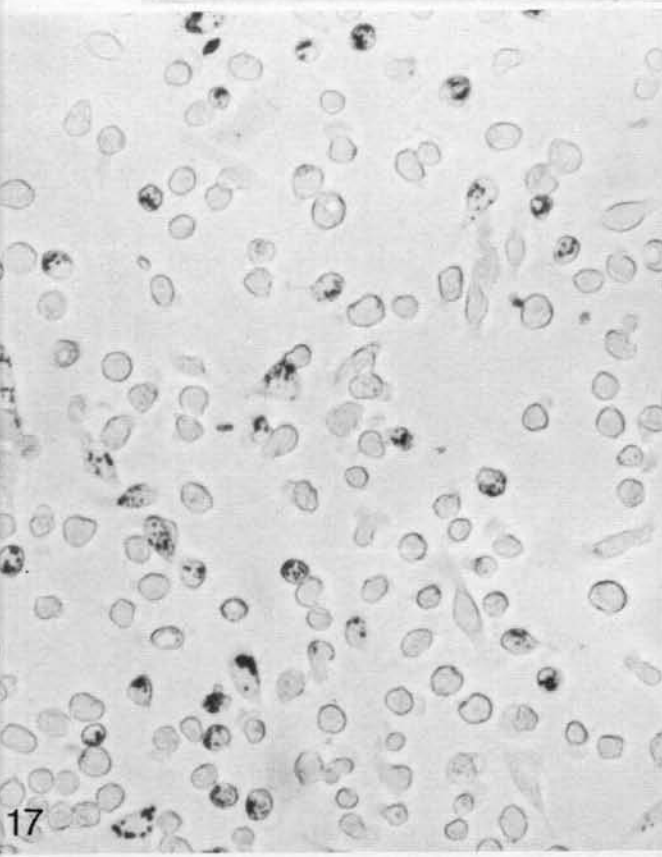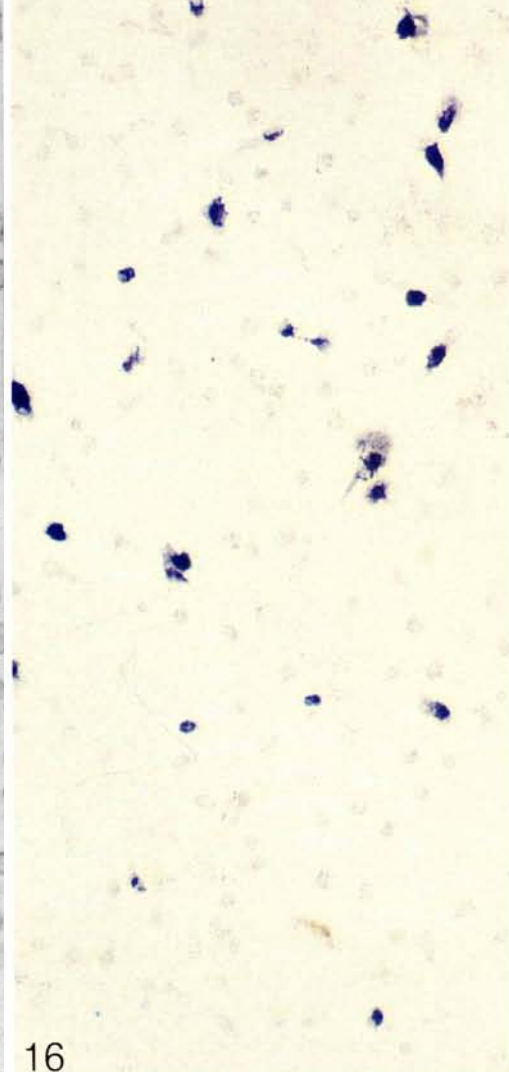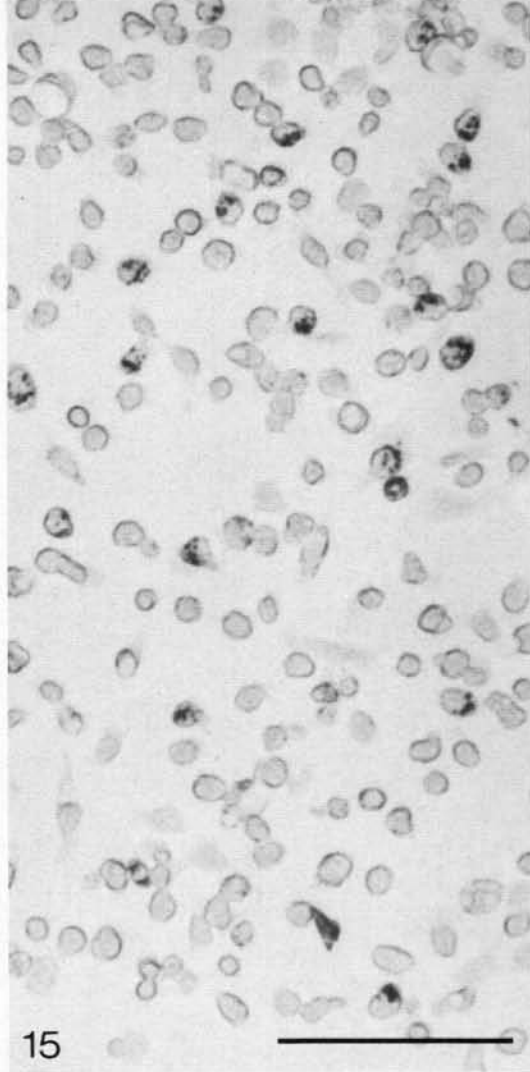
15

16

17

18

Table 10. *Staining for malic enzyme activity of mixed parietal endoderm cell cultures using melanin granules as an independent marker*

	Experiment No.	Genotype/melanin granule status of cells[+]	Duration of culture h	Total cells scored	Staining for malic enzyme activity			
					No. stained	No. unstained	No. ? stained	% inappropriately stained
NBT[++] incubation medium	1	Wild type/mg⁻	ca. 2½	941	896	45	0	4·8
		Null/mg⁺		59	0	58	1	0·0
	2	Wild type/mg⁻	ca. 2½	911	901	10	0	1·1
		Null/mg⁺		89	1	87	1	1·1
	3	Wild type/mg⁺	ca. 2½	120	95	25	0	20·8
		Null/mg⁻		880	1	879	0	0·1
	4	Wild type/⁺	ca. 2½	203	191	12	0	5·9
		Null/mg⁻		797	1	796	0	0·1
	5	Wild type/mg⁺	ca. 16	175	167	8	0	4·6
		Null/mg⁻		825	0	825	0	0·0
	6	Wild type/mg⁺	ca. 16	111	103	8	0	7·8
		Null/mg⁻		889	0	889	0	0·0
	7	Wild type/mg⁺	ca. 2½	255	216	39	0	15·3
		Wild type/mg⁻		745	719	26	0	3·5**
	8	Wild type/mg⁻	ca. 2½	839	823	16	0	1·9
		Null/mg⁺		161	1	160	0	0·6***
TNBT* incubation medium	9	Wild type/mg⁻	ca. 2½	832	816	16	0	1·9
		Null/mg⁺		168	6	162	0	3·6***
	10	Wild type/mg⁺	ca. 2½	160	157	3	0	1·9
		Null/mg⁻		840	21	819	0	2·6***

[+] Cells labelled with melanin granules are denoted as mg⁺ and those not labelled as mg⁻.
[++] Specimens were incubated in NBT medium for approximately 48 h.
* Period of incubation of cultures 8, 9 and 10 in TNBT medium was 15 h, 22½ h and 20½ h respectively.
** $\chi^2 = 43\cdot55$; $P < 0\cdot001$.
*** Not significantly different. However, the proportion of null cells that were stained in experiments 9 and 10 was significantly higher than in null whole-mount controls; $\chi^2 = 34\cdot6$ and $36\cdot6$, respectively: $P < 0\cdot001$.

mixed cultures, irrespective of whether they had been labelled with melanin granules or not (Table 10, experiment 1–4). No null cells were misclassified in the two experiments in which the period of co-culture of the cells was increased from approximately $2\frac{1}{2}$ to 16 h (Table 10, experiments 5 and 6). The data for wild-type cells are less straightforward because the proportion that failed to stain, and was therefore misclassified as null cells, tended to be higher when they constituted the melanin granule-labelled as opposed to the unlabelled population (compare experiments 1 and 2 with 3–6 in Table 10). One obvious explanation for this discrepancy is that labelling with melanin granules can interfere with the subsequent histochemical staining of wild-type cells. This possibility was supported by analysis of a further coverslip culture consisting of a mixture of unlabelled and melanin granule-labelled wild-type cells. The proportion of unstained cells was significantly higher in the labelled than in the unlabelled population in this culture (Table 10, experiment 7).

Staining of wild-type versus null tissues with TNBT incubation medium

Although NBT-containing incubation medium (Table 4) gave excellent discrimination between wild-type and null parietal and visceral endoderm from 10th–12th day conceptuses, it yielded poor and somewhat erratic differential staining in these tissues at earlier stages of development. This was manifest as an increased tendency towards punctate rather than diffuse staining in wild-type cells combined with failure of an unacceptable proportion of them to show greater deposition of diformazan than in nulls. The effect of using TNBT, a similar but somewhat more readily reduced tetrazolium (Altman, 1972; Pearse, 1972), instead of NBT was therefore examined. Despite producing a higher level of staining in null cells than NBT (compare Figs 19 and 20 with Figs 2 and 3), use of this tetrazolium ensured satisfactory differential staining in both the parietal and visceral endoderm at all stages investigated. A clear difference between wild-type and null embryos in staining of extraembryonic ectoderm, ectoplacental cone tissue and trophoblastic giant cells could be obtained using either NBT or TNBT. However, differential staining in the embryonic ectoderm was only seen consistently with TNBT, and was invariably less marked than in all other tissues of the early postimplantation embryo. The diformazan of TNBT tended to be distributed more uniformly throughout the cytoplasm of wild-type parietal endoderm cells than NBT and to render a higher proportion of such cells completely opaque (cf. Figs 2 and 19).

Staining was seen in only 7/2465 (= 0·28 %) cells in 12th day specimens of null parietal endoderm incubated in TNBT medium. The proportion of non-staining

Fig. 19. Part of a 12th day wild-type preparation of parietal endoderm stained with TNBT-containing incubation medium. Bar = 50 µm.

Fig. 20. Part of a 12th day null preparation of parietal endoderm treated identically to the wild-type specimen shown in Fig. 19. Bar = 50 µm.

Figs 19 & 20

R. L. GARDNER
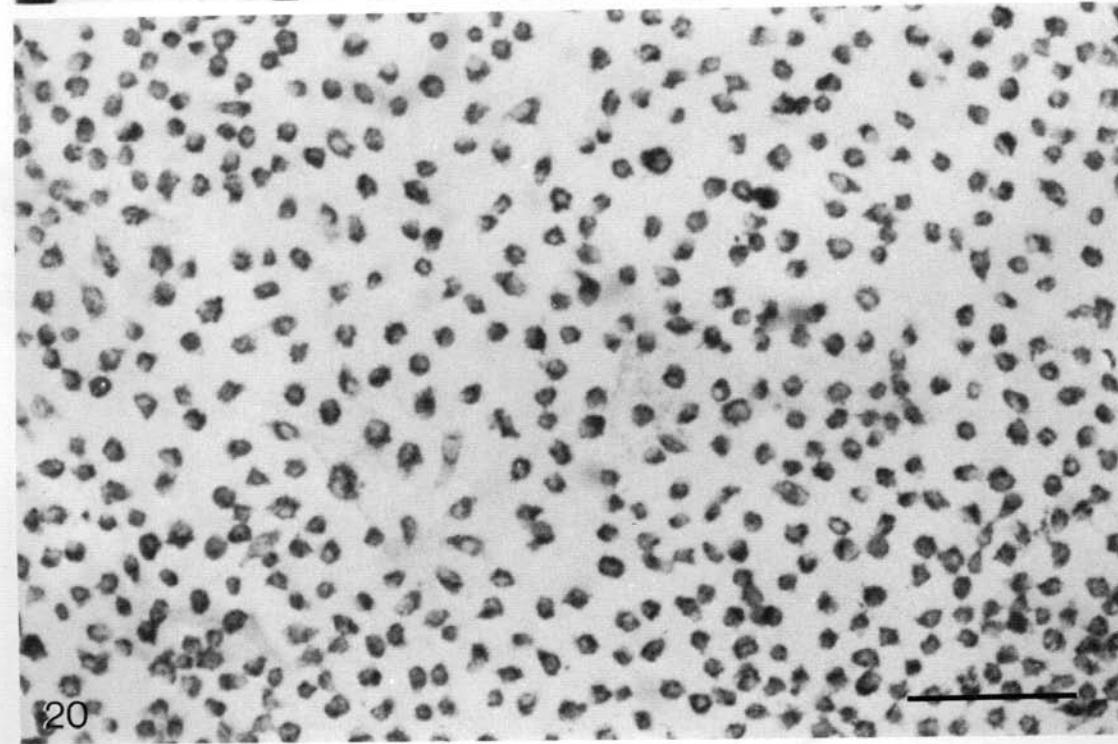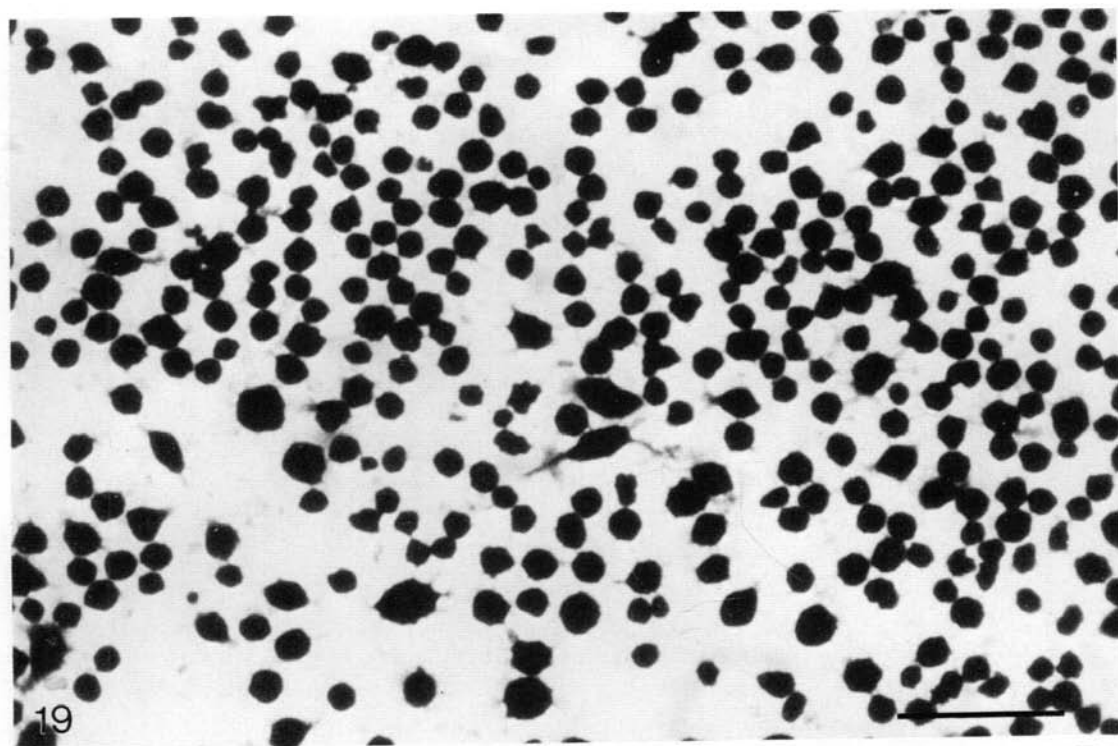
cells in corresponding wild-type specimens was 42/2200 (= 1·91 %). The in-
cidence of inappropriately stained cells in mid-gestation parietal endoderm using
this tetrazolium is therefore very similar to that found with NBT (see Table 5).
Furthermore, as in the case of NBT medium, no obvious elevation of back-
ground was seen in regions of null specimens which were in contact with wild-
type tissue throughout staining. However, somewhat different results were
obtained with TNBT as opposed to NBT medium for staining mixed parietal
endoderm cell cultures (Table 10, cf. experiments 1–7 and 8–10). The frequency
with which wild-type cells failed to stain was the same in these cultures as in
whole-mount control specimens (ca. 1·9 %, as noted above), regardless of
whether they were labelled with melanin granules or not. In contrast, the
frequency with which null cells stained above background was appreciably higher
in two of the three cultures (Table 10, experiments 9 and 10) than in correspond-
ing whole-mount controls. This appeared to be related to the duration of staining
of the cultures rather than the melanin granule status of the null cells.

DISCUSSION

Conditions have been found for staining whole-mount preparations of cap-
sular parietal endoderm and visceral yolk sac for malic enzyme activity that give
excellent discrimination between endoderm cells in wild-type specimens versus
mutant specimens that lack the cytoplasmic form of this enzyme. More than 98 %
of endoderm cells in preparations of wild-type membranes stained unequivocally
above background under these conditions, whereas in corresponding mutant
preparations less than 0·3 % did so. When treated similarly, one or both extra-
embryonic membranes from a substantial proportion of conceptuses developing
from blastocysts of one genotype injected with one or more cells of the opposite
genotype consisted of an impressive mosaic of stained and unstained cells.
Evidence that such mosaicism was due to the presence of both mutant and wild-
type cells in such membranes is as follows:

1. Though variable, the level of histochemical mosaicism in these membranes
was much higher than in any corresponding controls from conceptuses which had
developed from uninjected wild-type or mutant blastocysts.

2. The distribution of mosaicism within and between membranes that was
seen with primitive endoderm versus early ICM donor cells corresponded with
that found in earlier experiments in which allòzymes of GPI were used as cell
markers (Gardner & Rossant, 1979; Gardner, 1981, 1982; J. Rossant & R. L.
Gardner, unpublished observations). Thus, in the single primitive endoderm cell
injections, mosaicism could occur in both the visceral and parietal endoderm but
was confined to the latter in most cases. Furthermore, cells within the parietal
endoderm exhibiting the histochemical phenotype characteristic of the donor
were more abundant distally than proximally in the majority of cases (Table 8).
Visceral yolk sac mesoderm was never mosaic in conceptuses obtained in these

experiments. In contrast, mosaicism could occur in either the extraembryonic endoderm or visceral mesoderm plus amnion or, indeed, in all these tissues following transplantation of daughter pairs of early ICM cells. In all cases in which mosaicism was detected in the extraembryonic endoderm in early ICM cell injection experiments, it could clearly be discerned in both the parietal and visceral cell layers.

The above findings provide compelling grounds for equating mosaic patterns of staining with the presence of chimaerism in extraembryonic tissues. Nevertheless, one cannot assume that the histochemical phenotype of cells will necessarily be as closely correlated with their genotype in chimaeric specimens as in mutant versus wild-type controls. Transfer of enzyme molecules between living cells (Feder, 1976; Herrup & Mullen, 1979; Olsen *et al*. 1983) and diffusion of reduced co-enzyme or intermediate electron acceptor molecules during staining (Leeflang-de Pijper & Hulsmann, 1974) are two possible ways in which the correlation might be reduced in chimaeras. Operation of either of these factors would be expected to result in the occurrence of phenotypically intermediate cells in areas in which unequivocally stained cells were concentrated. In fact, no such cells are seen in either the predominantly stained areas in parietal endoderm or adjacent to coherent patches of staining in visceral endoderm (e.g. Figs 5, 6, 7, 9 and 14). Rather, cells in chimaeric extraembryonic endoderm typically resemble those that are characteristic of either mutant or wild-type controls in staining, regardless of their location.

Obviously, in order to test the efficacy of staining for malic enzyme as a marker in chimaeric situations it is essential to have an independent means of reliably distinguishing between mutant and wild-type cells *in situ*. In the absence of a way of achieving this *in vivo* staining was studied in mixed cultures of parietal endoderm in which all cells of one or other genotype had previously been labelled with melanin granules. Whether labelled or not, mutant cells were misclassified in such cultures with a similarly low frequency to that with which they stained above background in whole-mount controls using NBT incubation medium (cf. Tables 5 and 10). This was so even if the period for which they were co-cultured with wild-type cells was increased from $2\frac{1}{2}$ to approximately 16 h. Wild-type cells, in contrast, were usually misclassified more frequently in the mixed cultures when they constituted the labelled as opposed to the unlabelled population. This appears from the results obtained with cultures of labelled and unlabelled wild-type cells to be attributable to an adverse effect of melanin granules on histochemical staining. However, even when unlabelled, wild-type cells usually failed to stain in a higher proportion of cases than in corresponding whole-mount controls, suggesting that *in vitro* manipulation was also having an effect.

The difference in histochemical phenotype between $Mod\text{-}1^+/Mod\text{-}1^+$ and $Mod\text{-}1^n/Mod\text{-}1^n$ genotypes appears to behave cell autonomously, and therefore to provide a reliable *in situ* marker system for use in chimaeras. However, exploitation of the marker for studying cell lineage and deployment during

development obviously requires that the absence of cytoplasmic malic enzyme from one constituent cell population in a chimaera does not lead to distortion of these processes. This is very difficult to ascertain, particularly since the normal physiological role of the enzyme is not entirely clear (Frenkel, 1975). Nevertheless, in view of its probable metabolic roles, absence of the enzyme is more likely to manifest itself through effects on viability rather than via more specific developmental perturbation. Unlike the mouse, *Drosophila* appears to possess only a single locus coding for malic enzyme (Geer, Krochko & Williamson, 1979). Using various null alleles from natural populations, it has been found that flies exhibiting little or no activity of the enzyme are nevertheless viable, fertile and devoid of any obvious abnormalities (Voelker *et al.* 1981). In mice, a deficit of offspring carrying the null allele at the locus coding for the cytoplasmic enzyme was reported in certain matings between heterozygotes, and it appeared to have some association with sex (Lee *et al.* 1980*a*; Johnson *et al.* 1981). However, the sex ratio is normal in the mutant stock used in the present experiments, and its mean litter size comparable with that of $Mod-1^+/Mod-1^+$ mice which have a related genetic background (J. Green, personal communication). Hence, the possibility that viability is somewhat reduced in organisms carrying the mutant allele has yet to be substantiated. Even if this is found to be the case, it does not necessarily follow that the viability of mutant cells will be similarly affected in chimaeras (Lawrence, 1981). The cloning efficiency of mutant primitive endoderm cells injected into wild-type blastocysts was clearly not inferior to that of wild-type cells injected into mutant blastocysts (Table 6). In addition, there was no consistent difference in distribution of cells or in size range between mutant and wild-type clones in these reciprocal injection experiments. It is evident from studies that are in progress that the higher density and generally smaller size of parietal endoderm cells in mid-gestation mutant than wild-type embryos seen in the present experiments (see Table 5 and Figs 2 and 3) is related to genetic background rather than presence of the null allele (author's unpublished observations). Hence, there are no obvious grounds for suspecting that the deployment of cells in different tissues visualized with the aid of this marker system might be anomalous.

Detailed morphometric analysis of the clones obtained in the present cell injection experiments is still in progress. Therefore, only certain of their more general features will be discussed here, principally in relation to the parietal and visceral endoderm. Donor cells were typically arranged in one or more irregularly shaped patches in the visceral endoderm regardless of their location. Such coherent growth is consistent with the retention of the character of a continuous epithelium by this tissue throughout postimplantation development (Duval, 1892). In marked contrast, an extraordinary degree of admixture of donor and host cells was invariably seen in the parietal endoderm. It is worth emphasizing that this striking difference in arrangement of donor cells in the two layers of extraembryonic endoderm was also seen in every case in which progeny of single

transplanted primitive endoderm cells or daughter pairs of early ICM cells had colonized both tissues. Hence, it must reflect fundamental differences in growth characteristics of the tissues rather than the clones.

Nevertheless, the distribution of clonal descendants of early ICM cells that colonized the extraembryonic endoderm was typically different from that of primitive endodermal clones in both the present and previous experiments (Gardner, 1981, 1982; J. Rossant & R. L. Gardner, unpublished observations). Whether transplanted as singletons or daughter pairs, early ICM cells consistently colonized both the visceral and parietal endoderm and tended, furthermore, to be concentrated proximally in the latter tissue. Primitive endodermal clones, on the other hand, were confined to the parietal endoderm only in most cases where they showed a marked preference for colonization of the distal rather than proximal region of this tissue. The magnitude of this bias may in some cases have been somewhat greater than suggested in Table 7, because these data represent comparison between a distal field and a field from that part of the much more extensive proximal region showing the highest proportion of donor cells.

A scheme for the differentiation of endoderm in the late blastocyst has been proposed which provides an explanation for these disparate results (Gardner, 1982). It is based on the assumption that endoderm cells must retain contact with the underlying ectoderm in order to differentiate as visceral endoderm; loss of contact is presumed to trigger parietal differentiation. When primitive endoderm cells divide only one daughter is believed usually to retain contact with the ectoderm because the interface between the two tissues does not expand until somewhat later in development. Consequently, individual primitive endoderm cells would normally be expected to contribute progeny to both the parietal and visceral endoderm. The behaviour of transplanted early ICM cells which undergo endodermal differentiation is thus attributable to their being integrated efficiently into the incipient primitive endoderm layer in host blastocysts. Primitive endoderm cells are presumed to integrate much less readily following isolation and therefore usually become the first and hence the most distally migrating parietal endoderm cells. The trend towards preferential colonization of the proximal parietal endoderm by early ICM cells may simply be due to delay in division of donor relative to host cells in synchronous injection experiments. Clearly, further experiments in which the location of donor cells is analysed shortly after transplantation are necessary in order to test the adequacy of this explanation for the different distribution of the two types of clones. Likewise, the question whether the high degree of cell mixing seen in midgestation parietal endoderm is due to lack of coherent growth or secondary disruption of such growth can only be answered by shorter-term experiments.

While the greater part of both the parietal and visceral endoderm of midgestation conceptuses was analysed for chimaerism in the present investigation, the endodermal sinuses (Duval, 1892), which include the junctional zone between the two tissues, were not. Attempts are being made to obtain satisfactory

whole-mount preparations of placentae since the pattern of cellular deployment in the sinuses may help to explain the different growth characteristics of the two extraembryonic endoderm cell layers.

Apart from those of blood, all cells in the mesodermal layer of wild-type visceral yolk sacs appear to stain for malic enzyme activity, although they may do so less intensely than corresponding endoderm cells. Providing background staining is minimized by adequate prior incubation of fixed yolk sacs in PBS at 37 °C, wild-type donor cells can be identified unequivocally in the mesoderm of host mutant specimens. It is perhaps not surprising that the donor cells seem to be more diffuse-ly distributed in this layer than in the adjacent endoderm because it is composed of a rather variable number of cell layers as opposed to a single one (Wislocki & Padykula, 1953). Nevertheless, the extent of cell mixing in visceral mesoderm is clearly much less than in parietal endoderm. Mosaicism was also discernible in fragments of amnion from conceptuses showing chimaerism in the visceral mesoderm. Stained cells appeared to be segregated more conspicuously from un-stained cells in the ectodermal than the mesodermal layer of this membrane, although this point requires confirmation on rather better preparations.

Blood cells are the only cells that have consistently failed to stain in wild-type as well as null conceptuses. Embryonic ectoderm has shown the weakest staining of all other wild-type tissues that have been examined so far. The possibility that differential staining is nevertheless adequate for studying patterns of chimaerism in this tissue is currently being explored. Lack of enzyme activity in wild-type cells may not be the only problem in applying this technique more widely. The slow rate at which glutaraldehyde penetrates tissues (Hayat, 1981) obviously imposes limitations on the thickness of specimens that can be fixed adequately by brief exposure to a dilute solution of this reagent. More importantly, it is not known why lowering the concentration of substrate results in selective elimina-tion of staining of mutant cells. This practice might prove to be effective, for example, only for cell types in which the mitochondrial enzyme makes a relative-ly modest contribution to total malic enzyme activity.

In spite of the above reservations, the possibility of extending use of the marker to other tissues, including those of the definitive embryo, merits close investiga-tion because of its applicability to intact cells. The great advantage of this approach is that it enables overall patterns of chimaerism to be studied without disruption of normal cellular relations in permanent preparations which can, if necessary, be dissected further or sectioned in order to resolve finer details. It thus avoids the difficult and time-consuming task of reconstructing such patterns from serial sections which is unavoidable with all other potential generalized *in situ* cell marking techniques that are currently available in the mouse.

sections available to me. I should like to thank Mrs Marilyn Carey and Miss Judy Green for technical assistance, Dr Michael Dunnill for advice, and Dr Rosa Beddington, Mr Stan Buckingham, Dr David Cockroft, Professor Henry Harris, Dr John West and Mrs Jo Williamson for help in preparing the manuscript. The work was supported by The Imperial Cancer Research Fund and the Royal Society.

REFERENCES

AHERNE, W. A. & DUNNILL, M. S. (1982). *Morphometry*. London: Edward Arnold.
ALTMAN, F. P. (1972). *An Introduction to the Use of Tetrazolium Salts in Quantitative Enzyme Cytochemistry*. Colnbrook, U.K.: Koch-Light Laboratories Ltd.
ATIENZA-SAMOLS, S. B. & SHERMAN, M. I. (1979). *In vitro* development of core cells of the inner cell mass of the mouse blastocyst: effect of conditioned medium. *J. exp. Zool.* **208**, 67–71.
BARTHOLOMÉ, K., BRDICZKA, D. G. & PETTE, D. (1972). Purification and properties of extra- and intra-mitochondrial malate dehydrogenase (NADP: decarboxylating) from pig heart. *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1487–1495.
BERNSTINE, E. G. (1979). Genetic control of mitochondrial malic enzyme in mouse brain. *J. biol. Chem.* **254**, 83–87.
BIGGERS, J. D., WHITTEN, W. K. & WHITTINGHAM, D. G. (1971). The culture of mouse embryos *in vitro*. In *Methods in Mammalian Embryology* (ed. J. C. Daniel), pp. 86–116. San Francisco: Freeman.
DIXON, M. & WEBB, E. C. (1964). *Enzymes 2nd Ed.* London: Longmans, Green & Co.
DUVAL, M. (1892). *Le Placenta des Rongeurs*. Paris: Ancienne Librarie Germer, Baillière et Fils.
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.
ENGEL, W. & WOLFE, U. (1971). Synchronous activation of the alleles coding for the S-form of the NADP-dependent malate dehydrogenase during mouse embryogenesis. *Humangenetik* **12**, 162–166.
FEDER, N. (1976). Solitary cells and enzyme exchange in tetraparental mice. *Nature* **263**, 67–69.
FLITNEY, F. W. (1966). The time course of the fixation of albumin by formaldehyde, glutaraldehyde, acrolein and other higher aldehydes. *J. Roy. microsc. Soc.* **85**, 353–364.
FRENKEL, R. (1975). Regulation and physiological functions of malic enzymes. In *Current Topics in Cellular Regulation Vol. 9* (eds B. L. Horecker & E. R. Stadtman), pp. 157–181. New York: Academic Press.
FRENKEL, R. & COBO-FRENKEL, A. (1973). Differential characteristics of the cytosol and mitochondrial isozymes of malic enzyme from bovine brain: effects of dicarboxylic acids and sulfhydryl reagents. *Arch. Biochem. Biophys.* **158**, 323–330.
GARDNER, R. L. (1968). Mouse chimaeras obtained by the injection of cells into the blastocyst. *Nature* **220**, 596–597.
GARDNER, R. L. (1978a). The relationship between cell lineage and differentiation in the early mouse embryo. In *Genetic Mosaics and Cell Differentiation* (ed. W. J. Gehring), pp. 205–241. Berlin: Springer-Verlag.
GARDNER, R. L. (1978b). Production of chimeras by injecting cells or tissue into the blastocyst. In *Methods in Mammalian Reproduction* (ed. J. C. Daniel), pp. 137–165. New York: Academic Press.
GARDNER, R. L. (1981). *In vivo* and *in vitro* studies on cell lineage and determination in the early mouse embryo. In *Cellular Controls in Differentiation* (eds C. W. Lloyd & D. A. Rees), pp. 257–278. London: Academic Press.
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, R. L. (1983). Origin and differentiation of extraembryonic tissues in the mouse. *Int. Rev. Exp. Path.* **24**, 63–133.

GARDNER, R. L. (1984). Mammalian chimaeras – future perspectives. In *Chimaeras in Developmental Biology* (eds N. Le Douarin & A. McLaren) (in press). New York: Academic Press.

GARDNER, R. L. & JOHNSON, M. H. (1973). Investigation of early mammalian development using interspecific chimaeras between rat and mouse. *Nature New Biol. Lond.* **246**, 86–89.

GARDNER, R. L. & JOHNSON, M. H. (1975). Investigation of cellular interaction and deployment in the early mammalian embryo using interspecific chimaeras between the rat and mouse. In *Cell Patterning. Ciba Foundation Symposium 29 (new series)*, pp. 183–200. Amsterdam: Elsevier, Excerpta Medica.

GARDNER, R. L. & PAPAIOANNOU, V. E. (1975). Differentiation in the trophectoderm and inner cell mass. In *The Early Development of Mammals: British Society for Developmental Biology Symposium 2* (eds M. Balls & A. E. Wild), pp. 107–134. Cambridge: Cambridge University Press.

GARDNER, R. L. & ROSSANT, J. (1979). Investigation of the fate of 4·5 day *post coitum* mouse inner cell mass cells by blastocyst injection. *J. Embryol. exp. Morph.* **52**, 141–152.

GEARHART, J. & OSTER-GRANITE, M. L. (1978). Antibodies to allozymes as potential cell markers for chimeric mouse studies. In *Genetic Mosaics and Chimeras in Mammals* (ed. L. B. Russell), pp. 111–122. New York: Plenum Press.

GEARHART, J. & OSTER-GRANITE, M. L. (1980). An immunofluorescence procedure for the tissue localization of glucosephosphate isomerase. *J. Histochem. Cytochem.* **28**, 245–249.

GEER, B. W., KROCHKO, D. & WILLIAMSON, J. H. (1979). Ontogeny, cell distribution and the physiological role of NADP-malic enzyme in *Drosophila melanogaster. Biochem. Genet.* **17**, 867–879.

GREEN, M. C. (1981). Catalog of mutant genes and polymorphic loci. In *Genetic Variants and Strains of the Laboratory Mouse* (ed. M. C. Green), pp. 166–167. Stuttgart: Gustav Fischer Verlag.

HAYAT, M. A. (1981). *Principles and Techniques of Electron Microscopy: Biological Applications.* London: Edward Arnold.

HERRUP, K. & MULLEN, R. J. (1979). Intercellular transfer of β-glucuronidase in chimeric mice. *J. Cell Sci.* **40**, 21–31.

JANNING, W. (1972). Aldehyde oxidase as a cell marker for internal organs in *Drosophila melanogaster. Naturwissenschaften* **59**, 516–517.

JOHNSON, F. M., CHASALOW, F., LEWIS, S. E., BARNETT, L. & LEE, C.-Y. (1981). A null allele at the *Mod-1* locus of the mouse. *J. Hered.* **72**, 134–136.

JOLLIE, W. P. (1968). Changes in the fine structure of the parietal yolk sac of the rat placenta with increasing gestational age. *Am. J. Anat.* **122**, 513–532.

KIRKWOOD, K. J. & BILLINGTON, W. D. (1981). Expression of serologically detectable H-2 antigens on mid-gestation mouse embryonic tissues. *J. Embryol. exp. Morph.* **61**, 207–219. ꞌ

KUHN, D. J. & CUNNINGHAM, G. N. (1978). Aldehyde oxidase distribution in *Drosophila melanogaster* mature imaginal discs, histoblasts and rings of imaginal cells. *J. exp. Zool.* **204**, 1–10.

LAWRENCE, P. A. (1981). A general cell marker for clonal analysis of *Drosophila* development. *J. Embryol. exp. Morph.* **64**, 321–332.

LEE, C.-Y., YUAN, J. H. & MOSER, D. (1978). Cytoplasmic malic enzyme from mouse kidneys. *Mol. & Cell. Biochem.* **22**, 33–38.

LEE, C.-Y., LEE, S.-M., LEWIS, S. & JOHNSON, F. M. (1980a). Identification and biochemical analysis of mouse mutants deficient in cytoplasmic malic enzyme. *Biochemistry* **19**, 5098–5103.

LEE, C.-Y., CHASALOW, F., LEE, S.-M., LEWIS, S. & JOHNSON, F. M. (1980b). A null mutation of cytoplasmic malic enzyme in mice. *Mol. & Cell. Biochem.* **30**, 143–149.

LEEFLANG-DE PIJPER, A. M. & HULSMANN, W. C. (1974). Pitfalls in histochemical localization studies of NADPH generating enzymes or enzyme systems in rat small intestine. *Histochemistry* **39**, 143–153.

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

MEIJER, A. E. F. H. & DE VRIES, G. P. (1975). Semipermeable membranes for improving the histochemical demonstration of enzyme activities in tissue sections. V. Isocitrate: NADP$^+$ Oxidoreductase (Decarboxylating) and Malate: NADP$^+$ Oxidoreductase (Decarboxylating). *Histochemistry* **43**, 225–236.

NICHOLS, J. & GARDNER, R. L. (1984). Heterogeneous differentiation of external cells in individual isolated early mouse inner cell masses in culture. *J. Embryol. exp. Morph.* **80**, 225–240.

OLSEN, I., MUIR, H., SMITH, R., FENSOM, A. & WATT, D. J. (1983). Direct enzyme transfer from lymphocytes is specific. *Nature* **306**, 75–77.

OSTER-GRANITE, M. L. & GEARHART, J. (1981). Cell lineage analysis of cerebellar purkinje cells in mouse chimeras. *Devl Biol.* **85**, 199–208.

PEARSE, A. G. E. (1972). *Histochemistry: Theoretical and Applied*, 3rd Ed. Vol. 2. Edinburgh: Churchill Livingstone.

PEDERSEN, R. A., SPINDLE, A. I. & WILEY, L. M. (1977). Regeneration of endoderm by ectoderm isolated from mouse blastocysts. *Nature, Lond.* **270**, 435–437.

PONDER, B. A. J., WILKINSON, M. M. & WOOD, M. (1983). H-2 antigens as markers of cellular genotype in chimaeric mice. *J. Embryol. exp. Morph.* **76**, 83–93.

ROSSANT, J. & CHAPMAN, V. M. (1983). Somatic and germline mosaicism in interspecific chimaeras between *Mus musculus* and *Mus caroli*. *J. Embryol. exp. Morph.* **73**, 193–205.

ROSSANT, J., GARDNER, R. L. & ALEXANDRE, H. L. (1978). Investigation of potency of cells from the postimplantation mouse embryo by blastocyst injection: a preliminary report. *J. Embryol. exp. Morph.* **48**, 239–247.

ROSSANT, J., VIJH, M., SIRACUSA, L. D. & CHAPMAN, V. M. (1983). Identification of embryonic cell lineages in histological sections of *M. musculus M. caroli* chimaeras. *J. Embryol. exp. Morph.* **73**, 179–191.

SAUER, L. A. & DAUCHY, R. T. (1978). Identification and properties of the nicotinamide dinucleotide (phosphate)$^+$-dependent malic enzyme in mouse ascites tumor cells. *Cancer Res.* **38**, 1751–1756.

SHOWS, T. B., CHAPMAN, V. M. & RUDDLE, F. H. (1970). Mitochondrial malate dehydrogenase and malic enzyme: Mendelian inherited electrophoretic variants in the mouse. *Biochem. Genet.* **4**, 707–718.

SIMPSON, E. R. & ESTABROOK, R. W. (1969). Mitochondrial malic enzyme: the source of reduced nicotinamide adenine dinucleotide phosphate for steroid hydroxylation in bovine adrenal cortex mitochondria. *Arch. Biochem. Biophys.* **129**, 384–395.

SIRACUSA, L. D., CHAPMAN, V. M., BENNETT, K. L., HASTIE, N. D., PIETRAS, D. F. & ROSSANT, J. (1983). Use of repetitive DNA sequences to distinguish *Mus musculus* and *Mus caroli* cells by *in situ* hybridization. *J. Embryol. exp. Morph.* **73**, 163–178.

SOLTER, D. & KNOWLES, B. B. (1975). Immunosurgery of mouse blastocyst. *Proc. natn. Acad. Sci., U.S.A.* **72**, 5099–5102.

SPIELMAN, H., JACOB-MULLER, U. & BECKORD, W. (1980). Immunosurgical studies on inner cell mass development in rat and mouse blastocysts before and during implantation *in vitro*. *J. Embryol. exp. Morph.* **60**, 255–269.

STANNERS, C. P., ELICEIRI, G. L. & GREEN, H. (1971). Two types of ribosome in mouse-hamster hybrid cells. *Nature New Biol. Lond.* **230**, 52–54.

SZABAD, J., SCHUPBACH, T. & WIESCHAUS, E. (1979). Cell lineage and development in the larval epidermis of *Drosophila melanogaster*. *Devl Biol.* **73**, 256–271.

TURNER, V. S. & HOPKINSON, D. A. (1979). The use of meldolablue in isozyme stains after electrophoresis. *FEBS Letters* **105**, 376–378.

VOELKER, R. A., OHNISHI, S., LANGLEY, C. H., GAUSZ, J. & GYURKOVICS, H. (1981). Genetic and cytogenetic studies of malic enzyme in *Drosophila melanogaster*. *Biochem. Genet.* **19**, 525–534.

WEST, J. D. (1984). Cell markers. In *Chimaeras in Developmental Biology* (eds N. Le Douarin & A. McLaren) (in press). New York: Academic Press.

WHITTINGHAM, D. G. & WALES, R. G. (1969). Storage of two cell mouse embryos *in vitro*. *Aust. J. biol. Sci.* **22**, 1065–1068.

WISLOCKI, G. B. & PADYKULA, H. A. (1953). Reichert's membrane and the yolk sac of the rat investigated by histochemical means. *Am. J. Anat.* **92**, 117–151.