Clonal analysis of epiblast fate during germ layer formation in the mouse embryo

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Summary

The fate of cells in the epiblast at prestreak and early primitive streak stages has been studied by injecting horseradish peroxidase (HRP) into single cells in situ of 6.7-day mouse embryos and identifying the labelled descendants at midstreak to neural plate stages after one day of culture.

Ectoderm was composed of descendants of epiblast progenitors that had been located in the embryonic axis anterior to the primitive streak. Embryonic mesoderm was derived from all areas of the epiblast except the distal tip and the adjacent region anterior to it: the most anterior mesoderm cells originated posteriorly, traversing the primitive streak early; labelled cells in the posterior part of the streak at the neural plate stage were derived from extreme anterior axial and paraxial epiblast progenitors; head process cells were derived from epiblast at or near the anterior end of the primitive streak. Endoderm descendants were most frequently derived from a region that included, but extended beyond, the region producing the head process: descendants of epiblast were present in endoderm by the midstreak stage, as well as at later stages. Yolk sac and amnion mesoderm developed from posterolateral and posterior epiblast. The resulting fate map is essentially the same as those of the chick and urodele and indicates that, despite geometrical differences, topological fate relationships are conserved among these vertebrates.

Clonal descendants were not necessarily confined to a single germ layer or to extraembryonic mesoderm, indicating that these lineages are not separated at the beginning of gastrulation.

The embryonic axis lengthened up to the neural plate stage by (1) elongation of the primitive streak through progressive incorporation of the expanding lateral and initially more anterior regions of epiblast and, (2) expansion of the region of epiblast immediately cranial to the anterior end of the primitive streak. The population doubling time of labelled cells was 7.5 h; a calculated 43% were in, or had completed, a 4th cell cycle, and no statistically significant regional differences in the number of descendants were found. This clonal analysis also showed that (1) growth in the epiblast was noncoherent and in most regions anisotropic and directed towards the primitive streak and (2) the midline did not act as a barrier to clonal spread, either in the epiblast in the anterior half of the axis or in the primitive streak. These results taken together with the fate map indicate that, while individual cells in the epiblast sheet behave independently with respect to their neighbours, morphogenetic movement during germ layer formation is coordinated in the population as a whole.

Key words: germ layer formation, mouse embryo, cell fate, fate map, cell lineage, embryo growth, gastrulation, microinjection, HRP.

Introduction

The epiblast or primitive ectoderm of the mouse embryo is the sole source of all the fetal tissues, both somatic and germline, and it also forms the amnion ectoderm and all the extraembryonic mesoderm (Gardner and Papaionnou, 1975; Gardner and Rossant, 1979; Gardner et al. 1985). Allocation of cells in the ICM (inner cell mass) to either epiblast or hypoblast (primitive endoderm) during the preimplantation period at 4½ days of gestation has been inferred from chimaeras made by injecting single cells into blasto-
followed using blastocyst chimaeras because epiblast cells from 5½ days gestation and older embryos do not develop in the blastocyst environment (Gardner et al. 1985; reviewed by Rossant, 1986). Experiments in which murine epiblasts were transferred to ectopic sites demonstrated the potency of the epiblast to form derivatives of all three germ layers (reviewed by Beddington, 1986; Švajger et al. 1986), this capacity being present in gastrulating (Grobstein, 1952; Levak-Švajger and Švajger, 1974; Beddington, 1983a) as well as preprimitive-streak-stage embryos (Diwan and Stevens, 1976; Levak-Švajger and Švajger, 1971). No regional differences in the histogenetic capacity of the epiblast were found (Švajger et al. 1981; Beddington, 1983a). However, heterotopic transplants of epiblast at the late primitive-streak stage into host embryos showed some restriction of potency and also position dependency for the development of some tissue types: surface ectoderm developed from both orthotopic and heterotopic grafts in an anterior position, and lose mesoderm developed posteriorly; generally, heterotopic grafts colonized a smaller range of tissue types than expected from their grafted position and failed to form tissue types expected from their site of origin but not appropriate to their grafted position (Beddington, 1981, 1982). About half a day later in development, when the neural folds have elevated and the foregut indentation is evident, the epiblast/ectoderm has lost the capacity to form endoderm derivatives in ectopic sites, but is still able to form mesoderm (Levak-Švajger and Švajger, 1974). These results indicate that the epiblast as a whole, or in substantial pieces, retains considerable potency until late in gastrulation.

Labelled orthotopic grafts of the epiblast gave sufficiently consistent results (Beddington, 1981, 1982; Copp et al. 1986; Tam and Beddington, 1987; Tam, 1989) for partial maps of presumptive fate of the late-streak-stage epiblast to be drawn (Beddington, 1983b; Tam, 1989). Full thickness explants, i.e. explants containing both epiblast and visceral endoderm from mid-streak-stage embryos, or containing epiblast, mesoderm and endoderm from late-streak-stage embryos, showed pronounced regional autonomy in vitro and the fate map obtained (Snow, 1981) is consistent with the data from the orthotopic transplants of epiblast. These results, complementing the potency experiments, in which normal cell relationships were disrupted, suggest that maintenance of tissue architecture and local cell interactions are important in achieving normal cell fate.

While grafting and transplant experiments suggest that a gradual, regional restriction of potency occurs during gastrulation, prospective fate and time of commitment of individual cells cannot necessarily be inferred from experiments in which groups of cells were manipulated. It is theoretically possible that cell type specification occurs earlier and that gastrulation involves either sorting out or selection of previously specified cells, as has recently been suggested for the chick embryo (Stern and Canning, 1990). Clonal analysis of cell fate early in gastrulation would help to resolve this question, as well as testing whether presumptive fate can be mapped at the onset of gastrulation.

Gastrulation involves the formation of mesoderm and definitive endoderm from the epiblast: its first manifestation is the initiation of the primitive streak locally at the junction of the epiblast and extraembryonic ectoderm (Tam and Meier, 1982; Hashimoto and Nakatsuji, 1989). The appearance of the primitive streak visibly establishes the anterior–posterior axis of the embryo in the hitherto radially symmetrical cup-shaped epiblast, although there is morphological evidence that this axis is already specified at the time of implantation (Smith, 1980, 1985). Mesoderm forms from the edge of the primitive streak, according to Poelmann (1981a) by delamination without subsequent migration, while growth of the epiblast displaces the streak away from the already delaminated mesoderm. The pattern in which the somitomeres (paraxial mesoderm) are laid down between the midstreak and late streak stages supports this view (Tam and Meier, 1982). On the other hand, cinematography of gastrulating embryos has revealed mesoderm cells migrating anteriorly and distally (Nakatsuji et al. 1986). Proliferation kinetics are incompatible with the primitive streak acting as a stem cell population and exfoliating the entire mesoderm (Snow, 1977); but in the absence of cell labelling in situ, it is unclear whether the source of the mesoderm is the lateral epiblast (Poelmann, 1981a) or all but the distal epiblast (Snow, 1977; Snow and Bennett, 1978).

The replacement of the visceral embryonic endoderm, derived from primitive endoderm, by definitive endoderm derived from the epiblast has been partly elucidated: histology (Jolly and Féréster-Tadié, 1936; Snell and Stevens, 1966; Poelmann, 1981b; Lamers et al. 1987), labelling (Lawson et al. 1986; Kado kawa et al. 1987), orthotopic transplants (Beddington, 1981) and transplants to ectopic sites (Levak-Švajger and Švajger, 1974; Beddington, 1983a) all indicate that definitive endoderm is derived from the same population of cells as the notochord, i.e. the head process or anterior extension of the primitive streak, and forms at least part of the axial endoderm at the neural plate stage. Experiments in which single endoderm cells were labelled in situ and their descendants traced to early somite stages indicate that, already during gastrulation, there is a subpopulation of endoderm cells that will colonise the foregut (Lawson et al. 1986, 1987). This subpopulation was identified in the endoderm at the anterior end of the early primitive streak and traced to more anterior levels at midstreak and late streak stages. Although it has not been established that these cells contribute to the definitive foregut and its derivatives at midgestation, a way to check their putative origin in the epiblast would be to trace the descendants of epiblast cells labelled at the onset of gastrulation.

Gastrulation in the mouse is accompanied by an increase in cell proliferation rate in the epiblast which is evident at approx. 6½ days, in the period when the primitive streak is initiated (Snow, 1977; Lewis and Rossant, 1982). Rapid growth results in an expansion of
the epiblast and its derivatives from 660 cells at 6.5 days to 14,300 cells at 7.5 days in the Q strain (Snow, 1977). Spatial analysis of the mitotic index revealed a very rapidly growing region in the epiblast, anterior to the primitive streak; the calculated length of the cell cycle in this proliferative zone was as short as 3 h in comparison with 6–7 h in the rest of the epiblast (Snow, 1977). Poellmann’s (1980) estimate of cell cycle length in the frontal and lateral ectoderm, using \(^{3}H\)thymidine, is somewhat longer (7.6 h), but this may be an overestimate (Snow, 1985). There is, however, general agreement of an increase in growth rate during gastrulation compared with the early postimplantation period, and any description of the morphogenetic transformation involved in gastrulation must take this rapid expansion into account.

In this paper we describe the transformation of the epiblast of the prestreak and early-streak-stage embryo to the three germ layers of late-streak and neural plate stages by tracing the descendants of single cells labelled in situ with horseradish peroxidase (HRP), which is a reliable short-term lineage marker for the mouse embryo (Balakier and Pedersen, 1982; Pedersen et al. 1986; Lawson et al. 1986, 1987).

Materials and methods

Embryos

Noninbred Swiss mice of the Dub: (ICR) strain were used. Gestation was considered to have begun at midnight before the morning on which a copulation plug was found. Females were killed by cervical dislocation between 14.00 and 18.00 h on the 7th day of pregnancy (6.7-day embryos). Embryos were prepared for injection as previously described (Lawson et al. 1987). Because of the variation in developmental stage both between and within litters of nominal 6.7-day embryos, embryos were classified as ‘prestreak stage’ or ‘early-streak stage’. Prestreak-stage embryos appeared either radially symmetrical, or showed the first indication of bilateral symmetry in the thickened posterior epiblast presaging mesoderm formation. Mesoderm formation had begun caudally in early streak stage embryos: the distal tip of the mesodermal wedge indicating the anterior end of the primitive streak was located a quarter to one third of the length of the embryonic axis from the caudal junction of the epiblast and extraembryonic ectoderm.

Embryo culture

Embryos were cultured in Dulbecco’s modified minimal essential medium containing 50% rat serum as previously described (Lawson et al. 1986) with the following modification. One 6.7-day embryo was cultured in 1.0 ml medium with two 7.5-day embryos preincubated for approx. 5 h. The incubator was filled with 6% CO\(_2\) in air and the injected embryos cultured for an average of 22.7 h (range: 20–25 h). Coculture was later found to be unnecessary: single 6.7-day embryos developed normally in 1 ml medium as long as this was fully preequilibrated for temperature and CO\(_2\) concentration (approx. 2 h).

Cell labelling

The embryo injection chamber was a modification of one already described (Winkel and Pedersen, 1988). A sterile strip of coverslip, 2–3 mm wide, was supported lengthwise on the gasket of a single-chamber tissue-culture slide (Lab-Tek, Illinois) with the chamber removed. The embryos were aligned under the coverslip in Flushing Medium II (Spindle, 1980), containing 10% fetal calf serum. The medium–air interface was sealed with paraffin oil. A standard Zeiss microscope with fixed stage and fitted with a UD 40 objective without nose piece, adapted for interference contrast optics and epifluorescence, was attached to a silicon intensified target (SIT) video camera (Dage/MTI) and TV monitor. The embryo to be injected was restrained with a microforge-polished holding pipette (O.D.: 100–120 μm, I.D.: 60–65 μm). Injections into the epiblast at the distal tip of the egg cylinder without frequently labelling the overlying endoderm were possible if the embryo was restrained with a holding pipette with a subterminal aperture, which was applied to the embryo close to the distal tip (Beddington and Lawson, 1990). The apparatus and microelectrodes used for intracellular injection by iontophoresis were as described previously (Balakier and Pedersen, 1982). One epiblast cell per embryo was injected with 4% HRP (Sigma Type VI, lot no. 62F-9545)* plus 1% rhodamine-conjugated dextran (RDX, 70×10^3 M\(_{r}\); Molecular Probes, Portland, OR) in 0.05 M KCl using 3 nA depolarising continuous current for 10 s. The microelectrode was pushed through the endoderm layer with manual pressure on the micromanipulator; the epiblast cell was then penetrated using the capacitance compensation control to induce momentary vibration of the electrode tip. Both phases of microelectrode movement were monitored on the oscilloscope. The position of the injected, fluorescent cell was recorded from the TV monitor on Polaroid Type 667 Coaterless Black and White Land film. The time during which the embryo was exposed to fluorescent excitation was kept to a minimum (not more than 2×15 s); the SIT camera was used at maximum gain and the excitation intensity reduced to the minimum feasible for visualization. Only those embryos with an unequivocally and exclusively labelled epiblast cell as judged by the fluorescent signal 30–60 min. after injection were cultured.

In order to identify retrospectively the position of longitude of the injected epiblast cell in the egg cylinder, an additional injection (using 3 nA for 5 s) was made in each embryo into one visceral extraembryonic endoderm cell near the junction of embryonic and extraembryonic ectoderm and in the same plane as the injected epiblast cell (Fig. 1A,B). For analysis, the initial epiblast was considered as two tiers of 8 zones and a zone at the distal tip (Fig. 1C). The results from right and left halves of the epiblast cup were superimposed, thus giving a total of 11 arbitrary zones (Fig. 1A). Each zone represents approx. 65×50 μm epiblast basal surface in prestreak stage embryos and approx. 100×75 μm at the early streak stage.

Detection of HRP-labelled cells

Cells containing HRP were detected by treating intact embryos for 2 h in the dark with 0.05% Hancer-Yates reagent (Polysciences) in 0.1 m phosphate buffer, pH 5.5 (Streit and Rubi, 1977) containing 5% (w/v) sucrose and 0.02% H\(_2\)O\(_2\). Embryos were subsequently fixed in 2.5% glutaraldehyde in 0.1 m phosphate buffer, pH 7.2. After rinsing in buffer, the embryos were oriented for embedding by treating with phytohaemagglutinin and attaching to fibroblasts on Therma-phase. Numbers of embryos were 250 (Q strain) and 120 (C57BL/6 strain).

*Subsequent lots from this source were toxic. Tests for toxicity of the label can be carried out according to Lawson et al. (1986).
Fig. 1. (A) Schematic representation of a 6.7-day egg cylinder viewed laterally. The injection zones in the epiblast are indicated (I-XI). The craniocaudal axis runs from anterior to posterior via the distal tip of the egg cylinder. The open bar marks the approximate extent of the primitive streak in early-streak-stage embryos. H and D indicate the measured height and diameter of the embryonic part of the egg cylinder. The position of an injected epiblast cell (•) along the H axis from the junction of the embryonic and extraembryonic region (h) was measured directly; the position along the circumference from the anterior border (d) was judged retrospectively (see B) from a coincidentally injected extraembryonic endoderm cell (A). Ant, anterior; Post, posterior. Scale bar: 0.1 mm. (B) The position (d) of the injected extraembryonic endoderm cell was estimated from the position of the cluster of its labelled descendants after culture (d') on the assumption that the ratio of d to the total diameter, D, is constant during 24h culture. This assumption is based on the stable location of descendant clones in extraembryonic endoderm of embryos cultured from early-streak and later stages (see text for details).

\[ d = \frac{d'D}{D'} \]

The open bar marks the extent of the primitive streak. Scale bar: 0.1 mm (C) Exploded view of 6.7-day epiblast to show the tiers of injection zones. The bars projecting from zones I and V mark the anterior and posterior limits of the embryonic axis and indicate that zones I, V, VI, X and XI span the midline.

Results

The majority of prestreak-stage embryos developed to midstreak- and late-streak stages in culture, and most early-streak-stage embryos developed to the neural plate stage (Table 1). Of all these, 84% (136/162) had HRP-labelled cells (Table 1); this incidence does not differ significantly (χ² test, 1 d.f.) from the 90% (53/59) found in the uncultured controls (Table 2).

Histological analysis of control embryos (injected but not cultured) showed that estimating an injection as exclusively in epiblast on the basis of the fluorescent signal of RDX failed to exclude some (10/53) embryos with HRP-labelled endoderm at the site of epiblast injection (Table 2). This implies that approx. 19% of the cultured embryos would be expected to have artefactually labelled endoderm. The probable reason for this is that, compared with the detection of RDX by fluorescence, HRP in adjacent cells was detectable histochemically over a wider range of dilution.

Only 42% of HRP-labelled control embryos had a single labelled epiblast cell; most of the remainder had two (e.g. Fig. 2) and a few had 3 or 4 labelled cells (Table 2). Whenever more than one cell was labelled, they were connected apically by a cytoplasmic bridge, although such pairs, trios and quartets were often separated basally by one or more unlabelled cells. Since mitotic cells round up at the lumen (apically), this indicates not only that cell separation is incomplete when daughters are reassociated with the basal lamina, but also that there is cell mixing in the epiblast epithelium before and after the beginning of gastrulation.

An important feature of the approach for mapping the initially radially symmetrical epiblast layer was to inject a visceral-extraembryonic endoderm cell coincidently and in the same plane as the injected epiblast cell. This procedure made it possible to identify the 'position of longitude' of the injection site in the epiblast cup. Descendants of extraembryonic endoderm cells remained coherent and the centres of clones were located in the same (65%) or an adjacent (33%) octant in 98% of a series of embryos (n=89) injected between early streak and headfold stages, when the anterior–posterior embryonic axis is easily identified, and cultured for 24 h. No bias was found in the direction of an adjacent octant in which descendants were recovered. Similar behaviour of visceral extraembryonic endoderm was assumed for the embryo at the prestreak stage when it is overtly radially symmetrical.

Localization of descendants

The results of embryos injected at prestreak and early streak stages are presented separately.

nox coverslips (Wiley et al. 1985). Sections of glycolmethacrylate-embedded material were cut at 7 μm and stained with 0.17% methylene blue (Chroma: Med.Puriss.grade). Exact position and number of the injected cells were established from photographic reconstructions (Lawson et al. 1986). The position of labelled cells was plotted onto the outline of a half embryo with average linear dimensions.
Table 1. Development of 6.7-day embryos in vitro after intracellular injection of HRP

<table>
<thead>
<tr>
<th>Initial stage</th>
<th>Total embryos</th>
<th>0h</th>
<th>23h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height* (μm)</td>
<td>Diameter† (μm)</td>
<td>Height* (μm)</td>
</tr>
<tr>
<td>Prestreak</td>
<td>71</td>
<td>168±31</td>
<td>167±22</td>
</tr>
<tr>
<td>Early streak</td>
<td>91</td>
<td>258±35</td>
<td>228±35</td>
</tr>
</tbody>
</table>

* The distance from the junction of epiblast and extraembryonic ectoderm to the distal tip of the egg cylinder, or from the anterior junction of embryonic ectoderm and amnion to the tip (Fig. 1). Mean±s.D.
† The greatest width of the embryonic part of the egg cylinder when the embryo is viewed laterally (Fig. 1). Mean±s.D.

Table 2. Position and number of HRP-labelled cells in control embryos, expected from the RDX-signal to be labelled exclusively in epiblast

<table>
<thead>
<tr>
<th>No. HRP-labelled embryos expected</th>
<th>Total HRP-labelled</th>
<th>Epiblast only</th>
<th>Epiblast plus endoderm</th>
<th>Endoderm only</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>53</td>
<td>43 (81)</td>
<td>9 (17)</td>
<td>1 (2)</td>
<td>22 (42)</td>
<td>21 (40)</td>
<td>7 (13)</td>
<td>2 (4)</td>
</tr>
</tbody>
</table>

* Combined results of prestreak and early-streak stages.

(a) Ectoderm

The ectoderm at the midstreak and late streak stages was derived mainly from descendants of cells of the axial (zones I, VI, XI, X) and anterior paraxial (zone VII) epiblast of prestreak stage embryos (Fig. 3; Table 3). (The clone from zone V (Fig. 3) was atypical in that the initially very small embryo had only reached the early streak stage after culture). Epiblast cells from the anterior and proximal part of the egg cylinder (zones I, II, VI, VII) spread towards the primitive streak while those just anterior to the developing streak (zone X) expanded mainly axially, the posterior descendants being incorporated into the anterior half of the streak.

The same behaviour was reflected in the epiblast of the early-streak-stage embryo developing to the neural plate stage. Descendants of anterior axial (zones I, VI) and paraxial (zone VII) epiblast were spread towards the streak (Figs 4, 5A). In these embryos there was less contribution to ectoderm by cells from zone X, into which the anterior part of the primitive streak extended, and a relatively greater contribution from zones XI and VIII (Table 4). Because the primitive streak originates in zone V, the relationship of zone XI in the early streak stage embryo to the anterior end of the streak is the same as that of zone X at the prestreak stage, suggesting that the same subpopulation of cells was involved in the two stages. Axial descendants of progenitors from zone XI were frequently arranged in a narrow arc spanning the midline, with the ends directed caudally (Fig. 5B).

Labelled cells were found in amnion ectoderm only.
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Ectoderm

Fig. 3. Distribution of labelled descendants in ectoderm after injection into epiblast at the prestreak stage. The injection zones (Fig. 1) are indicated by broken lines in the upper figure which represents a lateral view of the epiblast at the time of injection: dots mark the position of epiblast progenitors (one per embryo) that had descendants in ectoderm. Each of the 11 lower figures shows the location of all descendants in ectoderm following injection in a particular zone at the prestreak stage. Each figure represents the longitudinal half of the embryonic part of a cultured embryo of average dimensions. The arrangement of the zonal figures in an upper tier (zone I-V), a lower tier (zone VI-X) and the distal tip (zone XI) follows the position of zones in the initial embryo; e.g. the extreme left figure of the upper tier represents zone I. The amnion (extraembryonic) is indicated in outline above the figure representing zone I. Above each zonal figure are the ratios of the number of embryos with descendants in ectoderm to the total number of embryos injected in the zone. The ratios on the left refer to the embryonic portion, those on the right (boxed) refer to the extraembryonic portion. A, Anterior; P, Posterior; Dots, epiblast progenitors (upper figure), descendants in ectoderm (lower figures); Open circles: descendants in the luminal layer of the primitive streak. Scale bar: 100 μm.

after injection into anterior proximal epiblast i.e. zones I and II (Figs 3,4).

(b) Mesoderm

The embryonic mesoderm at the midstreak stage was derived from most zones of the prestreak stage embryo (II-V; VIII-X). Zones not contributing to mesoderm were in the anterior half of the axis (zones I, VI, VII, XI) (Fig. 6; Table 3). At the midstreak stage the mesoderm forms a pair of wings between the epiblast and endoderm, with the base of each wing in the primitive streak and the tips extending towards the anterior junction of embryonic and extraembryonic ectoderm. The most anterior mesoderm, consisting of the tips of the wings, was derived mainly from zone V and would have been the first to traverse the very early primitive streak. The broader base of the mesoderm wings consisted of descendants of more anterior and
Table 3. Average number of HRP-labelled cells per embryo in different embryonic and extraembryonic regions after injection into a single cell in different zones of the epiblast at the prestreak stage and culture for one day

<table>
<thead>
<tr>
<th>Region</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
<th>XI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td>10.4</td>
<td>3.2</td>
<td>2.3</td>
<td>-</td>
<td>0.3</td>
<td>14.8</td>
<td>8.0</td>
<td>2.5</td>
<td>1.3</td>
<td>8.9</td>
<td>12.2</td>
</tr>
<tr>
<td>Ectoderm</td>
<td>-</td>
<td>5.4</td>
<td>1.3</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
<td>1.7</td>
<td>-</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>-</td>
<td>3.4</td>
<td>4.0</td>
<td>1.3</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
<td>9.0</td>
<td>0.3</td>
<td>2.6</td>
<td>-</td>
</tr>
<tr>
<td>Endoderm</td>
<td>0.1</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
<td>2.1</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>3.0</td>
<td>7.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Extraembryonic</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Amnion ectoderm</td>
<td>-</td>
<td>0.9</td>
<td>14.0</td>
<td>4.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<tr>
<td>mesoderm</td>
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</table>

Total/embryo 11.9 13.0 10.2 15.3 12.0 14.8 9.0 13.0 6.3 20.7 14.1

No. embryos 7 7 6 3 6 4 3 4 3 10 8

*Combines 'ectodermal' (luminal) (Fig. 3) and 'mesodermal' (non-luminal) (Fig. 6) elements.

Ectoderm

![Distribution of labelled descendants in ectoderm after injection into epiblast at the early-streak stage.](image)

Laterally situated progenitors (zones II, III, VIII), whose descendant populations had expanded before reaching the streak and evidently had traversed the streak over a larger front. The streak itself was colonized mainly by descendants from the anterolateral zone II (Fig 6; Table 3), with smaller contributions from lateral zones III and VIII. The head process was colonized exclusively from zone X.

Descendants of cells from most zones of the early-streak-stage epiblast contributed to mesoderm and to the streak (Table 4; Fig. 7). The pattern of contribution was predictable from the results from the prestreak stage, i.e. the most anteriorly positioned mesoderm cells arose from progenitors in the early streak (zones V and X) and were followed through the streak by originally more lateral precursors (zones III and IX) (Fig. 7). The posterior part of the streak and the region extending into the base of the allantois was colonized exclusively by descendants from the anteroproximal zones I and II (Figs 5,7), and the middle region of the streak mainly from the anterolateral zone VII. Descendants from zone X remained in the anterior half of
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The streak, as well as contributing to mesoderm and to the head process. In 3 embryos (injected in zones VI, VIII and XI), a few mesoderm cells were found not far from the midline (Fig. 7) and appeared to have been delaminated directly from ectoderm without traversing the primitive streak since they were closely associated with labelled ectoderm cells and there were no additional labelled cells in more posterior mesoderm.

(c) Extraembryonic mesoderm

The majority of the descendants from the posterolateral zone IV of both prestreak- and early-streak-stage epiblast contributed to yolk sac mesoderm, amnion mesoderm and the mesothelium covering the allantoic bud (Figs 6, 7; Tables 3, 4). A substantial contribution also came from zone V. Colonization of the allantoic primordium was rarely found, and then only by an occasional descendant from zones I and II (Fig. 7) that had labelled relatives in the extreme posterior part of the streak.

(d) Endoderm

The interpretation of the presence of HRP-labelled cells in endoderm (Figs 8, 9) is complicated by the expectation of false positives (Table 2). The incidence of embryos with labelled endoderm after injection at the prestreak stage (Table 5a) did not differ significantly from the controls (Table 2) for any zone except zone X ($\chi^2 = 17.1, P<0.001$). Similarly, from the incidence of embryos with labelled endoderm after injection at the early-streak stage (Table 5b), only epiblast of the posterior zones X, V and IX ($P<0.05$) more frequently had descendants in endoderm than the controls. The precise distribution of epiblast-derived endoderm cells cannot be determined from the distribution of labelled descendants from these zones, because the labelled endoderm cells were not necessarily derived from epiblast in all the embryos; similarly, a minor contribution from zones other than V, IX and X cannot be excluded. In spite of these reservations, the results justify the conclusion that at least some cells in the endoderm at the midstreak stage, as well as at later stages, are derived from epiblast at or near the anterior end of the early primitive streak.

Fate map

Fate maps of the prestreak and early streak stage epiblast have been constructed on the basis of the results described here (Fig. 10). Although crude, the maps for the two stages are similar: the differences can be ascribed to the more advanced final stage reached by early-streak-stage embryos as compared with the
Fig. 6. Distribution of labelled descendants in mesoderm after injection into epiblast at the prestreak stage. The presentation of the results is analogous to Fig. 3. The yolk sac (extraembryonic) is outlined above the amnion in the figures of posterior zones. Open circles in the embryonic region: descendants in layers of the primitive streak not abutting the lumen of the amniotic cavity. Triangles: descendants in the head process. Open circles in the extraembryonic region: mesoderm lining the chorion.

prestreak-stage embryos, e.g. there is relatively less presumptive ectoderm at the early-streak-stage because more epiblast becomes incorporated into the streak, and there is a relatively larger area of presumptive extraembryonic mesoderm for the same reason.

There is considerable overlap of presumptive regions in the fate map. This is partly due to biological variation, to normalizing the results, and to experimental error in identifying the site of injection. In addition, clonal analysis showed that sharp boundaries in fate do not exist at the cell level, not only with respect to the different structures in which cells might reside at the neural plate stage, but also with respect to the broader categories of ectoderm, mesoderm, endoderm and extraembryonic mesoderm (Fig. 11). When clonal descendants were classified on a zonal basis as contributing to one or more of these four categories (Table 6), they were found in more than one category in 22-50% of the labelled embryos after injection into any zone except zone X, where the incidence was higher (76%) and the frequency distribution was significantly different ($P<0.001$). This indicates that the different germ layers and extraembryonic mesoderm lineages are not established at the onset of gastrulation: the
Table 4. Average number of HRP-labelled cells/embryo in different embryonic and extraembryonic regions after injection into a single cell in different zones of the epiblast at the early streak stage and culture for one day

<table>
<thead>
<tr>
<th>Region</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
<th>XI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ectoderm</td>
<td>10.8</td>
<td>3.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.0</td>
<td>9.8</td>
<td>13.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Streak*</td>
<td>4.0</td>
<td>4.4</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td>2.6</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesoderm</td>
<td></td>
<td></td>
<td>12.3</td>
<td></td>
<td>3.3</td>
<td>0.6</td>
<td>0.2</td>
<td>3.6</td>
<td>3.5</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Endoderm</td>
<td></td>
<td></td>
<td>0.7</td>
<td>14.0</td>
<td>5.8</td>
<td>0.7</td>
<td>3.4</td>
<td>4.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraembryonic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amnion ectoderm</td>
<td>0.2</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraembryonic mesoderm</td>
<td>0.2</td>
<td>0.7</td>
<td></td>
<td></td>
<td>15.0</td>
<td>8.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total/embryo</td>
<td>15.1</td>
<td>9.6</td>
<td>14.3</td>
<td>29.0</td>
<td>18.0</td>
<td>13.0</td>
<td>14.2</td>
<td>8.2</td>
<td>13.4</td>
<td>22.6</td>
<td></td>
</tr>
<tr>
<td>No. embryos</td>
<td>12</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>11</td>
<td>15</td>
</tr>
</tbody>
</table>

* Combines 'ectodermal' (luminal) (Fig. 4) and 'mesodermal' (non-luminal) (Fig. 7) elements.

Fig. 7. Distribution of labelled descendants in mesoderm after injection into epiblast at the early-streak stage. The presentation of results is analogous to Fig. 4. The yolk sac and allantoic bud are combined in outline with the amnion. For further explanation of symbols, see Fig. 6.

individual cells in the epiblast may contribute to more than one layer, and those near the anterior end of the streak usually do so.

**Growth of the epiblast**
The average number of labelled cells per embryo after culture was 14.7, but the frequency distribution was
skewed (Fig. 12). No statistically significant difference between the number of descendants from different zones (Tables 3, 4) was found after analysis of variance of the log-transformed data. When initial pairs, trios and quartets were taken into account (Lawson et al. 1987), a population doubling time of 7.5 h was obtained and a calculated 43% of single progenitors were in or had completed a fourth cell cycle (Table 7). Despite its apparently homogeneous proliferation rate the epiblast cup expands nonuniformly by the asymmetrical spread of clonal descendants during growth of the embryonic region. This is illustrated by the contribution of the different zones to the extending anterior–posterior axis of the embryo. The embryonic axis increased 3.5-fold in length between the prestreak and neural plate stage: an examination of the origin of cells in the axis after culture for one day (Fig. 13) shows that the axis extended by (1) growth of the primitive streak and (2) expansion of an area of epiblast just anterior to the primitive streak. The primitive streak, which is initiated at the posterior end of the axis in zone V, accounted for approx. 30% of the embryonic axis at the early-streak stage and for approx. 50% at midstreak to neural plate stages. The primitive streak and associated axial epiblast at the midstreak stage consisted mainly of cells derived from lateral zones III and VIII and anterolateral zone II. Most of the cells of lateral origin had left the streak by the neural plate stage and had been replaced by cells from anterolateral zones II and VII and anterior zone I. The axial area just anterior to the streak (zone X in prestreak-stage embryos and zone XI at the early-streak stage) expanded symmetrically, occupying the anterior part of the streak and at least half of the axis anterior to the streak. The anterior axial
Fig. 9. Distribution of labelled descendants in endoderm after injecting into epiblast at the early streak stage. For further explanation, see Fig. 3.

Table 5. Incidence of embryos with HRP-labelled cells in different germ layers after injection into different zones at prestreak stage and early-streak stage and culture for one day

<table>
<thead>
<tr>
<th>Germ layer labelled</th>
<th>a. Prestreak</th>
<th>b. Early streak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Ectoderm</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Endoderm</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Amnion ectoderm</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Extraembryonic mesoderm</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

| Total embryos*      | 7  | 7  | 6   | 3  | 6  | 4  | 3   | 4   | 3  | 10| 8  |
| Ectoderm            | 11 | 5  | 1   | 0  | 0  | 3  | 5   | 6   | 1  | 5 | 15 |
| Mesoderm            | 4  | 4  | 3   | 0  | 3  | 1  | 3   | 1   | 4  | 7 | 1  |
| Endoderm            | 0  | 0  | 1   | 1  | 4  | 1  | 0   | 1   | 3  | 6 | 4  |
| Amnion ectoderm     | 2  | 2  | 0   | 0  | 0  | 0  | 0   | 0   | 0  | 0 | 0  |
| Extraembryonic mesoderm | 0  | 2  | 0   | 2  | 3  | 0  | 0   | 0   | 0  | 1 | 0  |

| Total embryos*      | 12 | 7  | 3   | 2  | 6  | 3  | 5   | 6   | 5  | 11| 15 |

*Number of embryos with cells in individual germ layers exceeds total because some embryos had labelled cells in more than one germ layer.
Cell fate in mouse epiblast 903

Prestreak
anterior posterior

Early streak
anterior posterior

- Ectoderm
- Mesoderm
- Headprocess / Notochord
- Endoderm
+ Extraembryonic mesoderm
- Amnion ectoderm

Fig. 10. Fate map of the epiblast of the prestreak and early-streak stages to show the derivation of germ layers up to the mid- to late-streak and neural plate stages respectively. The approximate extent of the primitive streak is indicated by the white bar.

Table 6. Frequency distribution according to injection zone of the incidence of clonal descendants* of epiblast in one or more of the following categories: ectoderm (including amnion ectoderm), embryonic mesoderm, endoderm, extraembryonic mesoderm. Results from injections at prestreak and early streak stages have been pooled.

<table>
<thead>
<tr>
<th>Epiblast zone injected</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X†</th>
<th>XI</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of categories with clonal descendants</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>13</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>10</td>
<td>10</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>136</td>
</tr>
</tbody>
</table>

* The labelled cells in a single embryo are considered to be clonal descendants. Labelled endoderm from all zones is included.
† Differs significantly, P<0.001.

In summary, the streak represents 75% of the new axial material present at the neural plate stage compared with the prestreak stage; its extension is accomplished by the transient incorporation of continually expanding lateral and progressively more anterior zones, and also by incorporating expanding epiblast that was initially in the axis just anterior to the streak. Most of the rest of the new material in the anterior half of the axis is also derived from the area just anterior to the streak.

In spite of the consistent behaviour of different areas of the embryo, the absence of coherent clonal growth as seen by the spread of HRP-labelled descendants of epiblast progenitors in individual embryos was striking. This lack of coherence was shown by descendants of all zones and in all three germ layers (Fig. 14). Although the present method of reconstruction does not allow accurate measurement of distances between labelled...
descendants in all instances, a rough estimate can be obtained in some specimens. For instance, after injecting a progenitor in zone 1, 13 labelled descendants were spread through a strip of ectoderm occupying approx. 360×50 μm of egg cylinder circumference and height. Moreover, no portion of the anterior–posterior axis, either the axial ectoderm (Table 8) or the primitive streak (Table 9) acted as a barrier to the spread of clonal descendants: axial epiblast progenitors frequently had descendants located bilaterally in ectoderm, and descendants that had traversed the primitive streak into mesoderm were found bilateral and contralateral, as well as ipsilateral, to their epiblast progenitors.

Discussion

Clonal analysis of germ layer formation has shown that
Fig. 13. Growth of the anterior–posterior axis. (A) The 6.7-day half-epiblast at prestreak and early streak stages is divided into 11 zones (see Fig. 1). Zones with no contribution to the axis after 24 h are stippled. (B) The axis has been straightened and the zones fanned out to accommodate the flattened form. (C) The eight rows of figures show the contribution of different zones to the axial region after one day in culture: in each of these figures, the axis is illustrated by a thin horizontal line that joins a white bar to the right designating the primitive streak. The length and position of a coloured bar indicate the extent along the axis in which descendants from that zone were found; the bar above each line representing the axis shows the distribution of zonal descendants in axial ectoderm and the bar below each line shows the distribution in the primitive streak and head process. The contribution of material in the original axis (zones I, VI, XI, X) is shown first followed by that in the original paraxial (zones II, VII, VIII, IX) and lateral (zone III) zones. Scale bar: 0.1 mm.
areas of the mouse epiblast are transformed during gastrulation in a reproducible and coordinated way such that a fate map of the presumptive germ layers and extraembryonic mesoderm can be drawn. However, the clonal analysis also showed that the morphogenetic movements occur in the presence of extensive, although not indiscriminate, cell mixing in the epiblast, and that descendants of a single progenitor may be spread widely, and also be present in different germ layers.

The fate map

The accuracy of a fate map depends on the resolution of the labelling technique as well as on the degree to which development is determinate at the moment of labelling. Single cell injection provides optimal resolution, but variations in initial embryo size and stage, as well as errors in retrospectively identifying the position of injection, reduce the accuracy of a composite fate map. Within these limitations reproducible results were obtained even from the overtly radially symmetrical prestreak-stage embryos. Therefore, at the onset of

Table 7. Calculated percentage distribution of the number of cell generations produced by single progenitors in the epiblast after 22.7 h

<table>
<thead>
<tr>
<th>Cell generation</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>6.9</td>
<td>7.3</td>
<td>7.6</td>
<td>35.0</td>
<td>40.0</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The method of calculation (Lawson et al. 1987) is based on the following assumptions: (1) the control and cultured populations have the same initial frequencies of labelled singletons, pairs, triplets and quadruplets, (2) the probability of division was the same, regardless of the initial number of labelled cells. The number of cultured embryos with one labelled cell (Fig. 12) represents the number of single cell progenitors that have not divided (cell generation=0) and, using the proportion of pairs in the initial population (Table 2), provides the number of undivided initial pairs in the group of cultured embryos with 2 labelled cells. The remainder of this group represents the number of single progenitors that have divided once. The procedure is applied iteratively to the rest of the population.

Fig. 12. Frequency distribution of the number of HRP-labelled cells per embryo after culturing for 1 day. Combined results from prestreak and early stage embryos.

Fig. 14. Spread of clonal descendants in early-streak-stage embryos cultured for one day. One representative embryo for each zone is shown. The presentation is analogous to Fig. 7. ○, ectoderm; ◊, contralateral ectoderm; *, primitive streak; +, head process; ■, mesoderm; □, contralateral mesoderm; ▲, endoderm; △, contralateral endoderm. Scale bar: 100 μm.
gastrulation the mouse embryo displays determinate development, in the limited sense that the fate of the descendants of a cell from a given region can be predicted probabilistically (Stent, 1985).

It must also be emphasised that the fate map obtained is valid for the first 24 h after labelling, before the majority of embryos have completed gastrulation: thus the fate map of presumptive mesoderm, endoderm and extraembryonic mesoderm could underestimate the actual areas whereas the fate map of presumptive ectoderm could overestimate the actual area. Grafting experiments have shown that at the late streak stage (7.5 days), only a strip of epiblast adjacent to the streak will contribute substantially to mesoderm; most of the epiblast at that stage will become neurectoderm and surface ectoderm (Tam, 1989). The initial developmental stage of the embryos used by Tam was equivalent to, or slightly younger than, the end point in our early streak stage series, but more advanced than the end point of most of the embryos in the prestreak stage series. In our experiments the ectoderm adjacent to the primitive streak after culture was derived from the same progenitors as more lateral and anterior ectoderm destined to form neuroectoderm and surface ectoderm. There could, however, be a shift in the relative contribution to ectoderm by axial and paraxial regions, if more descendants of paraxial than axial progenitors pass into and through the streak late in gastrulation. Therefore, it is likely that the fate map of the early streak stage correctly represents the regions of presumptive ectoderm, endoderm, mesoderm and extraembryonic mesoderm.

The substantial extent of overlap in the fate map reflects not only variation in the germ layer contributions of different progenitor cells within a zone, but also the pluripotency of the labelled epiblast cells. Only 56% of the clones had descendants in one germ layer (62% of the clones originating outside zone X and 24% of the clones from zone X). This may be an underestimate, since gastrulation had not reached completion, and some mixed clones might have lost their ectoderm component if cultured longer (i.e. some cells found in ectoderm would subsequently have entered the mesoderm or endoderm layers). Indeed, culture of early-streak stages for 36 h to early-somite stages increases the proportion of clones composed of a single category of cells, but only to 62% (unpublished results). This indicates that the ectoderm, mesoderm, endoderm and extraembryonic mesoderm lineages are not fully specified at the onset of gastrulation. If a fine mosaic of specified cells existed at this time throughout the epiblast, it would not be possible to draw a fate map and, if the specified cells were in the process of sorting out, as suggested for the chick embryo (Stern and Canning, 1990), the overlaps of the presumptive regions would contain a mosaic of pure clones, not the population of mixed clones that we observed.

Although it takes the form of a cup, the rodent epiblast is essentially similar to the epiblast of domestic mammalian species and other amniotes, in which it is a disc-shaped pseudostratified columnar epithelium. If the mouse epiblast is so viewed in two dimensions, by straightening the embryonic axis and spreading out the sides of the cup, the areas of the map are distorted, but their boundary relationships are not disturbed. The fate map of the early-streak stage can then be seen to be strikingly similar to that of the avian blastoderm at a comparable stage, and to the amphibian early gastrula (Fig. 15). Similar relationships have also recently been shown in the zebrafish (Kimmel et al. 1990). Thus, in spite of geometrical differences, topological fate relationships have been conserved in these vertebrate embryos.

**Cell mingling and clonal spread**

Ectoderm descendants of labelled epiblast progenitors did not form coherent clones. It is unlikely that the extensive cell mingling within the epithelial sheet was due to the injection procedure or to the presence of HRP in the cells, since visceral extraembryonic endoderm treated identically formed a coherent patch, in keeping with its behaviour when a genetic marker is used (Gardner, 1984). There is, at most, limited cell mingling in the preimplantation embryo, either in

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### Table 8. Incidence of unilateral and bilateral descendants in ectoderm after labelling axial progenitors*

<table>
<thead>
<tr>
<th>Injection zone</th>
<th>Unilateral</th>
<th>Bilateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>VI</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>X</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>XI</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>31</td>
</tr>
</tbody>
</table>

* Prestreak and early streak stages pooled.

### Table 9. Incidence of embryos with unilateral and bilateral descendants in mesoderm after labelling epiblast progenitors*

<table>
<thead>
<tr>
<th>Position of progenitor</th>
<th>Ipsi lateral</th>
<th>Contralateral</th>
<th>Bilateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral or paraxial</td>
<td>8</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Axial (zones V,X)</td>
<td>----8↑----</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

* Prestreak and early streak stages pooled.

† Embryos with unilateral descendants.
Cell fate in mouse epiblast

Fig. 15. Fate maps at the early gastrula stage of mouse, chick and urodele. The mouse epiblast at the early streak stage has been flattened and the overlaps between the presumptive germ layers in the fate map (Fig. 10) have been removed. The map of the chick epiblast (area pellucida) at stage 3 is according to Vakaet (1985). The urodele is viewed from the vegetal side (X=vegetal pole); much presumptive ectoderm and some mesoderm is therefore not visible (adapted from Nieuwkoop et al., 1985; Nieuwkoop and Sutasurya, 1979). The primitive streak of the mouse and chick and the dorsal lip of the blastopore of the amphibian are represented by a thick bar. •, ectoderm; ○, amnion ectoderm; □, mesoderm; △, endoderm; stippled, notochord; +, extraembryonic mesoderm.

Whatever the underlying cellular mechanism responsible for mingling, it is clear that clonal spread in the epiblast is not random, but anisotropic and directed towards the primitive streak.

Embryo growth and formation of the germ layers

The pattern of growth found by tracing descendants of progenitors from different regions of the epiblast is one of proliferation accompanied by extensive cell mingling, but with non-random distribution of descendants in the epithelial layer. The population doubling time, based on the number of labelled descendants, was 7.5 h, indicating that the epiblast, with its derivatives, increases more than eight-fold during one day in vitro.

A calculated 78% of cells went through 3 cell cycles within 23 h and more than half of these were in, or had completed, a fourth cell cycle. This implies a cell cycle time ranging between 6 and 8 h for most of the population. Descendants from progenitors in all parts of the epiblast tend to be aligned towards the primitive streak; the direction of clonal spread is very similar to the directional displacement of marked areas of the rabbit blastodisc (Daniel and Olson, 1966) and of the chick blastoderm (Spratt and Haas, 1965; Vakaet, 1984). Because the mouse epiblast is cup-shaped, lateral growth is mainly due to expansion towards the primitive streak of the material in the anterior part of the embryonic axis; axial growth is the result of expansion of epiblast just anterior to the streak and to continuous, but transitory, incorporation into the streak of the expanding lateral and progressively more anterior regions of epiblast.

Models for the growth of the embryo in utero during gastrulation have been presented previously (Snow, 1977; Poelmann, 1980). In Poelmann's model, which is...
based on cell counts, mitotic indices, degeneration indices and measurements of cell cycle duration, axial growth is unidimensional and axial cells, at least the 'frontal' cells anterior to the primitive streak, do not contribute to the expansion of the rest of the epiblast. In this model the bulk of epiblast growth is provided by the 'lateral epiblast' that expands the epiblast cup circumferentially and proximodistally. The results of the clonal analysis of HRP-labelled cells fail to support this model on two main counts: (1) axial growth is not uniform and 'frontal' cells at the early streak stage have produced most of the 'lateral' cells one day later; (2) expansion of the 'lateral' ectoderm is not equal in all directions: there is only limited spread of descendants anteriorly and proximodistally; the main thrust is posteriorly.

Snow's (1977) model is based on cell counts and mitotic indices. It rests heavily on the presence of a zone of high proliferative activity in a portion of the axis anterior to the primitive streak. The coherent expansion of the proliferative zone is seen as the driving force pushing the rest of the epiblast towards the primitive streak and causing exfoliation of mesoderm through the streak. The spread of clones labelled with HRP and the resulting fate map, as well as results from the rabbit (Daniel and Olson, 1966), are consistent with this model; however, clonal growth is not coherent and we did not find evidence of a proliferative zone. Although progenitors in the region just anterior to the streak (zone X in prestreak-stage embryos, zone XI in early-streak-stage embryos) produced relatively many labelled descendants (20.7 and 22.6 respectively, compared with an average of 13.4 for the remaining zones), the difference is not statistically significant, and certainly does not approach the magnitude expected from Snow's calculations. In order to match his proliferative data with the cell numbers generated by the epiblast between 6.5 days and 7.5 days in utero, Snow assumed that 25% of the daughters of each cell cycle of 3 h in the proliferative zone left the zone and adopted a cell cycle length of 6 h (compared with approx. 7 h in the remaining epiblast). If this reasoning applies to the cultured embryos, a progenitor labelled in the proliferative zone would be expected to have 8 times the number of descendants in 21 h as a progenitor from another zone, and the expected number of descendants from a proliferative zone progenitor might range from 16 to 64 or more. In the case of 64 descendants, about half of the cells would have gone through 7 cell cycles and would probably be unidentifiable because of dilution of the label (Lawson et al., 1986). A shorter culture period is required in order to test critically the presence of a proliferative zone in cultured embryos using HRP labelling.

An alternative possibility is that the primitive streak is responsible for aligning the epiblast cells: it could act as a sink, the source of which would be the proliferating population of the entire epiblast. Very little is known about the formation and maintenance of the primitive streak in mammals (Bellairs, 1986). Its initial appearance in a localised area at the junction of the epiblast and extraembryonic ectoderm defines the embryonic axis (Tam and Meier, 1982; Hashimoto and Nakatsuji, 1989); its formation is associated with (a) an increase in the frequency of mitotic spindles oriented perpendicularly to the cell sheet in the streak region, thus producing several cell layers (Snow and Bennett, 1978), (b) extensive disruption of the basal lamina (Poelmann, 1981a; Franke et al., 1983; Hashimoto and Nakatsuji, 1989), (c) increase in adhesive plaques and gap junctions (Batten and Haar, 1979) and (d) acquisition of vimentin and loss of cytokeratins by emerging primary mesenchyme cells (Jackson et al., 1981; Franke et al., 1982). The streak increases in length until the late streak stage, i.e., during most of the 24 h covered by this study, and mesoderm is being produced by it from its first appearance. Whereas the results from the clonal analysis can be largely accommodated by a model in which mesoderm is delaminated in situ (Poelmann, 1981a; Tam and Meier, 1982) with the cells of the expanding epiblast becoming incorporated into, and then being deposited by, the lengthening streak, the positions of the labelled cells relative to the site of injection make it likely that some cell migration by mesoderm, away from the streak, is also involved, as has been indicated by cinematography (Nakatsuji et al., 1986). It is noteworthy that clonal descendants in mesoderm were not obviously more scattered than those in the epiblast. However, in order to obtain an accurate picture of the transformations involved, computer-based three-dimensional reconstruction and analysis of the data is required.

The main body of the streak consists of a transitory population of cells during the first part of gastrulation. It is not clear whether this function is continued late into gastrulation: it has been suggested (Snow, 1985) on the basis of the development of explants and deficient embryos, and the normal reduction in the length of the streak, that the caudal 75% of the streak at midstreak and late streak stages is not a conduit for the passage of cells, but that the region is incorporated directly into allantois, hindgut, primordial germ cells, and tail bud. Although the contribution of the caudal part of the streak to extraembryonic mesoderm is complete by early somite stages, the middle part of the streak continues to produce lateral plate mesoderm from orthotopic grafts into late streak and older stage embryos (Tam and Beddington, 1987), and may be maintaining itself independent of further recruitment during early organogenesis (Tam and Beddington, 1986).

Whereas the cells forming the posterior part of the early streak are in transit and being continuously replaced by descendants of originally more lateral and anterior cells, the anterior end of the streak differs. Descendants from progenitors in zone X were found in the streak after culture as well as anteriorly in mesoderm and endoderm. It is thus possible that at least some of the cells at the anterior end of the streak form a stem cell population whose progeny will populate successively more posterior regions of the embryo.
Insertion of epiblast-derived cells into the endoderm layer had occurred by midstreak and late streak stages, primarily from epiblast at or near the anterior end of the presumptive or the overt streak. This result indicates an epiblast contribution to embryonic endoderm at an earlier stage than previously inferred from morphology (Jolly and Féręster-Tadé, 1936; Snell and Stevens, 1966; Poelmann, 1981b; Lamers et al. 1987) ectopic grafts (Levak-Svajger and Svajger, 1971, 1974) and general labelling of the endoderm (Kadokawa et al. 1987). The finding supports the putative epiblast origin of a subpopulation of endoderm cells that appears over the anterior end of the early primitive streak and then spreads, displacing embryonic visceral endoderm into the yolk sac (Lawson et al. 1987). Some of these cells have colonized the foregut by early somite stages and most of the remainder are localized within the region that will later be internalized into the embryonic gut (Kadokawa et al. 1987). The descendants of epiblast progenitors in endoderm found in the present study were not confined to the axial region, indicating that at least some epiblast-derived endoderm is inserted into visceral embryonic endoderm over a large area, as suggested by Kadokawa et al. (1987) and Tam and Beddington (1991), and not only from the head process. However, uncertainty over whether labelled endoderm cells were derived from epiblast in all embryos makes it impossible to give a detailed description of the localization of endoderm descendants. Also more prolonged culture is required to establish whether the descendants of epiblast in endoderm at midgastrulation will contribute to the gut and its derivatives, or are antecedents of definitive endoderm and not represented in the gut later in development. Similarly, longer term lineage tracing is necessary to document the allocation of epiblast cells to other organ rudiments.

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References


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