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Cellular and Genetic Analysis of Mouse Blastocyst Development

by

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I. INTRODUCTION

The major morphogenetic events that occur during preimplantation development of the mammalian embryo are formation of a compact mass of cells (the morula) and formation of the blastocyst. Development of the blastocyst involves differentiation of a unique cell type, trophoctoderm, that is necessary for implantation and nutrition of the embryo in the maternal environment, and the inner cell mass (ICM), which differentiates into primary endoderm and ectoderm before implantation (Snell and Stevens, 1966; Enders, 1971; Enders et al., 1978). In the mouse, trophoctoderm can form even when there are few or no ICM cells, and hollow vesicles of trophoctoderm can implant in vivo or grow out in vitro (Gardner, 1972; Ansell and Snow, 1975; Sherman, 1975; Sherman and Atienza-Samols, 1979). However, subsequent proliferation of trophoctoderm cells to form the ectoplacental cone and extraembryonic ectoderm depends on the presence of the ICM. The ICM itself gives rise to the fetus and to the extraembryonic membranes but cannot implant or survive in the uterus in the absence of trophoctoderm (Gardner, 1972; Gardner et al., 1973). Thus, the early postimplantation development of mouse embryos, and perhaps other mammalian embryos, depends on the successful differentiation and interaction of the two cell populations that form in the preimplantation period.

We have studied the development of mouse embryos by both cellular and genetic approaches. In the cellular analysis we have studied the

determination of cell fate in blastocysts and in cell populations derived from them in an attempt to estimate the time that these cells become committed to their fate. In the genetic analysis, we have taken advantage of existing mutations that are lethal to mouse embryos to discern essential features of early development. In this review we consider briefly the timing of cell determination in the ICM and the primary ectoderm, and we consider in detail the manifestation of defects in mouse embryos that are homozygous for the A^y allele of the agouti locus.

II. CELL DETERMINATION DURING EARLY MOUSE EMBRYOGENESIS

During the late morula (16- to 32-cell) stage the mouse embryo undergoes its first overt differentiation: blastomeres of the embryo differentiate into the trophectoderm and the ICM. On the basis of her studies with chimeras, Mintz (1965) suggested that the microenvironment of early embryonic cells plays an important role in trophectoderm-ICM differentiation. This idea was developed further by Tarkowski and Wróblewska (1967) to account for their observations of disaggregated 4- and 8-cell embryos. Their hypothesis was that blastomeres occupying outside positions at the morula stage form the trophectoderm and those in inner positions form the ICM.

The major prediction of this "epigenetic" hypothesis is that blastomeres of early embryos should be developmentally labile, i.e., capable of forming either trophectoderm or ICM, before the stage at which some come to occupy

internal positions. This prediction has been amply confirmed experimentally for 2-, 4-, and 8-cell blastomeres of mouse embryos (Tarkowski, 1959; Hillman et al., 1972; Wilson et al., 1972; Kelly, 1975, 1977; reviewed by Rossant, 1977; Rossant and Papaioannou, 1977).

Cell determination, or restriction in the developmental potential of cells, has been studied in early mouse embryos with microsurgical and immunosurgical techniques in an attempt to define the time that inner or outer cells become committed to their fate. By studying microsurgically isolated ICMs or trophectoderm tissues, Gardner and co-workers found that both tissues were already committed to mutually exclusive developmental fates by 3.5 days of gestation (d.g.) (Gardner, 1972; Gardner, 1975; Gardner and Papaioannou, 1975; Rossant, 1975a,b; Gardner and Rossant, 1976). With the advent of the immunosurgical technique (Solter and Knowles, 1975) it was possible to isolate inner cells from larger numbers of embryos and at earlier stages than with the technically more difficult microsurgical procedures. It is now clear from such studies that ICM determination occurs shortly before 3.5 d.g., as we will discuss. By contrast, cell determination in the trophectoderm has not been studied extensively, probably because of the technical difficulty of disaggregating the cells and placing them in inside positions. However, it has been shown that polar trophoblast moves into mural trophectoderm positions as blastocysts develop (Gardner and Papaioannou, 1975; Copp, 1978, 1979) and that mural trophoblast can be converted into polar trophoblast in early blastocysts by placing a donor ICM in contact with host mural

trophectoderm (Gardner et al., 1973). Cell determination of primary ectoderm has been studied by both microsurgical and immunosurgical techniques, but with apparently contradictory results, which we will discuss.

A. Cell Determination in the Inner Cell Mass

To assess the state of cell determination or commitment in a tissue, such as the ICM, it is necessary to move the cells away from their normal spatial or temporal environment. Rossant carried out two studies to assess the developmental potential of inner cells exposed to an outside environment. In the first study (Rossant, 1975a), ICMs isolated micro-surgically from 3.5-d.g. blastocysts were aggregated with morulae, and the aggregates were transferred to the uteri of foster mothers for further development. Analysis of glucose phosphate isomerase showed a weak ICM contribution to the trophoblastic fraction of 3 of 13 conceptuses. Rossant attributed this minor contribution to contamination from chorionic cells, which are derived from the ICM, and concluded that the ICM cells are committed to their fate by 3.5 d.g. In the second experiment (Rossant, 1975b), two microscurgically isolated ICMs were aggregated and placed in an empty zona pellucida, then transferred to the oviduct of a foster mother for further development. ICMs recovered one to three days later had endoderm-like outer layers but trophectoderm was absent, confirming that ICM is committed by 3.5 d.g.

In other studies ICMs isolated by immunosurgery from 3.5-d.g. blastocysts that had been cultured an additional 24 h (3.5 d.g. + 24 h) formed two-layered structures that resembled those seen by Rossant (1975a,b): an inner core of primary ectoderm surrounded by a layer of primary endoderm (Hogan and Tilly, 1978a; Wiley et al., 1978). However, during our study of immunosurgically isolated ICMs (unpublished observations) we observed that ICMs isolated from blastocysts (3.5 d.g. + 24 h) on rare occasions formed blastocyst-like fluid-filled structures after overnight culture. This suggested that at least a few ICM cells could still be developmentally labile at this stage.

In our further experiments we studied the stage dependence of trophectoderm formation by isolated inner cells. When ICMs were isolated from early blastocysts developing in vitro (69 h of culture from the 2-cell stage), one third of the ICMs were capable of forming blastocyst-like structures within 24 h of isolation (Spindle, 1978). Upon further cultivation these structures formed small but typical trophoblast outgrowths consisting of giant cells. This formation of trophectoderm by isolated ICMs did not appear to depend on the number of cells they contained; ICMs composed of two to three cells could form fluid-filled structures (A.I. Spindle, unpublished observations). The subsequent survival of ICMs, on the other hand, was strongly dependent on their size, as suggested by Snow (1976). The poor survival of inner cells isolated from early blastocysts could also be due to the toxicity of antiserum or guinea pig serum used in immunosurgery (Hogan and Tilly, 1978b; A.I. Spindle, unpublished observations).

We solved the ICM survival problem by the use of aggregation chimeras. All ICMs from 5-embryo chimeras survived and most formed trophectoderm and trophoblast outgrowths when inner cells were isolated from late morulae or early blastocysts that were beginning to cavitate (Spindle, 1978) (Table I). By contrast, this ability was reduced to low levels when ICMs were isolated at 69 h of culture from blastocysts that were partially expanded. ICMs isolated from fully expanded late blastocysts at 93 h of culture appear to have lost their capacity for forming trophectoderm (Spindle, 1978). A comparison of blastocysts that developed in vitro and in vivo revealed that the determinative state of ICMs of cultured embryos at 69 h of culture from the late 2-cell stage (113 h after injection of human chorionic gonadotropin) is similar to that of embryos obtained from the uterus at 10 A.M. on the fourth day of gestation (93 h after human chorionic gonadotropin). (This observation suggests that development of embryos is delayed one day during culture from the 2-cell to the blastocyst stage, as noted by Spindle, 1978.)

Trophoblast formation by immunosurgically isolated ICMs was also independently observed by Johnson et al. (1977), Handyside (1978), Hogan and Tilly (1978b), and Rossant and Lis (1979). Rossant and Lis (1979) also found that regenerated trophoblast cells were capable of inducing a decidual reaction in the uterus. The possibility that trophectoderm is regenerated by rapid proliferation of a few contaminating trophectoderm cells appears to be remote (Handyside, 1978; Spindle, 1978). Thus, it is now clear that at least some cells of the mouse ICM remain developmentally labile after trophectoderm differentiation.

Cell determination in the ICM appears to coincide with expansion of the blastocoele from a small to a large cavity. One major cellular event that occurs during this period is the sixth cleavage division, which doubles the total cell number of the embryo and increases the number of cells in the ICM (Handyside, 1978; Spindle, 1978). The relationship between cell division and commitment during this period of development deserves further attention, particularly in view of the indication (Braude, 1979) that gene expression necessary for blastocyst formation occurs just before the fifth cleavage division.

Because ICM cells of early blastocysts do not differentiate into trophectoderm when left inside the embryo, there appears to be a mechanism for them to receive information regarding their position in the intact embryo. One possible vehicle for the transfer of positional cues between outer and inner cells is the blastocoele fluid. This fluid is contained by the trophectoderm (Ducibella et al., 1975; Magnuson et al., 1977) and has an ionic composition distinct from that of the culture medium (Borland et al., 1977). We examined the role of the blastocoele microenvironment on trophoblast-ICM differentiation. Our results (unpublished) showed that 8-cell embryos or morulae injected into the blastocoele of giant chimeras could differentiate into morphologically normal blastocysts, provided that cell-cell contact between the donor embryo and recipient chimeras was prevented by leaving the donor zona pellucida intact. However, when donor-recipient cell contact was allowed by removing zonae from the donor embryos, the donor embryos attached to the host trophectoderm and

formed compact structures instead of blastocysts. Labeling donor cells with ^3H -thymidine excluded the possibility that trophoblast cells of donor embryos were incorporated into the recipient trophectoderm. From our results it is clear that blastocoele fluid alone does not prevent trophoblast differentiation in donor embryos. Whether cell-cell contact alone or both blastocoele fluid and cell contact are involved in inhibiting donor blastocoele formation remains to be determined. The nature of the compact structures formed by zona-free donor embryos is not yet clear. As judged from their ability to form trophoblast outgrowths in culture when they were removed from the recipients, the compact structures may be merely delayed or physically prevented from accumulating blastocoele fluid, or they may be ICMs that were not yet committed at the time of removal from the host. Further analysis is under way with ultrastructural and biochemical markers to identify cell types in these structures. This system may also be useful for studying the time of trophectoderm determination because it permits outer cells to be placed in an internal environment without disaggregating the donor embryo.

B. Cell Determination in the Primary Ectoderm

Determination of primary ectoderm was studied initially by microsurgical techniques. Gardner and Rossant (1976, 1979) investigated the stage of determination in primary ectoderm and primary endoderm by dissecting them from 4.5-d.g. blastocysts and injecting them into early blastocysts. Their results indicate that primary endoderm cells from

4.5-d.g. blastocysts contribute exclusively to the endodermal layer of the visceral yolk sac, whereas primary ectoderm cells contribute to the mesodermal layer of the visceral yolk sac and to the fetus, but not to yolk sac endoderm. These results imply that both cell types are determined by 4.5 d.g., that donor cells migrate to their appropriate position in the host ICM, or that donor cells can proliferate only in the correct position (Gardner and Rossant, 1979). It is impossible to distinguish between these alternatives without further experiments because micro-surgically isolated ectoderm and endoderm are placed back in approximately the same positions that they would occupy in normal blastocysts.

To test the developmental potential of primary ectoderm that is moved out of its normal environment, ectoderm was isolated from late blastocysts by double immunosurgery and its developmental capacity was studied in vitro (Strickland et al., 1976; Hogan and Tilly, 1977). In our experiments (Pedersen et al., 1977) immunosurgery was first performed on late blastocysts at 93 h of culture from the 2-cell stage. At the time of isolation most of the ICMs were partially covered by primary endoderm, and after overnight culture in suspension they were completely enclosed by endoderm. The second immunosurgery (to remove endoderm) was performed at 24, 48, or 72 h after the first. Ectoderm isolated at 24 and 48 h (but not at 72 h) regenerated a layer of endoderm during an additional 24 to 48 h culture in suspension (Table II). The survival of ectoderm in culture depended on the mass of tissue; ectoderm from single embryos or chimeras of two embryos died, whereas ectoderm

Table II

from 4-, 8-, and 10-embryo chimeras survived and developed a second layer of endoderm. Hogan and Tilly (1977) reported that ectoderm that was isolated from single blastocysts and cultured on fibroblast feeder cells attached to the substratum and differentiated into various tissues, including skin, nerve, beating muscle, cartilage, and fibroblasts, but did not regenerate primary endoderm. When ectoderm was cultured in suspension it did not survive. From these observations it appears that attachment and the presence of feeder cells are necessary conditions for survival and extensive differentiation. A major difference between the experimental conditions used by Hogan and Tilly (1977) and ours (Pedersen *et al.*, 1977) is the size of embryos from which ectoderms were obtained; they isolated ectoderms from normal-size blastocysts, whereas we obtained ours from chimeras made by aggregating eight to ten embryos. The increased size of ectoderm obtained from chimeras appears to ensure its survival in the absence of feeder cells; furthermore, suspension culture seems to be a necessary condition for regeneration of endoderm. Atienza-Samols and Sherman (1979) found that ectoderm cores isolated from single ICMs 48 h after the initial immunosurgery could regenerate a layer of endoderm. They found that culturing the ectoderm cores in conditioned medium improved their survival and doubled the incidence of endoderm regeneration from 10% to 20%. Their results likewise indicate a cell feeding requirement for ectoderm cell survival. Recently, Dziadek (1979) reported that endoderm could be regenerated by ectoderm isolated from chimeras of three embryos 24 to 40 h after the initial immunosurgery. The regenerated endoderm synthesizes α -feto-protein, which is a characteristic

product of visceral endoderm cells. As noted by Dziadek (1979) and by Gardner and Rossant (1979), the definitive identification of these endoderm cells requires tissue-specific markers. However, the morphological similarity of regenerated endoderm and visceral endoderm (Pedersen et al., 1977) strongly implies that they are identical. Furthermore, it seems unlikely that the regenerated layer is the definitive endoderm of the fetus, as suggested by Gardner and Rossant (1979), because the definitive endoderm would not be expected to form until 7 to 8 d.g. (Snell and Stevens, 1966; Diwan and Stevens, 1976), and the ectoderm isolated in our studies (Pedersen et al., 1977), by Atienza-Samols and Sherman (1979), and by Dziadek (1979) is probably equivalent to an earlier developmental stage.

The development of tissues isolated from 6.5- to 8-d.g. mouse and rat embryos and transferred to ectopic sites suggests that embryonic ectoderm gives rise to the definitive endoderm of the mammalian fetus; the definitive endoderm does not seem to arise from the primary (or visceral) endoderm (Grobstein, 1952; LeVak-Svajger and Svajger, 1974; Diwan and Stevens, 1976). The implication of these observations on cell determination in early mammalian development is that outer cell differentiation precedes inner cell commitment. This will be discussed together with the implications of studies on A^y/A^y mouse embryos.

III. MUTATIONS AT THE a LOCUS AFFECTING IMPLANTATION

The ease of observing coat color has facilitated study of the a locus in mice and has been partially responsible for the extensive use of this locus for genetic, embryological and physiological studies. The control of coat color by this and other loci has been recently reviewed by Silver (1979). The early identification of the yellow phenotype by mouse fanciers was followed by the discovery that yellow mice had both yellow and black progeny (Cuēnot, 1905). Early workers (Cuēnot, 1905; Castle and Little, 1910) deduced that yellow mice are heterozygous for the dominant yellow allele and that the homozygote is lethal. This was the first mutant allele to be identified in mice and led to further analysis of this locus and the genetic basis for coat color. Although our interest in mutations at the a locus is what the alleles affecting early development can tell us about the early embryo, we also consider the genetics of the a locus because this subject has not been reviewed.

A. Genetics

The a locus is in linkage group V, which has been identified with chromosome 2 (Miller et al., 1971). The G-bands on this chromosome are colinear with the linkage order and the a locus appears to be located in G-band 2HI (Searle et al., 1979). There are 14 a locus alleles affecting coat color, as reviewed by Green (1975). Only two of these

are known to affect early embryonic development: A^Y (yellow) and a^X , which is dark brown with a slightly paler belly. The yellow allele is dominant to agouti and the other alleles with respect to coat color but is recessive for lethality. The a^X allele, which was derived from irradiated spermatogonia, is recessive to A^Y , A^W , A , and a^t , is dominant to a for its coat color effects, and is recessive for lethality (Russell et al., 1963). Closely linked genes that have been used in studies of the a locus include Wellhaarig (we), waved hair, 12 centimorgans (cM) proximal; undulated (un), wavy tail and abnormal vertebral column, 5 cM proximal; brachypodism (bp), short, abnormal feet, 0.3 cM distal; and kreisler (kr), circling and head-shaking behavior, 3 cM distal. The minor histocompatibility locus, H-13, which is 2 cM proximal, is also a potentially useful marker (Snell et al., 1967).

In a study of a balanced lethal stock (A^Y/a^X), Russell et al. (1963) reported a low level of recombination between the A^Y and a^X alleles, showing that a^X is pseudoallelic to A^Y and a . They concluded that their order was $Kr - a^X - A^Y - un$ and that a^X and A^Y are separated by 0.5 cM. They also suggested that A^Y may be pseudoallelic to a because an agouti offspring was recovered from matings between A^Y/a parents. Similarly, Phillips (1966) found 0.6% recombination between A^S and A^W or a^t . The proximity of bp , which is only 0.3 to 0.4 cM from a (Runner, 1959; Phillips, 1966), raises the possibility that bp lies between a and a^X or A^S ; however, Phillips (1966) discounted this possibility because a crossover was obtained with bp that did not separate A^S and A^W . Because of the crossovers observed between a and A^S or a^X , Wallace (1965) and Phillips (1966) suggest that the a locus is a complex, multigenic locus.

B. Embryology

1. Histological Observations

The earliest embryological studies of the embryos of yellow mice showed that a portion of them died early in gestation, just after implantation. Kirkham (1917, 1919) serially sectioned oviducts and uteri of yellow mice and compared their embryos to those of white mice. Neither strain of mouse was characterized. The 2-cell embryos of yellow mice were normal, but 2 of 11 morulae in two litters had indistinct cell boundaries at 3 d.g. In six matings studied at 4 to 6 d.g., 11 of 43 embryos were "degenerate." Kirkham (1919) identified the abnormal blastocysts by their shrunken appearance, their small, crowded cells, and the small blastocyst cavity. In 21 matings of yellow mice studied at 3 to 20 d.g., Kirkham (1919) classified 39 of 94 embryos (29%) as degenerate, but found only 2 of 189 degenerate embryos (1%) in white mice. Despite their rapid degeneration, abnormal embryos induced a normal uterine decidual response. Kirkham (1919) concluded that the blastocysts that are lost during implantation in yellow mice are the homozygous embryos, and that they are defective in implantation. The use of white, rather than sibling non-yellow mice as controls in Kirkham's studies decreases the certainty of his identification, because strains vary in their rates of early embryo mortality, some showing higher spontaneous rates of preimplantation deaths than others (Leonard et al., 1971). In a similar study carried out on 13- to 19-d.g. embryos, Ibsen and Steigleder (1917) used the appropriate control matings ($A^Y/a \times a/a$, $a/a \times A^Y/a$, and

a/a x a/a) of an unspecified strain to show a 15.6% excess of early resorptions in 33 A^y/a x A^y/a matings. There were no differences in the frequency of late resorptions (death after 13 d.g.) in experimental and control matings. Ibsen and Steigleder (1917), as well as Little (1919), thus verified Kirkham's findings that abnormal embryos could induce a decidual reaction, but the late times chosen for histological analysis precluded a precise estimate of the time of A^y/A^y embryo death or the nature of their abnormalities.

Robertson (1942a,b) carried out the first study of yellow mouse embryos using an inbred strain, 101-A^y. His histological observations were concentrated on 2- to 6.5-d.g. embryos. Only 3 of 56 cleavage and early blastocyst stage embryos (5.4%) were abnormal. One of these was at the 8-cell stage, one was at the morula stage, and one was at the early blastocyst stage. Because of their rarity it seems unlikely that the two early abnormalities were related to A^y/A^y homozygosity, but Robertson (1942b) considered the abnormal blastocyst to be in transition between normality and the degeneration seen at later stages. This blastocyst had crowded cells and was small, with a collapsed blastocoele filled with trophoblastic processes. By comparison, 8 of 29 implanting blastocysts (27.6%) in 6 litters studied at 5 to 5.5 d.g. were small, lacked a blastocoele, had no obvious trophectoderm, endoderm, or ectoderm, and had pycnotic nuclei. Robertson (1942b) concluded that A^y/A^y embryos developed normally up to the late blastocyst stage but that their trophectoderm degenerated on contact with the uterine epithelium, leaving the ICM exposed. This view implies that the primary defect of

A^Y/A^Y embryos is in the trophoctoderm and that ICM is affected only indirectly, through loss of trophoblast functions that are essential for implantation or that protect or nourish the inner cells within the uterine environment. However appropriate this view may be, one should exercise caution in interpreting Robertson's results because only a small number of embryos were studied and there were no control observations to support the conclusion that the abnormalities observed were specifically related to A^Y homozygosity.

In another study using three inbred strains, Eaton and Green (1962) sectioned embryos from eight experimental matings (A^Y/a x A^Y/a) and nine control matings (a/a x A^Y/a or A^Y/a x a/a) at 5.5 to 7.5 d.g. In four experimental matings of C57BL/6J mice, 15 of 36 embryos (41.6%) were dead and in the other two strains, 13 of 34 embryos (38.2%) were dead at 5.5 to 6.5 d.g. Uterine crypts containing viable embryos were deeper than those with degenerate ones. In control matings of C57BL/6J mice, 6 of 55 embryos (10.9%) were dead at 5.5 to 6.5 d.g. Because the abnormal embryos evoked a normal decidual reaction and persisted in the uterus until after implantation (which occurred at 4.5 to 5.25 d.g.), Eaton and Green (1962) concluded that death of A^Y/A^Y embryos was not due to faulty implantation. Their conclusion is based in part on the advanced abnormal embryos they observed at 6.5 d.g. Correcting for the control deaths, there were 30.7% excess deaths in the embryos from C57BL/6J- A^Y/a x A^Y/a matings, which is slightly above the 25% Mendelian expectation. However, the criteria for distinguishing between abnormalities characteristic of control matings and those specific to

experimental matings were not given. The authors did, however, carry out matings to show that heterozygous yellow embryos survived to birth as well as wild-type, so it is unlikely that any excess deaths specifically represent A^Y/a embryos.

In their companion study, presumably carried out with the same strains of mice, Eaton and Green (1963) examined 262 embryos of $2U A^Y/a \times A^Y/a$ matings at 4.33 to 5.5 d.g., finding 71 abnormal embryos (27%). These fell into three classes: (1) expanded blastocysts with an expanded ICM and undifferentiated trophoblast giant cells, lying free in the uterine crypt (2%); (2) collapsed blastocysts with pycnotic nuclei in the ICM, partially attached at the abembryonic pole (13%); (3) blastocysts with partial blastocoele expansion, partially attached by a variable number of equatorial trophoblast giant cells (12%). Eaton and Green interpreted their results as indicating a correlation between the extent of equatorial trophoblast giant cell attachment and survival of class 3 embryos to early egg cylinder stages. The more advanced embryos (with more than 50% of their trophoblast attached to the endometrium) developed ectoplacental cone, embryonic and extraembryonic endoderm, and ectoderm; however, they did not increase in length during implantation, had virtually no mitotic figures, and began degenerating at 6 d.g. Eaton and Green conclude that trophoblast giant cell differentiation is the limiting factor in development of these abnormal embryos. This conclusion hardly seems justified on the basis of their histological observations because the hardier embryos may simply progress further in trophoblast development and die for other than the reasons suggested.

Furthermore, they state that the three classes of abnormal embryos were not found in control matings, but they presented no numerical data to support this conclusion, even though they found nearly 11% degenerating embryos in control matings in their earlier study (Eaton and Green, 1962). Therefore, caution is warranted in accepting their interpretations for the cause of A^Y/A^Y embryo lethality.

In a recent study of C57BL/6J- A^Y/a mice, Cizadlo and Granholm (1978a) compared cell numbers and embryo sizes of experimental and control litters. They found 3 of 24 embryos from $A^Y/a \times A^Y/a$ matings that were abnormal at 4.5 d.g. and 1 of 22 from the $A^Y/a \times a/a$ control matings that was abnormal. The abnormal embryos had cell numbers below the mean values, which were 123 ± 7 (SE) and 141 ± 11 for experimental and control matings, respectively. Although mean embryo cell numbers and trophoblast cell numbers were not significantly different between experimental and control matings, ICM cell (nuclei) numbers were lower in experimental matings (30 ± 2 versus 42 ± 5). Other quantitative features, such as number of corpora lutea, implantation sites per female, and number of sections per embryo were similar in experimental and control matings. Also, a lower proportion of the embryos in experimental matings underwent abembryonic trophoblast proliferation, as judged by the appearance of cells in this region. Even assuming that the observed differences are due to the A^Y allele, which cannot be stated with certainty, the authors' conclusion that A^Y homozygosity preferentially interferes with ICM cells does not appear to be justified, because of the effects they observed on trophoblast proliferation and effects on hatching of blastocysts (to be discussed).

2. Ultrastructural Observations

The histological observations of presumed homozygous yellow embryos are complemented by three electron microscopic studies of embryos from A^Y/a x A^Y/a matings. Calarco and Pedersen (1976) examined 17 morphologically abnormal embryos obtained at 3 or 4 d.g. from several C57BL/6J- A^Y/a x A^Y/a matings with particular attention to those showing excluded blastomeres and thought to be A^Y/A^Y because of their development in culture. They found that the excluded blastomeres resembled earlier cleavage stage embryos in having crystalloid inclusions; intracisternal A particles; small, vacuolated mitochondria; dense, rounded nucleoli; few polysomes; and little rough endoplasmic reticulum. The remaining cells of presumed homozygotes were ultrastructurally normal, and inner cells were found in all abnormal blastocysts. No observations were made of normal or abnormal embryos from control matings, and the identification of A^Y/A^Y embryos by blastomere exclusion has been questioned by other workers. Nowell and Chapman (1976) reported their observations of two grossly normal and one retarded, abnormally small blastocyst from matings of C57BL/6J- A^Y/a mice inter se. Normal cells at 4.5 d.g. had abundant free ribonucleoprotein particles, contorted nuclei with prominent nucleoli, junctional complexes between adjacent trophectoderm cells, and other ultrastructural characteristics of normal embryos at the late blastocyst stage (Enders, 1971; Enders et al., 1978). Cells of the abnormal embryo and of one presumed normal embryo had enlarged nuclei, empty spaces in the cytoplasm, and contorted and vacuolated mitochondria. Because

of their limited observations one cannot safely conclude that any of the features noted by Nowel and Chapman (1976) actually characterize the homozygous yellow embryo at the late blastocyst stage. Using the same C57BL/6J-A^Y/a strain, Cizadlo and Granholm (1978b) studied 24 embryos obtained from four A^Y/a x A^Y/a matings (20 of 24 obtained at 3.5 d.g. and 4 of 24 at 4.25 d.g.), and five embryos from five a/a x A^Y/a matings. The six abnormal embryos from A^Y/a x A^Y/a matings included one degenerate cleavage stage embryo, two morulae with isolated blastomeres that had condensed cytoplasmic material, two morulae with increased numbers of intracisternal A particles and relatively unreticulated nuclei, and one blastocyst with a degenerating trophoblast cell and other vacuolated blastomeres. These embryos were ultrastructurally normal in other respects, like the embryos from control matings. It is puzzling that all the advanced embryos in their experimental sample (morulae or blastocysts) showed ultrastructural abnormalities, because only 25% would be expected to be A^Y/A^Y according to Mendelian expectations, and no abnormalities were observed in the small control sample. Because of these limitations and the previously stated reservations regarding embryo genotype, the ultrastructural features of homozygous yellow embryos remain poorly understood; therefore, the existing observations, which are all on preimplantation stage embryos, must be interpreted with caution. The uncertainty in identifying A^Y/A^Y embryos by their ultrastructural features before their death reflects the inherent limitations in static observations of developing systems. These problems have been overcome to some extent by experimental approaches.

3. Experimental Observations

A variety of experimental approaches have been used in attempts to identify A^Y/A^Y embryos and to determine the mechanism of lethality, including ovarian transplantation, hormone treatment, embryo culture, and chimera formation.

The earliest experimental analysis was by Robertson (1942b), who transferred ovaries of A^Y/a mice to ovariectomized agouti littermates and histologically studied the embryos obtained by mating these agouti females to A^Y/a males. Among 57 embryos in 18 matings, there were no abnormalities in cleavage stage or preimplantation blastocysts, but 5 of 17 implanting blastocysts (29%) and 9 of 28 egg cylinders (32%) were abnormal. The abnormalities appeared at the same time (5 d.g.) as in embryos developing in A^Y/a females, but the affected embryos survived longer, developed approximately twice the number of cells, formed Reichert's membrane and a rudimentary ectoplacental cone, and completely eroded the uterine epithelium. These features were not seen with the abnormal embryos in A^Y/a mothers. Thus, by comparison with the usual $A^Y/a \times A^Y/a$ matings, the non-yellow uterine environment appeared to be slightly more favorable for the development of A^Y/A^Y embryos; nevertheless these died shortly after implantation and were disorganized masses of cells undergoing resorption by 6.5 d.g. (Robertson, 1942b). As noted, however, the positive identification of A^Y/A^Y abnormalities was not possible in Robertson's studies owing to lack of control observations.

In an extension of earlier work, Eaton (1968) reported that administering progesterone (5 mg/mouse) at 4.5 d.g. to A^Y/a females that were mated to

A^y/a males led to an increase in the number of "escaper" embryos at 5.5 d.g. The "escapers" constituted only 2% of the embryos in untreated mothers, but the incidence increased to 7%, 13%, and 23% in those treated with 5, 7.5, and 15 mg progesterone, respectively. Although Eaton suggests that the progesterone treatment was rescuing embryos that would otherwise fail to attach and would die before 5.5 d.g., the small number of embryos in each treatment group does not permit such a conclusion. It seems equally possible that the additional "escapers" were drawn from the normal class of embryos, particularly in view of his observations that the higher amounts of progesterone (15 to 20 mg) given at 4 d and 20 h led to whole litters of "escapers," but 5 to 16 mg progesterone administered over several days produced no increase in "escapers." This issue could have been resolved by observations on control matings, but unfortunately these were not reported.

Another category of experimental studies on mutant embryos involves their culture and analysis in vitro (reviewed by Bennett, 1975; Wudl et al., 1977; Gluecksohn-Waelsch, 1979). Pedersen (1974) first used this approach with yellow mouse embryos by obtaining 8-cell, morula, or blastocyst stage embryos from C57BL/6J or C57BL x SEC F1 hybrid mice and culturing them for 24 to 120 h. No abnormalities were consistently seen in 8-cell embryos; however, after 24 h of culture, there were 11% more abnormal embryos in A^y/a x A^y/a matings than in A^y/a x a/a matings. Also, Pedersen (1974) found 15% more abnormal morulae and blastocysts obtained without culture from experimental matings than from control matings. These abnormal embryos, like those found after 24 h culture of 8-cell embryos, had delayed or arrested cleavage, partial arrest and exclusion of blastomeres, and extensive

fragmentation or disorganization. Approximately two thirds of the abnormal embryos had exclusion of some blastomeres, and it was suggested that this defect characterizes the A^Y/A^Y embryo in C57BL mice. The only abnormalities seen in embryos from hybrid mice were the small, collapsed blastocysts found in experimental matings. Pedersen's (1974) evidence for a defect expressed in A^Y/A^Y embryos at preimplantation stages is weakened by the small numbers of matings studied in both experimental (10 to 13) and control (5 to 10) matings, and by the relatively low frequency of excess abnormalities in experimental matings after the incidence of abnormalities in appropriate controls was subtracted. A more solidly established feature of embryos from $A^Y/a \times A^Y/a$ matings was the failure of approximately one fourth of cultured blastocysts to escape from the zona pellucida. Pedersen (1974) reported that removing the zona pellucida with pronase enabled blastocysts from experimental matings to attach and grow out, but there was an 18% excess of abnormal outgrowths, as compared with controls. The principal abnormality observed was the small size and number of nuclei in the outgrown trophoblast monolayer and the absence of ICM cells in these outgrowths, in contrast to normal embryos.

In another attempt to detect early defects in A^Y/a embryos, Pedersen and Spindle (1976) compared the cell numbers in morphologically normal and abnormal embryos of $A^Y/a \times A^Y/a$ matings. The morphologically abnormal embryos had half as many cells ($n = 33$) as normal ($n = 60$), but their labeling index with ^3H -thymidine was the same. The smaller number of cells in abnormal embryos may be related to the delayed cleavage of embryos that developed morphological abnormalities. The time-lapse analysis cited by Pedersen and Spindle (1976) suggested that abnormal embryos divided from the 2- to 4-cell

stages 2 to 4 h later than normal embryos. For confirmation of these preliminary observations it would be necessary to carry out further time-lapse analysis of both control and experimental matings. It was apparent from our results (data not shown) that except for the delays noted in early cleavage, both normal and abnormal embryos cleaved with 11- to 17-h cell cycle times, and that there was variation between embryos in the asynchrony of blastomere divisions. These results are consistent with those obtained for normal embryos by Kelly et al. (1978) with time-lapse or periodic examination in several inbred strains of mice.

Studying the in vitro development of embryos from C57BL-A^y/a mice, Granholm and Johnson (1978) also found a correlation between early cleavage delay and the incidence of hatching failure in embryos from experimental and control matings. The excess of unhatched embryos in experimental matings was greatest (26%) among those that lagged in development at the 4- to 8-cell stage, perhaps owing to cleavage retardation in A^y/A^y embryos. However, there was also a 15% excess of unhatched embryos in the advanced group, which suggests that under their experimental conditions neither hatching failure nor lagging could be used to identify A^y/A^y embryos exclusively. In other work, Johnson and Granholm (1978) reported that among control matings of C57BL-A^y/a mice there were as many embryos with excluded blastomeres (17.5%) as in experimental matings (15.9%). More morphologically abnormal embryos were found at the morula stage in 61 control matings than in 16 experimental matings, although there was a 15.6% excess of abnormal embryos at the blastocyst stage in the experimental matings. These observations led Johnson and Granholm (1978) to conclude that morphological abnormalities are not adequate for identifying individual A^y/A^y embryos.

Papaioannou and Gardner (1976, 1979) came to a similar conclusion from studies of the A^y allele in AG/Cam mice. They transferred morphologically normal and abnormal embryos to foster mothers and found no correlation between morphological abnormalities and early postimplantation death of embryos from experimental matings. However, the high incidence of excluded blastomeres in both their control (32%) and experimental (37%) matings suggests that they may have been recognizing persisting polar bodies of morphologically normal embryos, or that abnormalities were induced by the superovulation protocol. Also, the manifestation of early abnormalities in A^y/A^y embryo development may be highly strain dependent, as suggested by the comparison of embryos from C57BL and C57BL x SEC F1 hybrid mice (Pedersen, 1974).

Despite their inability to recognize A^y/A^y mice before implantation, Papaioannou and Gardner (1979) made chimeras between embryos of experimental (A^y/a x A^y/a) and control matings. Their results, obtained by transferring the ICM from embryos of experimental matings into a control blastocyst (or vice versa) show that death of homozygous yellow mouse embryos is not due to cell lethality or to effects exclusively in the ICM. On the contrary, their results suggest that the trophectoderm of presumed A^y/A^y embryos may be specifically defective, and that ICM derivatives may survive for longer periods than previously realized when they are in chimeras with normal ICM and trophectoderm cells. Papaioannou and Gardner (1979) concluded from the observed rates of survival and chimerism that presumed A^y/A^y ICM derivatives are able to develop at least until 10.5 d.g. The implications of these and other observations on presumed A^y/A^y are considered below.

IV. CONCLUSIONS

It is apparent from the studies described here on cell commitment in the mouse ICM and primary ectoderm that inner cells become committed to their fate sometime after the beginning of outer cell differentiation. ICM becomes committed during a period of several hours between the beginning and end of blastocoele formation. After this time the isolated inner cells lose the ability to form trophectoderm and instead form primary endoderm when exposed to outside conditions. Primary ectoderm, which is enclosed by polar trophectoderm and primary endoderm in the intact late blastocyst, retains the ability to form primary endoderm for 40-48 h in ICMs isolated from chimeric embryos. After this time, the fate of isolated ectoderm may still be labile, but inner cells seem to require cell feeding from endoderm or other sources for survival. The apparent differentiation of outer cells before the commitment of inner cells may imply that outer cells play a role in the commitment of inner cells as originally suggested by Tarkowski and Wróblewska (1967). The ICM is known to affect the fate of trophectoderm differentiation (Gardner, 1972; Gardner et al., 1973); however, the nature of positional cues in the early embryo is poorly understood. It appears that blastocoele fluid by itself does not mediate the commitment of pluripotent cells to an inner fate. In the further analysis of cell commitment in the early mouse embryo, the role of cell-cell contact and even cell-extracellular matrix interaction should not be overlooked (Hay and Meier, 1976; Wolpert, 1978). It may be necessary to define the substance(s) that indicate position in early mouse embryos in order to study the biochemical basis for cell commitment and for the transition from one decision-making state to another during subsequent stages of development.

Although the analysis of the a locus has been carried out from a very different perspective than the analysis of cell commitment, several relevant points can be made from the study of the mutant embryos. First, although no single definitive study has been carried out to describe the time and mode of death of A^y/A^y embryos in utero, a comparison of all the studies done with yellow mouse embryos leads to the conclusion that the homozygotes die at approximately 5.5 to 6.0 d.g. This is after the commitment of ICM and may approximately coincide with the time of commitment of primary ectoderm cells. However, the timing of A^y/A^y embryo death probably has little to do with commitment of ICM or ectoderm, in view of the interpretation from Gardner and Papaioannou's chimeric analysis (1979) that ICMs of A^y/A^y embryos can survive as late as 10.5 d.g. Further work is clearly necessary to determine whether A^y/A^y cells can survive throughout gestation.

The intriguing possibility that trophectoderm of A^y/A^y embryos is specifically defective also deserves further work. Such a defect could account for the preponderance of hatching failures in matings containing A^y/A^y embryos. A carefully controlled morphological and biochemical analysis of yellow mouse embryos at 4.5 to 5.5 d.g. could further elucidate the effect of homozygosity on trophectoderm and ICM tissues before resorption occurs. Such a study would require identifying the homozygotes before extensive deterioration occurs. The cleavage delay in A^y/A^y embryos may not be large enough to serve this purpose, given the variation in the timing of early developmental events in normal embryos. Also, hatching failure is a retrospective means of identifying presumed homozygotes, and embryos that fail to hatch may have already begun to deteriorate. A means for identifying

preimplantation stage A^y/A^y embryos would clearly facilitate these studies. Similar analyses of embryos homozygous for the a^x allele could also add perspective to the pattern of defects found in A^y/A^y embryos.

The relevance to implantation of the studies reviewed here derives mainly from the fact that successful postimplantation development in utero requires both trophectoderm and ICM survival and differentiation. The understanding of cell commitment in early mammalian embryos should be greatly facilitated by the application of genetic analysis, as in other developing systems. Because it is difficult to select for recessive mutations that affect mammalian embryos (Pedersen and Goldstein, 1979), existing mutations at the a locus and other loci should continue to be valuable for understanding preimplantation and early postimplantation development.

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TABLE I STAGE DEPENDENCE OF TROPHECTODERM FORMATION BY ISOLATED INNER CELLS^a

Stage of embryo	n	Developmental fate of inner cells	
		Trophectodermal vesicle formation	Trophoblast outgrowth
Late morula ^b	82	59	65
Early blastocyst ^b	116	73	73
Mid-blastocyst ^b	51	1	5
Late blastocyst ^c	27	0	0

^aInner cells were isolated by immunosurgery from chimeras of five embryos at the stage indicated and scored for vesicle formation (24 h later) or trophoblast outgrowth and giant cell formation (120 h later). Data from Spindle (1978).

^bInner cells were isolated at 69 h of culture from the 2-cell stage. The transition between early blastocyst and mid-blastocyst was arbitrarily taken as blastocoele expansion equal to or greater than one-half the volume of the blastocyst, as estimated in the dissecting microscope.

^cInner cells were isolated at 93 h of culture from the 2-cell stage.

TABLE II TIME DEPENDENCE OF ENDODERM FORMATION BY ECTODERM ISOLATED FROM ICMs^a

Time of ICM culture (h)	n	Ectoderm with endoderm
24	28	22
48	14	14
72	31 ^b	1

^aICMs were isolated from late blastocyst chimeras of 10 embryos using immunosurgery and cultured for 24 to 72 h; ectoderm was then isolated by immunosurgery, cultured for 48 to 72 h, and scored for endoderm formation. Data from Pedersen et al. (1977).

^bMost ectoderms degenerated within 48 h without forming endoderm.

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