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Cell lineage and differentiation during growth of the early mammalian embryo

BY R. L. GARDNER
Imperial Cancer Research Fund Developmental Biology Unit, Department of Zoology, South Parks Road, Oxford OX1 3PS

The aim of this overview is to examine the relationship between growth and cellular diversification in the mammalian conceptus during the early stages of its development. First, what is known about the lineage and differentiation of cells will be presented against a background of the gross morphological transformations that take place. This will be followed by an appraisal of the pattern of growth of the conceptus, together with certain aspects of its metabolism. Finally, the effects on growth and differentiation of experimentally altering the number of cells in the conceptus or postponing its implantation in the uterus will be considered. Most of the work discussed relates to the mouse because there is presently a dearth of information on conceptuses of other species. Nevertheless, the fragmentary comparative findings which are available enable a basic similarity to be discerned, even between groups as diverse as rodents, ungulates and primates, including man.

As in most non-mammalian species, development starts with a series of more or less synchronous cell divisions whereby the cytoplasm of the fertilized egg becomes subdivided into an increasing number of progressively smaller cells. This process of cleavage leads to the formation of a solid spherical ball of cells called a morula which begins to cavitate just before the thirty-two-cell stage in the mouse, and slightly later in the human. As cavitation proceeds the conceptus can clearly be seen to be bounded by an attenuating epithelium, the trophectoderm, to the inner surface of which the remaining cells are attached locally as the inner cell mass (ICM). This partitioning of cells into two distinct populations marks the transition from morula to blastocyst stage (Fig. 1). Its origins have been traced back to the late eight-cell stage in the mouse when each cell forms a stable microvillous pole on its free outer surface (Handyside, 1980). Depending on the orientation of subsequent division these polarized cells typically produce either one polar and one apolar daughter or two polar daughters (Johnson & Ziomek, 1981). Polar cells remain external in the morula whilst apolar cells come to reside internally (Johnson *et al.* 1981; Johnson & Maro, 1986).

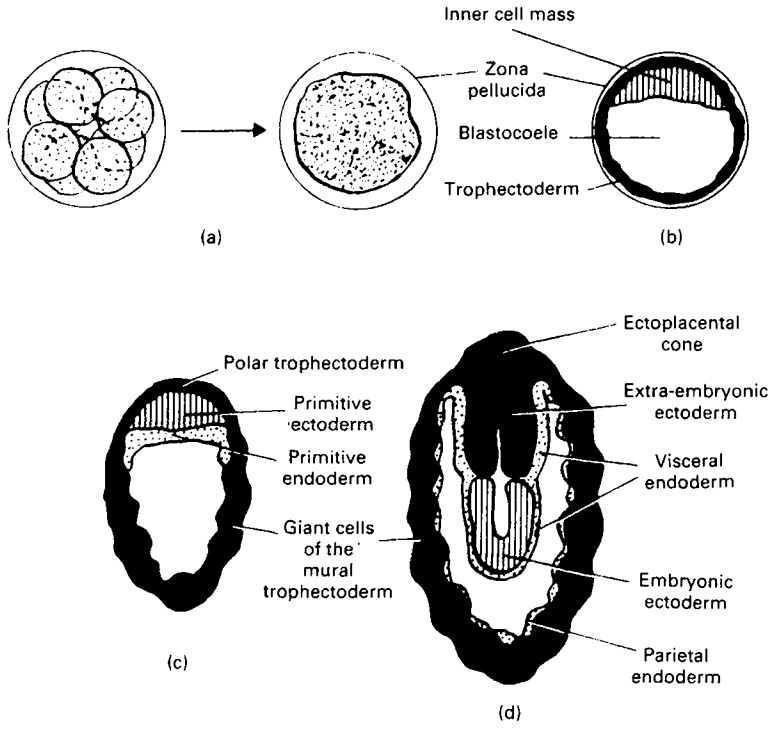

Fig. 1. Stages in the early development of the mouse conceptus (a) eight-cell to morula (2·5 d post-coitum), (b) pre-implantation blastocyst (3·5 d post-coitum), (c) implanting blastocyst (4·5 d post-coitum), and (d) early post-implantation conceptus (5·5 d post-coitum). Derivatives of the trophectoderm (■), primitive endoderm (▨) and primitive ectoderm (▩). (From Adamson & Gardner, 1979, with permission.)

Once development has progressed beyond the early blastocyst stage considerable variation between species emerges which seems to reflect differences in mode of implantation and placentation. In the mouse, expansion of the blastocyst is relatively modest and is accompanied by further cellular differentiation within both the trophectoderm and ICM. All trophectoderm cells except those overlying the ICM eventually stop dividing but continue to replicate their DNA periodically, thereby gradually transforming into mononucleate giant cells (Fig. 1). ICM cells lying adjacent to the blastocoele form a distinct mass of tissue, the primitive endoderm, which is obviously differentiated with respect to the deep cells of the primitive ectoderm (Fig. 1).

While differentiation of the primitive endoderm can clearly take place before implantation (Gardner *et al.* 1988), onset of giant cell formation in the trophectoderm is linked to attachment of the blastocyst to the uterine epithelium (Dickson, 1963). Once implantation is under way cells of the primitive endoderm become organized into distinct parietal and visceral layers (Fig. 1). Thereafter, no further cellular differentiation is discernible until approximately 2·5 d after the beginning of implantation when local thickening of the now elongated and cavitated primitive ectoderm heralds the start of gastrulation (Snell & Stevens, 1966). Extensive reorganization of this tissue follows whereby the primordium of the fetus is laid down and additional extra-embryonic

structures including the allantois and amnion are formed. Hence, roughly 8 d after fertilization all components of the mature conceptus are present in at least a rudimentary form.

CELL LINEAGE

Various methods have been used to trace the lineage of cells through cleavage to the blastocyst stage. These include intracellular injection of the enzyme horseradish peroxidase (Balakier & Pedersen, 1982; Gearhart *et al.* 1982; Pedersen *et al.* 1982) or, where selective labelling of external cells in morulae is desired, exploiting their ability to endocytose fluorescent microspheres (Fleming, 1987). These methods work admirably in short-term studies but, being dependent on labels that are susceptible to dilution through mitosis or metabolism, cannot be used to determine the ultimate fate of early embryonic cells. The need for indelible cell markers in long-term lineage studies led to the harnessing of genes for this purpose by combining cells from cleaving embryos or blastocysts of different genotype or reconstituting blastocysts from genetically dissimilar component tissues (Tarkowski, 1961; Mintz, 1962; Gardner & Lyon, 1971; Gardner *et al.* 1973; Kelly, 1977). Provided they are returned to the uterus by the blastocyst stage these various types of chimaera can continue to develop normally for analysis at any stage later in gestation or post-natally.

As noted earlier the first discrete lineages are established by the blastocyst stage. The fate of the trophectoderm and either the entire ICM or its component primitive endoderm and ectoderm has been determined both by reconstituting blastocysts and by transplanting tissues or individual cells between them. Several points of interest emerge from studying the lineage relationships that such experiments have revealed (Fig. 2). First, each tissue of post-implantation conceptuses produced by reconstituting blastocysts from genetically dissimilar trophectoderm and ICM or, more recently, trophectoderm, primitive endoderm and primitive ectoderm (Gardner, 1988), is typically composed of cells of only one genotype. This means that each tissue of the blastocyst is uniquely responsible for the formation of a particular sub-set of the various cell populations present in the later conceptus. Second, the fate of both the trophectoderm and primitive endoderm is entirely extra-embryonic, neither lineage making any discernible cellular contribution to the fetus itself (Fig. 2). Third, while the primitive ectoderm is the precursor tissue of both the entire soma of the fetus and its germ-line (Gardner *et al.* 1985), it also gives rise to several extra-embryonic components, notably the mesoderm of the yolk sac and chorio-allantoic placenta, including their vasculature, as well as the amnion. Hence progeny of the great majority of cells in the blastocyst eventually play a supporting role in mammalian development by mediating attachment and exchange between fetus and mother rather than participating directly in embryogenesis. It is in recognition of the fact that before gastrulation development is primarily concerned with the differentiation of extra-embryonic tissues that use of the term pre-embryo has been advocated for these early stages.

The fourth point is that single-cell transplantation experiments provide no evidence that tissues of the early conceptus are mosaics of cells committed to forming specific subsets of their derivatives. Rather, individual clones can span the entire spectrum of components of the later conceptus to which the parent tissue gives rise (Gardner, 1985). Finally, the ICM is clearly responsible for maintaining the proliferation of the overlying

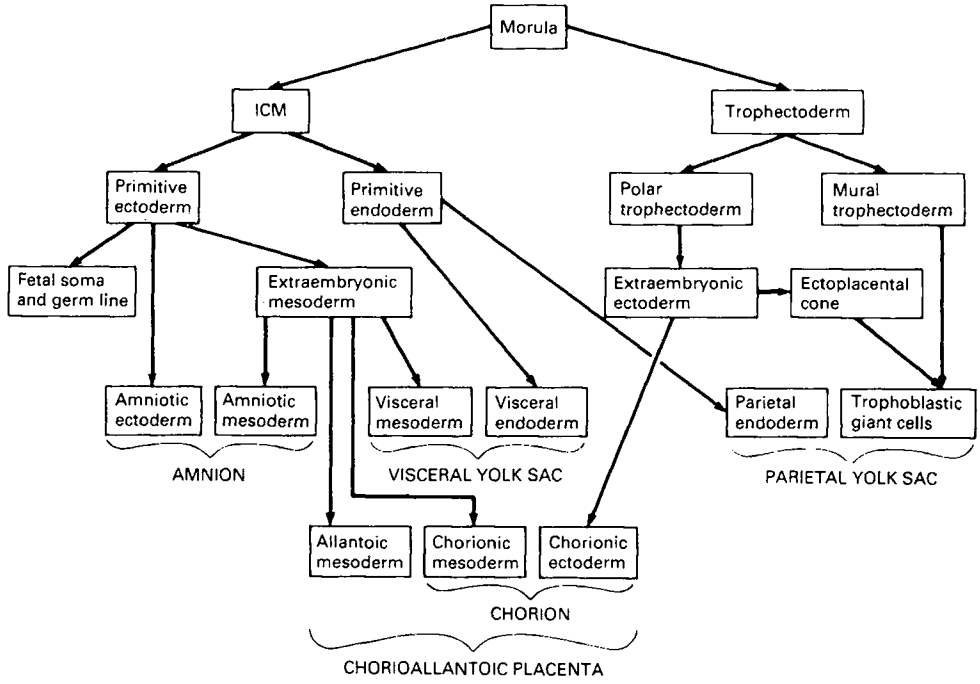

Fig. 2. Lineage scheme showing the origin at the blastocyst stage and relationship between the various components of the later mouse conceptus. (From Gardner, 1983, with permission.)

polar trophectoderm cells both during implantation and for an uncertain period thereafter (Gardner, 1989; Gardner & Beddington, 1988).

Although transplantation of cells or tissue between conceptuses has been invaluable in analysing cell lineage, it does have certain limitations. One of the most important is that the blastocyst is the most advanced conceptus that will continue to develop normally in vivo following manipulation in vitro. Hence, extending analysis to later stages means that transplantations have either to be asynchronous or host conceptuses placed in culture for further development. Colonization of blastocysts with cells from early post-implantation conceptuses has been successful for both polar trophectoderm and primitive endoderm derivatives (Rossant *et al.* 1978). Unfortunately, results with the primitive ectoderm, which is of particular interest since it contains all the fetal precursor cells, have been negative. This tissue has, however, been used in transplantation experiments in vitro which have served broadly to define the fate of various of its sub-regions following the onset of gastrulation (Beddington, 1981). An important limitation of these experiments is that normal development of mouse post-implantation conceptuses can be sustained in culture for not more than 1·5–2 d even when they have not been manipulated. This obviously restricts their scope to ascertaining the short-term fate of cells. Hence, there is no advantage in using genetic rather than exogenous markers in such experiments. Indeed, markers like horseradish peroxidase offer the distinct advantage of enabling cells to be labelled in situ rather than isolated and transplanted (Lawson & Pedersen, 1987).

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Because of the foregoing problems the possibility of using replication-defective retroviruses to analyse later lineages by genetically labelling individual cells in conceptuses in utero has excited considerable interest. However, while the feasibility of this approach has been established (Sanes *et al.* 1986), its potential has yet to be fully evaluated.

GROWTH AND METABOLISM

There is no net growth of the conceptus during the pre-implantation phase of its development, the volume of cells being roughly halved during each cleavage. Both the dry mass of the conceptus and its total protein content actually decrease between fertilization and the morula stage, but recover following blastocyst formation (Brinster, 1967; Hensleigh & Weitlauf, 1974; Sellens *et al.* 1981). However, once implantation begins a marked reduction in length of cell cycles occurs (Snow, 1976) and is accompanied by a dramatic increase in size and change in form of all tissues of the conceptus apart from the already post-mitotic mural trophectoderm (Fig. 3). As discussed later, onset of this prolonged phase of rapid growth can be postponed indefinitely by delaying implantation, arguing that it depends on the conceptus establishing intimate contact with maternal tissue. A curious feature is the timing of entry into this growth phase. It occurs when the conceptus is attached to degenerating uterine epithelium and surrounded by a completely avascular cup of primary decidual cells (Rogers *et al.* 1982) which has been shown to constitute a significant barrier to the passage of macromolecules from the maternal circulation (Parr & Parr, 1986). While such large molecules may not be involved directly in nutrition of the conceptus some act as carriers of smaller ones, notably vitamins (Adiga & Murty, 1983). Nutrition of the conceptus is said to be histiotrophic during this pre-placental period because it is believed to depend on secretory or breakdown products of uterine epithelial and decidual cells (Amoroso, 1952). The conceptus does not possess either a circulation or placenta until the visceral yolk sac has developed, approximately 4 d after the beginning of implantation. The chorio-allantoic or definitive placenta does not begin to function until even later, during the second half of gestation. Therefore, it remains somewhat of a mystery how the various tissues of the early post-implantation conceptus, particularly the primitive ectoderm which is farthest removed from maternal interstitium, obtain an adequate supply of nutrients for engaging in such rapid growth.

The mouse egg is, like that of other mammals, very small compared with its avian or amphibian counterpart and contains little yolk. Not surprisingly, therefore, the mammalian conceptus is crucially dependent on the provision of nutrients via the maternal reproductive tract even before it implants. The newly fertilized mouse egg contains substantial mRNA synthesized before fertilization, much of which appears to be degraded selectively late in the two-cell stage when the genome of the embryo first becomes active (Flach *et al.* 1982). Nevertheless, certain enzymes synthesized on maternal templates persist through cleavage to the blastocyst stage or even beyond (Auerbach & Brinster, 1967; West *et al.* 1986). Therefore, there is a gradual rather than abrupt switch from maternal to embryonic genome which may in the case of lactate dehydrogenase be triggered by implantation (Monk & Petzoldt, 1977). Recent findings suggest that activation of the embryonic genome occurs somewhat later in the human than the mouse, between the four- and eight-cell stage (Braude *et al.* 1988).

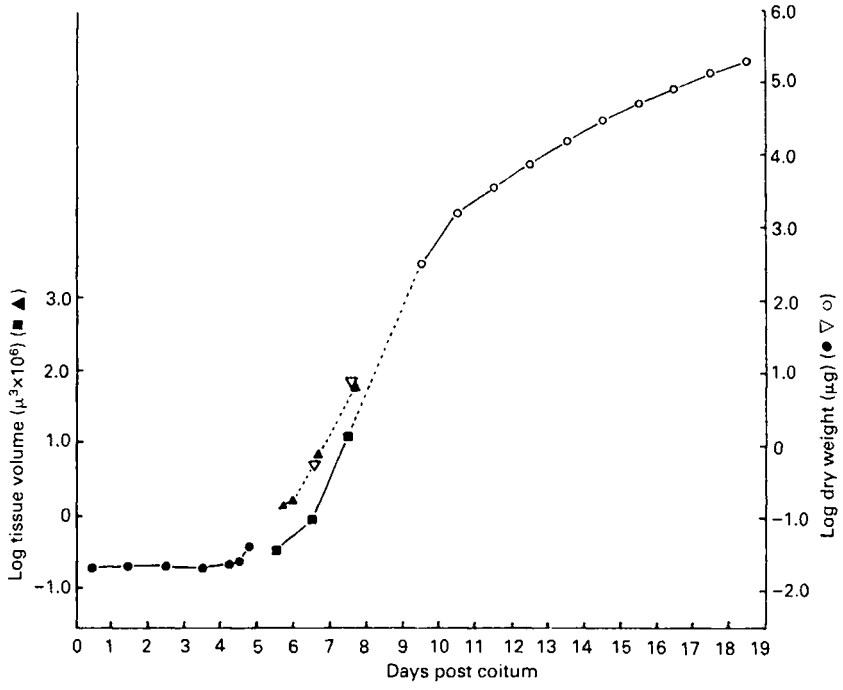

Fig. 3. Growth curve of the conceptus from conception to birth in the mouse. (From McLaren, 1976, with permission.)

Energy metabolism of the conceptus has been investigated principally in two ways. One has been to define both the micromolecular and macromolecular composition of media that best support development of conceptuses in vitro. The other has been to determine the activity of various enzymes. While this is usually done on homogenates there has recently been a move to non-invasive methods, prompted particularly by the problem of assessing the suitability for return to the uterus of human conceptuses produced by in vitro fertilization (Leese, 1987). Obviously, the most rigorous test of the normality of cultured conceptuses is to see whether they can produce viable young. This requires returning them to the uterus which is, of course, an option only in the case of pre-implantation stages. Other criteria including size, protein content and state of differentiation have been used to assess the development of post-implantation conceptuses in culture (Brown & Fabro, 1981).

During the initial stages of its development in vitro the pre-implantation mouse conceptus shows an absolute requirement for pyruvate or compounds which can be readily converted to it, such as lactate, phosphoenolpyruvate or oxaloacetate (Brinster, 1970). Hence, notwithstanding its high glycogen content, the conceptus is unable to utilize glucose appreciably before the eight-cell stage. A marked increase in metabolism of glucose and accompanying decline in glycogen is evident following blastocyst formation (Brinster, 1970). Recently, a similar early inhibition of glycolysis has also been reported in the pre-implantation human conceptus (Wales *et al.* 1987). According to Barbehenn *et al.* (1974) inhibition in the mouse is primarily at the 6-phosphofructokinase

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step. However, these workers also found evidence of a defect in the citric acid cycle and in glycogen mobilization early in cleavage.

Following explantation, 6–8 d conceptuses showed a 20- to 30-fold higher production of labelled lactate than CO_2 from [U-^{14}C]glucose when incubated in an atmosphere of 5% carbon dioxide in air (Clough & Whittingham, 1983). In addition to this high rate of aerobic glycolysis, the pentose phosphate shunt appeared very active relative to the citric acid cycle. The partial pressure of oxygen has yet to be determined in the immediate environment of such conceptuses in situ. Nevertheless, the fact that they are geared to such an energetically inefficient mode of metabolism when both their cell number and total protein content are increasing very rapidly suggests it is likely to be low. This is also indicated by improved development of rat conceptus exposed to lower O_2 tensions in vitro (New *et al.* 1976).

CHANGES IN GROWTH

Pregnancy can occur concurrently with lactation in the mouse and rat if mating takes place during post-partum oestrus (Aitken, 1977). It is, however, often prolonged in these circumstances to an extent that is roughly proportional to the number of suckling pups (Mantalenakis & Ketchel, 1966). This is because implantation is postponed while lactational demands on the dam are heavy. The effect of lactation can be mimicked by ovariectomizing females early in pregnancy and giving them progesterone daily (Bergstrom, 1978; Gardner *et al.* 1988). Delay of implantation can be terminated at any juncture simply by injecting the females with a single small dose of oestradiol or, in the case of lactation, by removing the suckling young. Regardless of the means by which delay is induced conceptuses develop on schedule to the advanced blastocyst stage when they gradually enter a metabolically quiescent state in which growth and cell division are almost completely suspended (Gardner *et al.* 1988). The decline in DNA synthesis that accompanies the entry of blastocysts into delay is seen first in the distal mural region of the trophectoderm and then in the proximal region before being discernible in polar tissue and, finally, in the ICM (Given, 1988). This is the reverse of the order in which synthesis is resumed following the termination of delay (Given & Weitlauf, 1981). While there has been much discussion as to how development of the blastocyst might be held in abeyance during delayed implantation, the mechanism has so far defied analysis. However, the fact that delayed implanting mouse blastocysts have a high ATP:ADP ratio argues that their quiescence is not simply due to their being in a 'low energy' state (Nieder & Weitlauf, 1984).

An intriguing question is whether lactational delay of implantation has any consequences for later development. Vorherr *et al.* (1984) claim that the post-implantation phase of gestation is significantly shorter following lactational delay than in uninterrupted pregnancy in the rat. They attribute this to acceleration of fetal growth following re-direction to the gravid uterus of the extra nutritive reserves that are no longer required by the dam to meet the heavy demands of lactation. This implies that the nutritive state of the dam can exert a profound effect on the rate of fetal growth and maturation since a reduction in duration of post-implantation gestation of up to one-third was reported. However, no evidence that the interval between implantation and parturition is shorter in lactationally-delayed than in non-delayed pregnancy was found in a recent study in the mouse (T. J. Davies and R. L. Gardner, unpublished observations).

In the mouse, aggregation chimaeras invariably yield fetuses and offspring that are within the normal size range, regardless of whether they are formed by the union of two, three, four or, in one study, as many as nine entire pre-implantation conceptuses (Gardner, 1984). Size regulation is also seen in mice obtained following halving of conceptuses, for example, by destruction of one cell at the two-cell stage. Essentially similar results have been found following manipulation of cell number during early development in other mammals. Evidently, the mammalian conceptus possesses a considerable capacity for growth adjustment at some stage in gestation. Attempts to establish when and how size regulation occurs following alterations in cell number before implantation have been based on examination of development following destruction of one cell at the two-cell stage and aggregation of either two or four conceptuses at the eight-cell stage. Little or no regulation occurs before implantation and this has interesting consequences for the structure of the blastocyst whose ICM:trophectoderm cell ratio is, in accordance with the 'inside-outside' hypothesis, higher than normal in double- and quadruple-sized specimens and lower than normal in the half-sized ones (Buehr & McLaren, 1974; Rands, 1985). While there is some disagreement as to precisely when down-regulation in size takes place, it is clearly completed by the beginning of gastrulation when the proportions of the trophectoderm and ICM derivatives are also within the normal range. The only difference found by Lewis & Rossant (1982) when comparing 'double' and 'standard' conceptuses during early post-implantation development was that the mean cell cycle was longer in the 'doubles'. These workers therefore suggested that down-regulation may be achieved by a generalized slowing of growth of the conceptus rather than by death or withdrawal from cycle of the surplus cells. They also argued that the timing of morphogenetic events which take place before size regulation may depend on absolute cell number because they were completed precociously in 'double' conceptuses. However, this is in direct conflict with observations of Rands (1986*a*) who found no evidence of accelerated morphogenesis in 'quadruple' conceptuses before normal size had been restored. Also at variance with the more extensive findings of Rands (1986*b*) is the claim by Lewis & Rossant (1982) that upward size regulation in 'half' conceptuses is completed at the same time as down-regulation in 'doubles'. According to Rands (1986*b*), 'half' conceptuses do not attain normal size until several days later and, most interestingly, fall behind controls once more during the latter part of gestation. Rands (1986*b*) has pointed out that down-regulation coincides with the stage at which growth of the conceptus is accelerating and up-regulation possibly with the stage at which it is decelerating (see Fig. 3), and that both might, therefore, be achieved by delaying these changes.

Almost complete growth regulation has also been found following induction of dramatic loss of cells in 8th-day conceptuses by maternal injection of mitomycin C (Snow & Tam, 1979). What is particularly interesting about development after such an insult is that various morphogenetic processes can be dissociated from each other as well as from growth. A review of this topic can be found elsewhere (Snow *et al.* 1981), as can one on the role of growth factors in early development (Slack, 1989).

CONCLUDING REMARKS

Most of the findings discussed here come from work on the mouse, a species which has many advantages from an embryological point of view. However, one consequence of

the development of in vitro fertilization as a treatment for infertility is that research on early human embryos is now possible, albeit on a rather modest scale. So far, most of the work done on this material has been directed towards the solution of clinical problems. This is likely to continue to be the case in the United Kingdom so long as such research remains under voluntary rather than statutory control.

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