Complete dissipation of coherent clonal growth occurs before gastrulation in mouse epiblast

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SUMMARY

Observations on chimaeric mice argue that there must be considerable dispersal and intermingling of the clonal descendants of epiblast founder cells at an early stage in the development of the tissue. However, it has not been established when or how this occurs. Here we have used a genetic marker that enables donor cells to be visualized in situ to examine the early postimplantation distribution of clones obtained by transplanting epiblast founder cells into host blastocysts. We have also determined the spatial relationship between sister cells in non-chimaeric postimplantation epiblast by ionophoretic injection of a fluorescent macromolecule. Both experimental approaches support the conclusion that breakdown of coherent clonal growth accompanies epithelialization of the epiblast and is essentially complete by the onset of gastrulation.

Furthermore, the clonal analysis shows that descendants of different epiblast founder cells continue to intermingle extensively well into organogenesis. We suggest that this sustained intermingling of cells in the epithelial epiblast, which does not occur in the adjacent visceral endoderm, depends on cells losing contact with the basal lamina when they divide. These findings have implications both for patterning of the early amniote embryo and for the growth of tall columnar epithelia in general.

Key words: Epiblast growth, Clonal analysis, Epiblast founder cell, Intercellular bridge, Visceral endoderm, Coherent clonal growth, Fluorescein complexon, Tetramethylrhodamine dextran lysin biotin, Chimaera, Mouse

INTRODUCTION

Whilst there is little relative movement of cells during the preimplantation phase of development in the mouse (Mintz, 1965; Garner and McLaren, 1974; Kelly, 1979; Balakier and Pedersen, 1982), chimaeric fetuses and offspring typically show mosaicism that is both ubiquitous and fine-grained (Gearhart and Mintz, 1972; Ford et al., 1975; McLaren, 1976; Soriano and Jaenisch, 1986). Even clones formed by transplanting single early epiblast cells between blastocysts can yield melanocytes at all axial levels from the head to the tail (Gardner and Lyon, 1971; Gardner et al., 1985). Therefore, very extensive and sustained dispersal and intermingling of the descendants of individual epiblast founder cells must occur (Gardner, 1986). While it is difficult to envisage how such clones could become distributed so widely once gastrulation begins, before this stage the epiblast adopts an epithelial organization which might be expected to militate against cell dispersal. Support for the lack of cell mixing in the epiblast prior to gastrulation is based on limited observations on early postimplantation chimaeras (Rossant et al., 1983; Rossant, 1985). However, since these chimaeras were made by transplanting whole inner cell masses (ICMs) rather than dissociated cells between blastocysts, and the donor and host blastocysts were from different species of mouse, they are of doubtful relevance to the normal situation.

Single epiblast cells labelled in situ at the beginning of gastrulation form non-coherent clones which, depending on their initial location, may undergo considerable axial extension (Lawson et al., 1991). Interestingly, even at this early stage sister cells – and occasionally additional close relatives that could be identified by spread of the lineage label via persisting intercellular bridges – were frequently not adjacent to injected cells (Lawson et al., 1991). This suggests, contrary to the interspecific chimaera study, that cell mingling may indeed begin before gastrulation.

The only epithelium of the early mouse conceptus whose early postimplantation growth has been analysed clonally is the visceral endoderm in which clones clearly retain coherence (Gardner, 1984, 1985; Cockroft and Gardner, 1987; Lawson et al., 1991). However, there is no reason to suppose that all simple epithelia will grow in the same way. In tall columnar epithelia like those of the small intestine, embryonic neuroepithelium and mammalian epiblast, cells evidently relinquish contact with the basal lamina when they divide (Jinguji and Ishikawa, 1992; Reinsch and Karsenti, 1994). The scope for cell mingling would therefore seem to be greater in these epithelia than in the shorter ones in which cells remain attached to the basal lamina throughout the mitotic cycle (Zelligs and Wollman, 1979). However, without clonal analysis the extent of cell mingling in different epithelia cannot be assessed.
We have used two distinct strategies to investigate clonally the growth of mouse epiblast before gastrulation. The first was to transplant early epiblast cells from blastocysts carrying a marker transgene into non-transgenic host blastocysts so that the distribution of the resulting clones could be visualized in situ at early postimplantation stages. The second, which avoids any artefacts that may be associated with the dissociation and transplantation of cells, was to exploit spread of a fluorochrome-tagged macromolecular label from injected to uninjected cells via persisting intercellular bridges (Goodall and Johnson, 1984; Lawson et al., 1991), to determine the spatial relationship between sister cells in situ.

MATERIALS AND METHODS

5th day postcoitum (p.c.) implanting blastocysts recovered from PO female mice mated to males that were homozygous for the ROSA26-B-geo transgene (Friedrich and Soriano, 1991) were dissected microsurgically so as to isolate the inner cell mass (ICM) in its entirety or separate the epiblast from the primitive endoderm (Gardner, 1982). Once isolated, the tissues were dissociated and either single cells or tightly adherent presumed sister pairs were injected into 4th day p.c., blastocysts from matings between non-transgenic PO mice, as described elsewhere (Gardner, 1978; Gardner and Rossant, 1979). The injected blastocysts were transferred to the uteri of 3rd day p.c. pseudopregnant PO females for recovery of postimplantation conceptuses between the recipients’ 6th and 9th day p.c. Conceptuses recovered on the 6-8th day p.c. were fixed and stained for lacZ activity (Beddington and Lawson, 1990) after reflection of Reichert’s membrane, whilst both the amnion and the visceral yolk sac were torn open before fixation of those recovered on the 9th day. Following staining for up to 48 hours, conceptuses were rinsed, photographed and then stored in Zamboni’s fixative prior to detailed examination as whole mounts or after wax embedding and serial sectioning.

For the in situ lineage-labelling of individual epiblast or visceral endoderm cells, POxPO conceptuses were recovered on the 6th or 7th day p.c. The label, 10 kDa tetramethylrhodamine-dextran-lysine-biotin (TMRDLB, Molecular Probes, Inc), was injected ionophoretically as described elsewhere (Gardner et al., 1992) using alternation of differential interference contrast and epifluorescence optics until penetrated cells fluoresced very strongly. 1-5 well-spaced cells were injected per 6th day conceptus and 1-7 cells per 7th day conceptus. After each injection, conceptuses were viewed at intervals by fluorescence microscopy to check for spread of the label, which invariably occurred within a few minutes, or not at all. Only if one or more uninjected cells became stained unequivocally above background was the label recorded as having spread. Fluorescein Complexon (FC, Kodak), a fluorochrome of 710 Da that does not exhibit obvious intracellular binding (Fraser and Bryant, 1985), was injected into 7th day epiblast cells similarly, as a 5% solution in 0.1 M KCl.

Finally, to confirm the location of mitoses within epiblast and visceral endoderm, several 7th day p.c. pregnant females were killed 2.0-2.5 hours after they had been injected intraperitoneally with nocodazole at 1 μg/gm body weight. The conceptuses were then fixed in situ in Zamboni’s fluid after the mesometrial part of the decidua had been trimmed away and embedded in methacrylate (Polysciences, JB4 embedding kit) for sectioning at 4 μm.

RESULTS

Epiblast versus primitive endoderm chimaeras

Clones were generated by transplanting into 4th day p.c. non-transgenic blastocysts, single or very firmly attached (presumed sister) pairs of very early epiblast, primitive endoderm or unselected cells from 5th day p.c. blastocysts that were heterozygous for the ROSA 26-B-geo transgene. All except one of the resulting 50 conceptuses that showed staining for lacZ activity could be classified readily as epiblast or primitive endoderm chimaeras (Table 1). The exception had weak staining throughout the egg cylinder and in the core of the ectoplacental cone and was, therefore, presumed to be a degenerating chimaera. In each of three chimaeras recovered on the 7th day p.c., donor cells formed the entire epiblast of a tiny supernumerary egg cylinder that shared a common parietal yolk sac with a normal-sized cylinder composed wholly of host cells (Fig. 1A). In the remaining 28 epiblast chimaeras, the donor cells contributed variably to a single conceptus. In 6th day p.c. epiblast chimaeras, the donor cells were typically localized in one or two fairly circumscribed foci (Fig. 1C,D). In striking contrast, no vestige of coherent clonal growth of the donor cells was discernible in any 7th day epiblast chimaera. This was particularly obvious in specimens with a relatively minor donor contribution where the
degree of intermingling of donor and host cells appeared similar throughout the tissue, giving it an essentially uniform, ‘pepper-and-salt’ appearance in whole-mount preparations (Fig. 1A). The 7th day epiblast chimaeras were judged to be either pregastrulation or at a very early primitive streak stage because, unlike all of the 8th and 9th day specimens, staining was either absent altogether from the extraembryonic region or confined to its most distal extremity. Not only did a ubiquitous donor contribution persist in all 8th and 9th day epiblast chimaeras as expected but, surprisingly, the very intimate admixture of donor and host cells was also maintained. Thus, even in 9th day chimaeras, very fine grained mosaicism was evident both in the developing brain and elsewhere (Fig. 2C,D).

The spectacular dissipation of coherent growth of clones observed in the epiblast contrasted markedly with the situation in the adjacent primitive endoderm-derived visceral endoderm. Here, on the 7th as well as the 6th day, donor cells were segregated in one or more coherent patches regardless of their location within the tissue. Whilst coherent growth of the visceral endoderm ceased thereafter in the embryonic region where its cells suffer displacement or death during their replacement by epiblast-derived fetal endoderm (Lawson et al., 1987), it persisted in the extraembryonic region (Fig. 2B).

**In situ labelling of epiblast cells**

Two patterns of organization of the epiblast were distinguishable amongst 28 6th day conceptuses used for TMRDBL injections. 21, all of which lacked a proamniotic cavity (pac), showed no sign of epithelial organization, either grossly, or according to the shape and arrangement of labelled cells. The latter were most commonly stellate rather than elongated and, from the disposition of sisters identified by spread of the label, the tissue was clearly multi-layered. Furthermore, mitotic cells were seen adjacent to the basal lamina as well as away from it. In contrast, in the remaining 7, of which 5 had a pac, the epiblast had an epithelial organization with cells arranged radially like the spokes of a wheel. In these conceptuses, individual labelled cells were typically elongated orthogonally to the basal lamina with their apices located centrally where mitoses appeared to be restricted and multilayering was no longer discernible. In 38 of the 80 successful 6th day epiblast cell injections, the label spread unequivocally to one or more uninjected cells (Table 2). In all except 5 of the 38 cases where the label spread, it did so to an adjacent cell. 3 of the remaining 5 cases, where spread was to a non-adjacent cell, were in epiblast that had become epithelial.

Unequivocal transfer of label was recorded in 37 of 97 epiblast cells that were injected successfully in a series of 22 7th day p.c. conceptuses, and the frequency with which it spread to a non-adjacent cell was significantly higher than in the 6th day injections (Table 2; Fig. 1E). Not infrequently, such non-adjacent pairs of cells were connected apically by a thin process that was clearly delineated by fluorescence microscopy. Whilst in 7th day conceptuses the epiblast was

**Table 1. Development and chimaerism following transplantation of cells between +/ROSA26 and +/- blastocysts**

<table>
<thead>
<tr>
<th>Day p.c. of analysis</th>
<th>No. of blastocysts transferred</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
<th>No. of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All recipients</td>
<td>Pregnant recipients</td>
<td>No. of decidua</td>
<td>No. of concepts</td>
<td>Total</td>
<td>Epiblast</td>
<td>Endoderm</td>
<td>Chimaeras</td>
<td>Total</td>
<td>Epiblast</td>
<td>Endoderm</td>
</tr>
<tr>
<td>6th</td>
<td>131</td>
<td>126</td>
<td>79</td>
<td>70</td>
<td>14*</td>
<td>8</td>
<td>5</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7th</td>
<td>40</td>
<td>35</td>
<td>34</td>
<td>33</td>
<td>20</td>
<td>15**</td>
<td>5</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8th</td>
<td>36</td>
<td>24</td>
<td>23</td>
<td>21</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9th</td>
<td>54</td>
<td>48</td>
<td>35</td>
<td>34†</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>261</td>
<td>233</td>
<td>171</td>
<td>158</td>
<td>50</td>
<td>31</td>
<td>18</td>
<td>32</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*1 conceptus that was stained throughout was classified as a degenerating chimaera.

**Includes 3 cases where donor cell(s) had formed the entire epiblast of a small supernumerary egg-cylinder.

†One conceptus was abnormal, being composed of a non-chimaeric empty yolk sac vesicle.
invariably a tall epithelium surrounding a prominent pac, the arrangement of cells revealed by the injection of label proved surprisingly variable. Labelled cells did not always have their long axis strictly orthogonal to the basal lamina. Furthermore, where a labelled uninjected cell was adjacent to an injected one, the two were not necessarily in contact throughout their length. They could be together basally but splayed well apart apically or vice versa, or even in contact both apically and basally whilst clearly separated in their mid-region.

It proved difficult to obtain selective labelling of mitotic cells in the apical region of 7th day epiblast. However, in none of the 11 cases where this was achieved could an extension from such cells to the basal lamina be detected by fluorescence microscopy.

Although spread of TMRDBL was more frequent in 6th and 7th day visceral endoderm than in corresponding epiblast injections, only in a single instance was it to a cell that was not adjacent to the injected one (Table 2).

Fluorescence was confined to the injected cell in 8 of 53 injections of FC in 7 day epiblast. In more than half the cases where the FC spread (25/45 =56%), it did so radially from the injected cell to its immediately surrounding neighbours and beyond. In the remaining 20 cases, spread was preferential, either to a single adjacent cell (11 cases) or to a single non-adjacent cell (9 cases).

Finally, mitoses were very abundant in the epiblast of sectioned 7th day p.c. conceptuses recovered from females that had been injected with nocodazole and were invariably located apically in the tissue well away from the basal lamina (Fig. 3A-C). In contrast, all mitotic cells in both the adjacent and extraembryonic visceral endoderm were in contact with the basal lamina (Fig. 3C,D).

**DISCUSSION**

In the transplantation experiments, donor and host cells were largely segregated in the epiblast of chimaeras recovered on the 6th day when, in most cases, this tissue was not yet epithelial. In chimaeras recovered 1 day later when the epiblast was invariably organized as an epithelium around a more or less prominent pac, donor cells were distributed throughout it and intimately admixed with host cells, a situation that persisted through gastrulation. In striking contrast, and in accordance with the results of previous studies (Gardner, 1984, 1985; Cockroft and Gardner, 1987; Lawson et al., 1991), donor cells colonizing the adjacent visceral endoderm were arranged in coherent patches in chimaeras recovered at all stages. Therefore, particularly since both types of donor cell often came from the same blastocysts, the dispersal of clones in epiblast between the 6th and 7th day is clearly tissue-specific and not just a peculiarity of the particular combination of genotypes that were used to make the chimaeras.

To avoid any artefacts that might be associated with cell

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**Table 2.** In situ labelling of individual epiblast vs visceral endoderm cells with TMRD in 6th and 7th day p.c. conceptuses

<table>
<thead>
<tr>
<th>Day p.c.</th>
<th>Tissue</th>
<th>No. of conceptuses</th>
<th>Total no. of cells injected</th>
<th>No. of cases of spread of label</th>
<th>To adjacent cell</th>
<th>To non-adjacent cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>6th</td>
<td>Epiblast</td>
<td>28</td>
<td>80</td>
<td>38*</td>
<td>33</td>
<td>5†</td>
</tr>
<tr>
<td>7th</td>
<td>Epiblast</td>
<td>22</td>
<td>97</td>
<td>37**</td>
<td>19</td>
<td>18†</td>
</tr>
<tr>
<td>6th</td>
<td>Endoderm</td>
<td>11</td>
<td>62</td>
<td>43</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>7th</td>
<td>Endoderm</td>
<td>6</td>
<td>45</td>
<td>29</td>
<td>28</td>
<td>1</td>
</tr>
</tbody>
</table>

*A single uninjected cell became positive in 31 cases, two cells in 6 cases and 3 in the remaining case. In all 7 cases where 1 uninjected cell was positive, one was adjacent to the injected cell and, in 6 cases, also in direct contact with the other(s). In the 7th, the label had spread both to a cell adjacent to the injected one and, via a discernible intercellular bridge, equally to a second one that was not adjacent to either.

**A single uninjected cell became positive in 34 cases and two uninjected cells in the remaining 3. In two of the latter, the pair of uninjected cells were adjacent and next to the injected one and in the 3rd, separate but with one member adjacent to the injected cell.

†Difference significant P>=0.001 by contingency $\chi^2$.

+ Injected cells were a mixture of squamous (23), cuboidal (7) and low columnar (15). The single case of spread to a non-adjacent cell occurred following injection of a cuboidal cell.
dissociation and transplantation, single epiblast versus visceral endoderm cells were also labelled in situ with TMRDBL. The aim was to exploit spread of the label from injected to uninjected cells as a way of determining the spatial relationships between the products of recent mitoses. The findings accord well with chimaera data. Thus, in the case of the visceral endoderm, spread of TMRDBL was overwhelmingly to an adjacent cell on both 6th and 7th day. In the epiblast, by contrast, this label spread occasionally to non-adjacent cells on the 6th day, more often in epiblasts that were already epithelial, and significantly more frequently on the 7th day. This implies that, by the onset of gastrulation, sister cells in the epiblast often become separated before completing the cycle in which they were born. The validity of this conclusion obviously depends on the premise that TMRDBL can only spread between cells via the intercellular bridge which forms during cytokinesis and continues to unite the products of mitosis for some time thereafter (Mullins and Bieseie, 1977). The only other way in which molecules have been shown to move directly from the cytoplasm of one cell to that of another is via gap junctions. However, the molecular mass of TMRDBL (10 kDa) is about ten-fold greater than the upper size limit estimated for mammalian gap junctions (Flagg-Newton et al., 1979). Furthermore, whilst in 7th day epiblast cell injections, FC (710 Da) frequently spread to several cells in a radially symmetrical pattern following injection, TMRDBL fluorescence was seldom recorded in more than one uninjected cell. Since gap junctional communication may be suspended during mitosis (Goodall and Maro, 1986; Xie et al., 1997), the relatively common failure of spread of FC or its spread to only one cell may reflect the rapid growth rate of the epiblast at this stage. This in turn suggests the sister pairs identified by spread of TMRDBL are either relatively young or, alternatively, that considerable time is required before they become sufficiently reintegrated into the epithelium for gap junctional communication with their neighbours to be restored.

Collectively, the present findings argue that dissipation of coherent clonal growth begins in the epiblast when it embarks on the transition from a solid ball of cells to an epithelium on the 6th day p.c. and is essentially complete by the onset of gastrulation some 24-30 hours later. Although the increase in cell number in the epiblast before gastrulation is roughly double that in the adjacent visceral endoderm (Snow, 1976), this difference seems insufficient to account for their strikingly dissimilar patterns of growth. However, the two tissues also differ in form, the visceral endoderm varying regionally between a squamous and a low columnar epithelium and the epiblast being a tall, pseud stratified one (Kaufman, 1992).

This means that in the epiblast, unlike the visceral endoderm, dividing cells must relinquish contact with the basal lamina if they are to maintain tight junctions with their neighbours throughout mitosis (Zelligs and Wollman, 1979; Reinsch and Karsenti, 1994). Consistent with this is the finding that mitotic cells are invariably located apically in epithelial epiblast (Snow, 1977; and present study) and that no extension of such cells to the basal lamina was detectable under conditions of fluorescence microscopy that enabled visualization of the thin intercellular bridge linking sister cells apically.

As cells that are about to divide move apically, neighbours clearly spread basally to occupy the space they have vacated (see Fig. 3). Therefore, once a cell has divided, its two daughters must find routes whereby they can extend to re-establish contact with the basal lamina. As is shown schematically in Fig. 4, this would seem to offer considerable scope for separation of sister cells, particularly if cytokinesis involves incursion of neighbouring cells into the cleavage furrow so that the newly formed cell pair becomes separated once the bridge between them is released onto the luminal surface (Jinguji and Ishikawa, 1992). There are two features of the epithelial epiblast that would greatly facilitate dispersal of clones by this means. One is that, since all cells in the tissue are cycling rapidly and asynchronously (Solter et al., 1971; Snow, 1976), small separations of apically produced daughter cells may rapidly increase as the descendents of subsequent cell divisions insert between them. The other is that because the tissue is a narrow cylinder with a hemisphere at one end, interphase cells are wedge-shaped (Kaufman, 1992). Hence, unless newly formed sister cells share the same route for extension, they will normally become more widely separated from each other once they have reattached to the basal lamina than whilst they are still at the apical surface of the epithelium. Furthermore, given the small size of the pregastrulation epiblast, the distances required for complete dispersal of clones are modest.

The present findings contrast with those in lower vertebrates such as Xenopus and the zebrafish in which there is little cell mingling in any part of the embryo before gastrulation (Jacobson and Klein, 1985; Kimmel and Law, 1985) when morphogenetic movements such as epiboly and convergent-extension occur. They also have wider implications for development in mammals and, by extension, amniotes generally. First, the rapid and sustained intermingling of cells that characterizes the early growth of the epiblast would seem to be incompatible with either the occurrence of regional differentiation or the perpetuation of positional information within the tissue prior to gastrulation. Therefore, specification of the locus of the primitive streak, for example, is likely to depend on information that resides elsewhere. The visceral endoderm is an attractive candidate: not only is it adjacent to the epiblast but it continues to grow in a coherent way that would allow positional information to be conserved (Gardner, 1984; Lawson et al., 1987). There is, furthermore, some evidence for localized gene expression before
gastrulation in this tissue (reviewed in Gardner, 1998). Second, because fine-grained mosaicism persists through to organogenesis, the number of epiblast founder clones contributing to the individual tissue primordia of the fetus will approach the maximum possible according to the total number of cells that are allocated to them. Consequently, genetic mosaicism arising in cells of the epiblast lineage prior to gastrulation, due to chromosomal non-disjunction or other errors that might impair their later differentiation, will be distributed to many or all tissue primordia. This may not disrupt development as catastrophically as if one or more tissues were composed largely or entirely of defective cells, as would be the case if the epiblast continued to grow coherently. However, where the physiology rather than the viability of cells is primarily compromised by mosaicism, such extensive dispersal and intermingling of epiblast founder clones would tend to maximize any adverse effects.

Many epithelia that occur either transitorily in embryos or persist to maturity belong to the tall columnar or pseudostratified categories and are therefore likely to resemble the epiblast in their later differentiation, will be distributed to many or all tissue primordia. Consequently, genetic mosaicism would tend to maximize any adverse effects.

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