J. Embryol. exp. Morph. Vol. 42, pp. 293–303, 1977
Printed in Great Britain

Gastrulation in the mouse: Growth and regionalization of the epiblast

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SUMMARY

Histological determination of cell numbers in the mouse embryo between 4½ and 7½ days *post coitum* show that growth during this period, in which gastrulation occurs, is not uniform. Prior to primitive streak formation mean cell generation time is about 9 h. Coincidental with the appearance of the primitive streak the embryo enters a period of rapid growth, lasting about 24 h, during which the mean cell generation time must be about 5 h in order to account for the increase in cell numbers. A more detailed study, in which variations in mitotic activity in different regions of the embryo have been analysed, has identified a small region, the so-called 'proliferative zone', constituting about 10 % of the whole epiblast, in which cell generation time may average as little as 2–3 h over a 24 h period. The cell generation time for other epiblast regions is estimated at about 6·5 h.

It is calculated that the proliferative zone, in the 24 h period commencing with primitive streak formation, could generate about half the cells in the 7½-day embryo. The topographical consequences of such a rapidly expanding region in the embryo are discussed in the light of other, circumstantial evidence, and it is postulated that the cells generated in the PZ may constitute the ectoderm of later stage embryos.

INTRODUCTION

The morphological changes associated with primitive streak formation and mesoderm production in the mouse were first correctly described by Fraser (1883). More detailed microscopical analyses were later provided by Jenkinson (1900), Sobotta (1903, 1911) and Huber (1915) for both mouse and rat. The development of these two rodent species is essentially identical, differing only in scale and timing. Very little was added to that knowledge in the succeeding 60 years.

Recently interest in the rodent early post-implantation stages has been revived as a result of the following series of observations. (1) In female mouse embryos changes in the genetic expression of one X-chromosome occur during this period of development (Gardner & Lyon, 1971; Kozak & Quinn, 1975). (2) Chromosome replication behaviour changes, both in sex chromosomes and in autosomes (Takagi, 1974). (3) The propensity for making teratocarcinomas

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is lost sometime during, or shortly after, gastrulation (Solter & Damjanov, 1974), and in the same period. (4) The embryonic ectoderm (the epiblast of Fraser, 1883) loses its ability to form teratomas containing mesodermal and endodermal derivatives (Skreb, Svajger & Levak–Svajger, 1976). (5) In mouse aggregation-chimaeras a downward regulation in embryo size takes place shortly before primitive streak formation (Buehr & McLaren, 1974).

The concensus of these papers, particularly those involving teratoma production, when taken in conjunction with the elegant cell lineage studies of Gardner and his colleagues (see Gardner & Papaioannou (1975) for references), suggest that the epiblast is a homogeneous population of pluripotent undifferentiated cells. Skreb *et al.* (1976) concluded also that it is unlikely that the tissue is spatially regionalized into presumptive ectoderm, mesoderm and ectoderm, a conclusion apparently supported by my own findings (Snow, 1976).

This report extends my earlier observations and also describes a detailed analysis of mitotic activity in the epiblast which indicates that this tissue is not homogeneous but is regionalized in a way which may permit the epiblast to be mapped as a mosaic of presumptive ectoderm, mesoderm and endoderm cells in much the same way as the blastulae of more primitive vertebrates.

MATERIALS AND METHODS

Embryos of the randomly bred Q strain of mice were used throughout. The methods for determining cell number have been described in detail elsewhere (Snow, 1976). Briefly, two approaches were used:

(i) Embryos were serially sectioned and camera lucida drawings were made of each section, outlining the tissues present. The area of each tissue in each section was measured using a planimeter and its volume calculated from a knowledge of section thickness. Additive reconstruction of the embryo from these data gives tissue volumes. Cell volume was determined from several high power camera lucida drawings of measured areas of various regions in each embryo. Cell number in these areas was calculated using Abercrombie's (1946) formula for converting total nuclear counts to nuclear (=cell) midpoints. Cell numbers are then estimated by simple arithmetic.

(ii) Many embryos at the egg-cylinder stages are very regular in shape and estimates of tissue volume can be made by regarding the embryo as a cylinder with either a half spheroid cap on one end or a plug in one end. In these instances measurements of cylinder length and several cross-sectional diameters (which reveal that egg-cylinders vary from circular to elliptical in cross-section) are used in the appropriate equation for calculating cylinder and hemispheroid volume. This method is only applicable to regular-shaped embryos when it yields values which do not differ significantly from serial reconstructions. In this paper (and in the earlier publication, Snow (1976)) the values from both methods have been pooled.

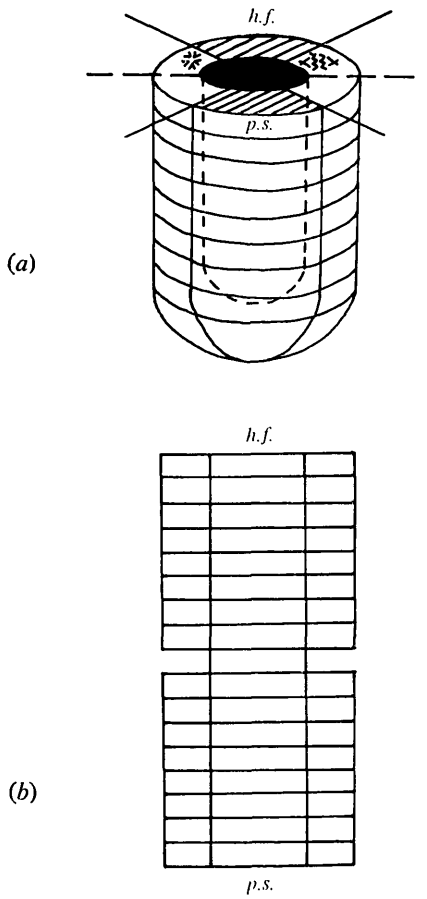

Fig. 1. (*a*) The model of the embryonic part of the egg cylinder on which the metaphase/anaphase index analysis is based. (*b*) The plan on which the M/A index is mapped. Detailed description of these models is given in the text. *p.s.*, Primitive streak; *h.f.* head fold.

For the analysis of mitotic activity $6\frac{1}{2}$-, 7- and $7\frac{1}{2}$-day embryos were cut in cross-section only and serial camera lucida drawings were made of every one. Only metaphases and anaphases were scored (see Discussion). The distribution of divisions was mapped on a common plan constructed as follows. Fig. 1(*a*) shows a diagram of the embryonic portion of the egg-cylinder represented as a stack of cross-sectional slices. If the primitive streak can be identified each mitotic division can be mapped into an appropriate sector of each section as indicated. The primitive streak sector and the head fold sector (on the opposite side of the cylinder) are mapped as quadrants and the lateral epiblast as octants. To reduce this data to the common plan each embryo is regarded as being composed of eight slices, the lower surface of the first slice being level with the bottom of the pro-amniotic or amniotic cavity, and the upper surface of the

eighth slice being the junction of epiblast and extra-embryonic ectoderm. Since embryos were routinely cut in 10 μm sections there were invariably more than eight sections and sometimes as many as 30. These were distributed uniformly to the eight slices, e.g. the slices of a 16-section embryo have 2 sections per slice, an 18-section embryo 2·25 per slice and so on. Mitotic divisions in sections which spanned the junction between adjacent slices were apportioned according to the fraction of the section lying in each slice, e.g. a dividing cell may be scored as 0·75 in slice 3 and 0·25 in slice 2. Each division is therefore only recorded once.

The map showing distribution of mitotic activity is then made by imagining the egg-cylinder cut down its lateral sides (between the octants) and opened out flat as indicated in Fig. 1*b*. The longitudinal axis of the embryo is indicated by the primitive streak (*ps*) and head fold (*hf*) ends of the map. The hatched compartment in the centre represents that group of cells directly below the bottom of the amniotic cavity – it is not necessarily of comparable size to the other compartments in the mid line because of variation in the shape of the amniotic cavity; it is rarely smaller.

The metaphase/anaphase (M/A) index which is recorded on this map is the percentage of cells in each region in those stages of mitosis. The number of cells in each compartment is calculated from a knowledge of the numbers of cells in each sector of each section which in turn is derived from high magnification camera lucida drawings of various regions in each embryo as described previously (Snow, 1976). For the calculation of average M/A indices for each embryonic stage the number of cells and the number of divisions per compartment are totalled separately for a number of embryos. The M/A index is then calculated from these figures.

RESULTS

In Q-strain mice the primitive streak appears at about $6\frac{1}{2}$ days *pc* and the period of development studied is from $5\frac{1}{2}$ to $7\frac{1}{2}$ days.

Cell numbers

The data are shown in Table 1, which includes my previously published findings (Snow, 1976). Since it is currently believed that the primary endoderm of the egg-cylinder does not contribute to the later stage embryo and foetus (Gardner & Papaioannou, 1975) the point of interest in this table is the growth of the $5\frac{1}{2}$-day epiblast and the production from it of post primitive streak ectoderm and mesoderm. It is not meaningful to assess the rate of growth of ectoderm and mesoderm separately by the methods used in the cell number analysis (see discussion) so in Table 2 the total numbers of cells generated from the epiblast is shown, together with the average cell generation times necessary to account for this increase. Growth is not uniform: there is an increase in cell proliferation rate which coincides with primitive streak appearance, and persists for nearly 24 h.

Table 1. *Total cell numbers in the embryonic germ layers*

Age (days *p.c.*)	No. of embryos (no. of litters)	Number of cells		
		Endoderm	Mesoderm	Epiblast/ ectoderm
5½	14 (5)	95	—	120
6	6 (3)	130	—	250
6½	13 (5)	250	—	660
7	7 (2)	430	1220	3290
7½	16 (5)	680	6230	8060

Table 2. *Mean cell cycle times required to account for the growth of the epiblast*

Age (days *p.c.*)	5½		6		6½		7		7½
No. of cells	120		250		660		4510		14290
No. of divisions		1·04		1·32		2·71		1·58	
Mean cell cycle		11·5		9·1		4·4		6·7	

Table 3. *Metaphase/anaphase index (%) for whole embryos*

Age (days) ...	5½	6	6½*	6½†	7	7½
Ectoderm	4·2	4·6	3·8	6·9	4·6	4·1
Mesoderm	—	—	—	—	1·5	2·4

* Pre-primitive streak
† Primitive streak present.

Anterior (*h.f.*)

6·8	7·4	5·9
4·6	3·3	6·0
3·9	5·4	6·0
4·7	4·3	9·4
6·4	9·6	7·2
7·2	13·2	3·2
4·9	15·2	4·9
4·9	10·0	9·5
	9·2	
5·8	9·0	6·0
5·3	11·2	5·2
7·0	2·2	3·2
8·5	2·8	2·9
4·6	4·8	3·2
4·5	8·3	3·6
5·0	4·1	7·0
4·6	1·3	6·8

(*p.s.*) Posterior

Fig. 2. Map of the M/A index in the various regions of 6½-day primitive streak-stage embryos.

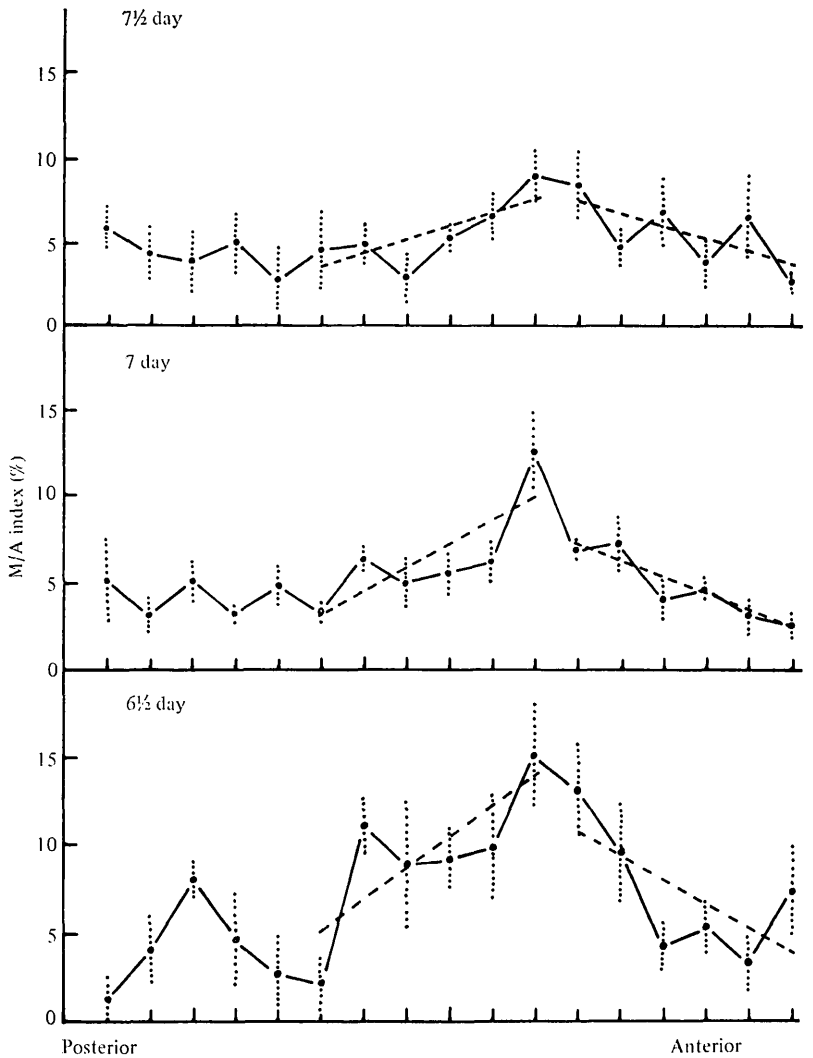

Fig. 3. The M/A index along the posterior–anterior axis of 6½-, 7- and 7½-day embryos. The data is taken from maps such as that shown in Fig. 2. The regression lines (– – –) away from the proliferative zone were plotted according to the formula $y = b_0 + b_1 X$. The correlations and regressions are significant at the 1 % level in all but the posterior slope at 7½ days which is significant at the 5 % level. The vertical lines (. . . . .) represent ±1 standard error.

Mitotic activity

In the epiblast/ectoderm of these embryos the mitotic divisions are located at the luminal surface of the pseudo-stratified tissue, except in the primitive streak region of the more advanced 6½-, and 7-day embryos. Here mitoses can be found at all levels of the tissue including adjacent to the endoderm.

Table 3 shows the overall metaphase/anaphase index for the epiblast and its

Table 4. *The proliferative zone; size and mitotic activity compared to the remaining epiblast*

Age (days) ...	6½	7	7½
Proportion of epiblast (%)	9	7	7
No. cells (approx.)	60	230	600
M/A index (%), PZ	15	10·3	9·2
M/A index, other epiblast	6·4	4·4	3·9

derivatives. One obvious implication of these data is that ectoderm proliferates more rapidly than mesoderm. The difference between the two classes of 6½-day embryos is also noteworthy.

Closer analysis of six primitive streak-stage 6½-day embryos, and of four each at 7 and 7½ days reveals an uneven distribution of mitotic activity in the epiblast/ectoderm. Figs. 2 and 3 show these data. At 6½ days there is an obvious region in the mid line, slightly towards the anterior end with a significantly higher M/A index than the remaining epiblast ($P < 0{\cdot}001$). I have called this region the proliferative zone (PZ); it can also be identified at 7 and 7½ days but because of a technical artifact is not so obvious in 7½-day embryos. The artifact is one of sample size. For the 6½-day embryos each of the compartments in the mid line represents about 20 cells per embryo whereas at 7½ days it is about 250 cells (yielding total cell numbers for the map compartments of 120 and 1000). As a result the PZ overlaps more compartments at 6½ days than at the later stages. Re-examination of each embryo, and of others cut in longitudinal section, has permitted closer delimitation of the PZ. Table 4 indicates the approximate size of this zone, the number of cells involved and the M/A index compared to that of the remaining epiblast/ectoderm. Throughout this period of development it is a small region occupying less than 10 % of the embryo which has about a 2½ times greater mitotic activity than the rest.

DISCUSSION

Gastrulation in many species involves the co-ordinated movement of coherent sheets of cells. In those organisms in which such movements have been followed with suitable cell markers maps have been constructed which designate areas on the blastula according to their developmental fate. Such maps have not been made for mammalian embryos although Daniel & Olsen (1966) demonstrate cell movements in the rabbit embryonic shield which are similar to those which occur in the chick (Spratt & Haas, 1960). On the contrary, the micro-surgical subdivision of the rat epiblast and subsequent demonstrations that each piece is capable of generating teratomas containing ectodermal, mesodermal and endodermal derivatives suggests that it may be unjustified to consider the epiblast as composed of *areas* of presumptive ectoderm, mesoderm and endoderm.

Table 5. *Estimated cell cycle times (h) for various regions of the embryo (see text for details)*

Age (days) ...	6½	7	7½
Epiblast/ectoderm	4·8	7·2	8·1
Mesoderm	—	22·2	13·9
Proliferative zone	2·2	3·2	3·6
Other epiblast	5·1	7·5	8·5

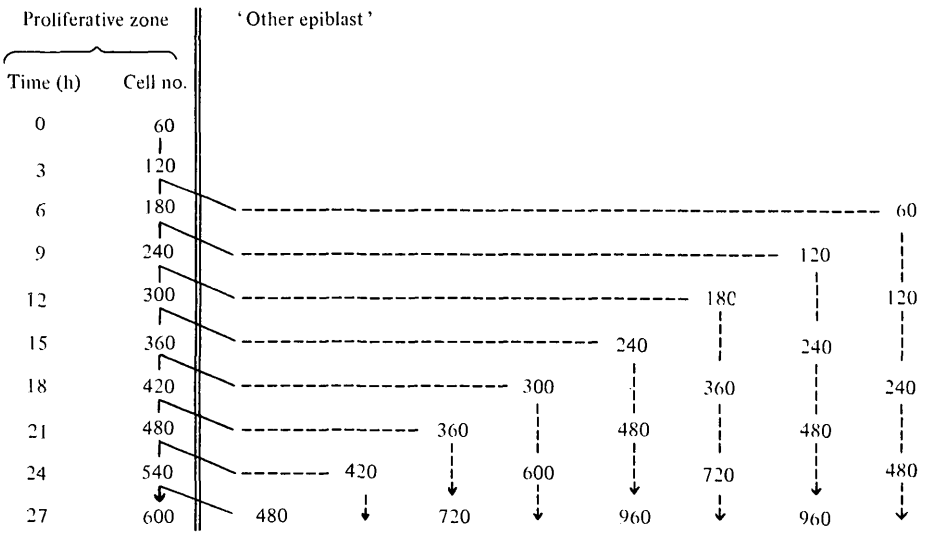

Fig. 4. Model for the growth of the proliferative zone and of the cells generated in that site. A linear increase in size of the PZ has been assumed with the balance of cells produced migrating out of the zone into the 'other epiblast' where they proliferate more slowly, with a 6 h cycle time. A total of 5940 cells is generated. If it is assumed that the growth of the PZ is geometrical, with an increase of 30% each cycle, then such a model produces some 4500 cells.

The data described above nevertheless show a marked regionalization within the epiblast with respect to mitotic activity. Can this indicate other differences between the cell populations? The precise whereabouts in the 7½-day embryo of cells generated in the PZ is not known at present but there is circumstantial evidence to suggest that they may be ectodermal. In homozygous t^{w18} mutant embryos the proliferative zone region behaves normally but the remaining epiblast shows severe mitotic disturbances (Snow, in preparation). The t^{w18} allele manifests itself at the primitive streak-stage by the production of aberrant mesoderm. The ectodermal component of the embryo seems normal (Artzt & Bennett, 1972; Spiegelman & Bennett, 1974; Spiegelman, 1976).

Are the kinetics of cell proliferation in the epiblast consistent with this hypothesis?

The M/A index was scored with a view to using the data to calculate cell

cycle times in the various regions of the embryo. The duration of metaphase/anaphase in mouse and rat is about 20 min (Knowlton & Widner, 1950; Luschbaugh, 1956; Dustin, 1959; Leblond, 1959; Caltaneo, Quastler & Sherman, 1961) and is a much less variable entity than mitosis duration (see Cleaver, 1967). Cell cycle times can be calculated from the equation

$$T = \frac{\text{M/A duration}}{\text{M/A index}} \times 100$$

provided the following conditions pertain: (1) the cell population is randomly dividing, (2) all cells divide, and (3) cells are not lost from the population.

Although individual embryos show regions of synchrony (see below) data pooled from several embryos approximates to a randomly dividing population. In accounting for the cell number increase it seems unlikely that a non-dividing population exists so it is assumed that condition (2) is met, and as the boundaries of the cell populations are clearly defined cell loss by death is the only complication in condition (3). Although some cell death is observed it is not extensive and not associated particularly with stage or region. It does not occur in all embryos. For the ensuing discussion it is disregarded.

Cell cycle estimates from the M/A data result in the figures shown in Table 5. The close similarity between these estimates and those from the cell number analysis (Table 2), with respect to the whole epiblast, is reassuring, particularly as the analyses are separate and independent of one another. The PZ emerges as a site of extraordinarily rapid cell proliferation. Mesoderm on the other hand seems to divide very slowly and in the 24 h under consideration would contribute little to its own increase. Hence most of the early mesoderm must be derived directly from the epiblast. The M/A index in the primitive streak is too low for simple exfoliation of daughter cells from this region to account for mesoderm production. For instance, at $6\frac{1}{2}$ days the primitive streak quadrant contains about 165 cells with an estimated cycle time of 6·8 h. In 24 h they generate about 2000 cells, which is approximately the size of this ectodermal quadrant at $7\frac{1}{2}$ days. In order for cells to be shed as mesoderm a considerable movement of cells into the primitive streak quadrant is required.

Fig. 4 illustrates a scheme of development in which an average cell cycle time of 3 h is assumed for the PZ over a period of nine divisions. Cells produced but not remaining in the PZ emerge into the 'other epiblast', where they divide with a cycle time of 6 h on average. About 6000 cells originate from the initial population of 60. The remaining epiblast, with an average cycle time of around 7 h, would generate about 6500 cells. These figures are surprisingly similar to the numbers of cells counted in ectoderm and mesoderm at $7\frac{1}{2}$ days and the total of 12500 cells produced would place the resulting $7\frac{1}{2}$-day embryo well within the range of cell numbers actually observed.

Topographically it seems likely that the cells generated in the PZ will form a coherent sheet of cells and not intermingle with the remaining epiblast during

gastrulation. Evidence for this is seen in the epiblast itself. The tissue is a pseudostratified epithelium, and in individual embryos occasional small groups of cells exhibiting a high degree of division synchrony can be found. It seems most plausible that these cells represent clones with a common ancestor, indicating that over a period of several divisions progeny cells do not move away from one another to any great extent. (The significant feature of the PZ is that this site *always* shows high mitotic activity whereas the synchronous clones found in one embryo are rarely observed in the same site in other embryos). Exfoliation of mesoderm cells through the primitive streak is therefore seen as the result of the expansion from the PZ site forcing the remaining epiblast to evaginate.

Such a scheme of development would bring mouse gastrulation very much into line with the observation on chicks and rabbits. In those species morphogenetic movements have been demonstrated taking place from an anterior position in a posterior median direction. Furthermore Chen (1932) and Derrick (1937) have shown regions of high mitotic activity in an anterior region of the avian blastoderm and Daniel & Olsen (1966) a somewhat less obvious proliferation centre in a similar site in the rabbit. The precise role of such proliferation centres in gastrulation is however equivocal since Spratt (1966) not only failed to find such a site in the anterior regions of the chick embryo but found most mitotic activity in the primitive streak itself. Furthermore Cook (1973) has shown that much of gastrulation in *Xenopus* can be completed in the absence of cell division.

For this model to be consistent with the observations of Skreb *et al.* (1976) it would be necessary to assume that although the epiblast *in situ* is regionalized into areas whose developmental fate can be predicted the cells are not determined but are still capable of considerable regulation in development.

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(Received 16 May 1977, revised 12 June 1977)