Formation of the Blastocyst: Determination of Trophoblast and Embryonic Knot *

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I. Introduction 59
II. Trophoblast and Embryonic Knot Representing Distinct Populations of Cells (Differentiation of Trophoblast and Embryonic Knot) 60
III. Determination of Trophoblast and Embryonic Knot 62
1. Theory A: Determination Depending on the Position of Blastomeres (Inside-Outside Model) 62
Observations Supporting Theory A 62
2. Theory B: Determination Depending on Polar/Bilateral Organization of the Egg (Morula) 64
Observations Supporting Theory B 65
a) Experiments 65
b) Morphological and Histochemical Data 66
IV. General Discussion 70
References 74

I. Introduction

In mammalian development, the earliest apparent differentiation of cells into two distinct lines manifests itself in the early blastocyst, when trophoblast and embryonic knot ("inner cell mass") become distinguishable. Recently more and more attention is being paid to this process by developmental biologists, geneticists, cell biologists, reproductive biologists, and oncologists. This is apparently for one of the following reasons: (1) Blastocyst formation seems to result from the first process of determination in mammalian ontogeny, which means that the genome has not been under the influence of any determinative stimuli before. (2) The system appears to be far less complex than later stages so that it may be especially suitable for experimentation. (3) Determination of trophoblast, a tissue of some peculiar properties, may exhibit interesting specific features. (4) Trophoblast is in the focus of interest of reproductive biologists as well as oncologists because of its role in mediating contact between embryo and mother, and because of its invasive growth.

Attempts of investigators to elucidate the mechanism of determination of trophoblast and embryonic knot led them to design elegant experiments which, in turn, resulted in formula-

* Dedicated to Professor Dr. F. Seidel.

tion of different theories to be described in this paper. Excellent reviews of part of the arguments summarized here have been given by *Mulnard* (1966), *Seidel* (1969), *Graham* (1971), *Gardner* (1973), and *Herbert* and *Graham* (1974).

Terminology

"Embryonic knot": In the present paper, we will use this term rather than the more commonly used term "inner cell mass" because the latter would fit only one of the theories to be described here, and because it would not be suitable for discussion of a possible onset of determination before certain cells acquire an inside position.

"Polarity": The term is being used in a general sense not referring specifically to polar organization along the animal-vegetal axis.

II. Trophoblast and Embryonic Knot Representing Distinct Populations of Cells (Differentiation of Trophoblast and Embryonic Knot)

Considering the process of blastocyst formation to be connected with a process of cell differentiation would require data showing that trophoblast and embryonic knot (or at least one of both) are in fact composed of distinctly differentiated cells. The following observations give support to this view:

1. Trophoblast and embryonic knot cells, when isolated from 3 1/2-day mouse blastocysts, differ in their ability to induce a decidual reaction in the pseudopregnant uterus: trophoblast cells do induce this reaction, whereas embryonic knot cells do not (*Gardner*, 1971, 1972a).

2. The same isolated trophoblast and embryonic knot cells also differ in their tendency to stick together and to form a common structure in vitro: embryonic knot cells do so, whereas trophoblast cells stay apart; the latter form fluid-filled vesicles, whereas the former produce only solid cell clusters. Embryonic knot cells, when injected into the cavity of another blastocyst, will become integrated and form part of the body of the embryo, even rat embryonic knot cells injected into a mouse blastocyst: this is not the case with likewise injected trophoblast cells (*Gardner*, 1971, 1972a; *Gardner* and *Johnson*, 1973).

3. Trophoblast cells of blastocysts are connected with each other by junctional complexes. Well-developed junctions can already be seen between blastomeres in the outer layer of cleavage stages, i.e., between presumptive trophoblast cells, whereas between inner cells of morulae or between trophoblast and embryonic knot of blastocysts they are more rare and remain primitive. Differences in density of the cytoplasm and in number as well as structure of certain organelles have also been described (rat: *Schlafke* and *Enders*, 1967; *Dvořak*, 1971; mouse: *Calarco* and *Brown*, 1969; rabbit: *Hessedahl,* 1971).

4. Mitosis rate seems to be different in both types of cells, the embryonic knot showing the higher values. There is already a difference between inside and outside cells of morulae

as judged from combination of cell number and ^{3}H-thymidine incorporation studies (*Barlow* et al., 1972).

5. In histochemical studies, differences between trophoblast and embryonic knot have been found relatively often, e.g. differences in phosphatase activity. Problems of interpretation of histochemical findings are in part discussed elsewhere (cf. also *Denker*, 1970).

6. Differences in cell surface properties of trophoblast and embryonic knot are possibly indicated by the fact that, in mouse blastocysts, both types of cells exhibit slightly different susceptibility to lysis by cytotoxic antisera (*Moskalewski* and *Koprowski*, 1972); furthermore, certain viruses injected into the blastocyst develop in the trophoblast but not in the embryonic knot (*Glass* et al., 1974).

7. Evidence has been presented for the expression of different esterase isoenzymes in trophoblast and embryonic knot of mouse blastocysts. The trophoblast-type isoenzyme A was detectable already in the late morula; this might be the earliest well-established biochemical criterium for beginning differentiation of trophoblast (*Sherman*, 1972).

The above mentioned data give evidence for morphologic, biochemical, and physiologic differences between trophoblast and embryonic knot. This may indicate that a process of differentiation has taken place, if this term is used in a merely descriptive sense – differentiation meaning the establishment of differential properties of cells. Direct evidence for differential gene activity of trophoblast and embryonic knot cells, however, is still lacking but might be found in the near future because it is already established that the genome is active in preimplantation embryos: Some indirect evidence is derived from investigations on synthesis of different classes of RNA and on changes in enzyme activities during preimplantation (although technical problems of determination of intracellular pools of precursors are not completely solved, and changes in enzyme activity do not necessarily reflect changes in genetic activity) (for review see *Church* and *Schultz*, 1974; *Graham*, 1973; *Woodland* and *Graham*, 1969). Effects of actinomycin D and α-amanitin demonstrate that RNA synthesis is indeed required for cleavage and blastocyst formation (*Golbus* et al., 1973; *Manes*, 1973). Direct evidence for genetic activity is derived from the fact that t^{12}/t^{12} homozygous mouse embryos die at the late morula stage (*Mintz*, 1964a) (apparently after onset of trophoblast differentiation, *Hillman* et al., 1970), and by the finding that the paternal phenotype of glucose phosphate isomerase isoenzymes is expressed in the blastocyst stage (*Chapman* et al., 1971), and the information for hypoxanthine-guanine phosphoribosyltransferase is apparently transcribed as early as the morula stage (*Epstein*, 1972).

Of the two classes of cells, trophoblast and embryonic knot, trophoblast gives better evidence that it undergoes real differentiation until the blastocyst stage: The trophoblast develops junctional complexes, secretes blastocyst fluid, is able to induce a decidual reaction, produces a specific esterase isoenzyme (see above) and a protease or protease activator (*Denker*, 1971a, 1974). The embryonic knot cells, on the contrary, seem to remain in a more primitive state (cf. also *Gardner*, 1971).

III. Determination of Trophoblast and Embryonic Knot

1. Theory A: Determination Depending on the Position of Blastomeres ("Inside-Outside Model") (Fig. 1A)

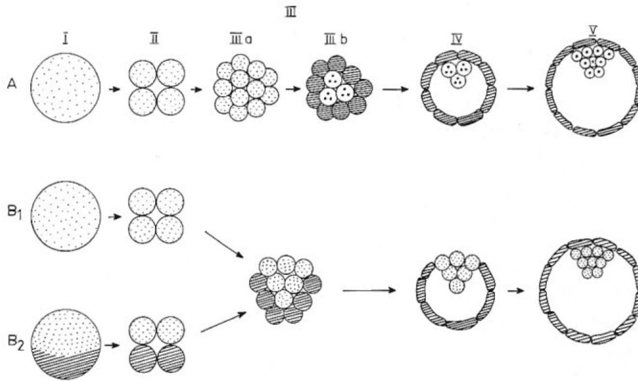

Fig. 1. Diagram to illustrate the two theories on determination of trophoblast and embryonic knot. Theory A (inside-outside model): In the beginning, the developmental potentials of all blastomeres are equal (A II, IIIa). When, in the course of cleavage, some cells become completely surrounded by others, these inside cells are being determined to form embryonic knot (inner cell mass) (A IIIb); the other (outside) blastomeres will differentiate into trophoblast.
Theory B: Determination depends on localized factors of polar (bilateral) distribution, which, in this diagram, are assumed to be trophoblast-determining. These factors are either (B_2) localized in a certain area of egg cytoplasm (B_2 I) and become segregated during cleavage so that they will be found only in certain blastomeres (B_2 II); or (B_1) factors are of unknown, maybe even exogenic, origin, but their action is nevertheless locally restricted. In both cases (B_1 and B_2), morulae show polarity (III) as a result of polar action of determining factors

According to this theory, there are no differences between blastomeres either in their status of determination or in their developmental potentials, until one or several of them become completely surrounded by others, i.e., in or after the eight-cell stage. The completely surrounded cells will form the embryonic knot (inner cell mass), whereas the whole of the outer layer will become trophoblast. The ability to differentiate into trophoblast is inherent to all blastomeres at the beginning; the information (the determinative stimulus) to form, instead, embryonic knot is provided by the specific milieu "inside" a morula or blastocyst (*Tarkowski* and *Wróblewska*, 1967).

Observations supporting theory A have mostly been made in the course of experimentation in the mouse. Two or more cleavage stage mouse embryos up to the morula stage can be fused together to form one single chimeric blastocyst developing into one single embryo (*Tarkowski*, 1961, 1965; *Mintz*, 1962, 1965). This demonstrates a remarkable regulative capacity of the early mammalian embryo. Even rat and mouse morulae can be combined this way to form interspecific chimeras (*Stern*, 1973; *Zeilmaker*, 1973). If, in these embryos, future trophoblast and embryonic knot cells (or one of both groups) had already been determined in a fixed and unchangeable manner, only a specific sorting out of cells

during fusion could explain the formation of one single chimeric blastocyst. At first, this type of explanation has in fact been given (*Tarkowski*, 1961). Labeling experiments, however, did not give evidence for any specific sorting-out process (Fig. 2) (*Mintz*, 1964b, 1965; *Hillman* et al., 1972).

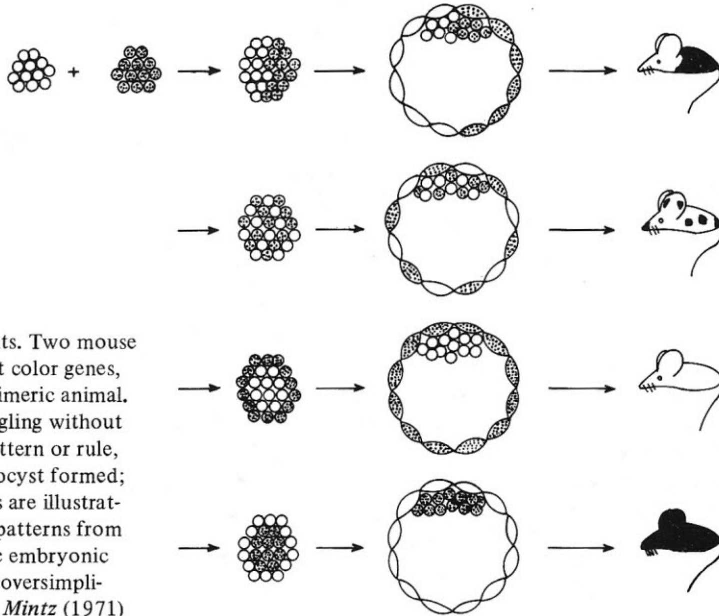

Fig. 2. Fusion experiments. Two mouse morulae, differing in coat color genes, are fused to form one chimeric animal. Blastomeres are intermingling without exhibiting any regular pattern or rule, as to be seen in the blastocyst formed; four extreme possibilities are illustrated. Resulting coat color patterns from blastocysts with chimeric embryonic knot (first two lines) are oversimplified; for exact details see *Mintz* (1971)

In fact, blastomeres put outside a morula tended to become incorporated into the trophoblast rather than the embryonic knot. Several variants of this experiment have been performed, all giving principally the same result. This obviously indicates that the developmental fate of blastomeres does, or at least can, depend on their position – inside or outside (Fig. 3) (*Hillman* et al., 1972). Note that in this type of experiment it is not known whether the blastomeres used for recombination are presumptive trophoblast or embryonic knot cells, so that conclusions can be drawn only from statistical analysis of proportions of labeled cells in the trophoblast (or embryonic knot) of the chimera.

Very impressive are experiments in which all blastomeres of a ^{3}H-thymidine-labeled 8-16 cell embryo were brought into an inside position by the way that this embryo was being surrounded by another 14 unlabeled embryos (Fig. 3) (*Hillman* et al., 1972). By this means, all cells of the labeled inside embryo could be forced, in some cases, to form part of the embryonic knot, even those which normally would have formed trophoblast.

Another way of labeling cells was used in experiments by *Wilson* et al. (1972) and *Stern* and *Wilson* (1972): silicone oil microdroplets were put into the cytoplasm. Peripheral cells of late morulae or early blastocysts of the mouse were labeled this way and the embryos fused with either identical stages or eight-cell embryos. In the resulting chimeras, part of the labeled outside cells were found in the embryonic knot. This again demonstrates that

either the fate of outside (presumptive trophoblast) cells of the late morula/early blastocyst is not fixed yet, or that not all of these cells are presumptive trophoblast (cf. p. 72).

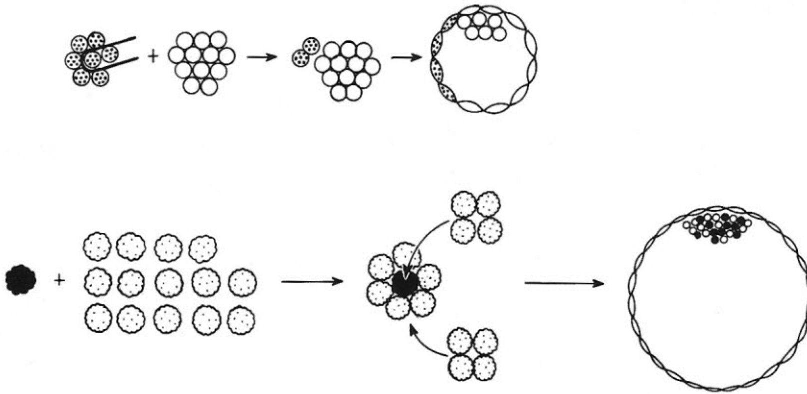

Fig. 3. Fusion experiments: Demonstration of decisive role of inside or outside position of blastomeres (after *Hillman* et al., 1972). Mouse embryos. Labeling was done with ^{3}H-thymidine.

Above: Two labeled blastomeres of 8-cell stage on outside of 8- to 16-cell unlabeled embryo. Position of labeled cells was determined in resulting blastocyst. In 40 experiments performed, 92% of all labeled cells were found in trophoblast.

Below: Labeled 8- to 16-cell embryo is being completely surrounded by 14 unlabeled embryos of same stage (note that each symbol here represents one whole embryo). Result is a giant blastocyst able to give rise to a well-shaped embryo. In 4 out of 7 experiments, labeled cells were found exclusively in embryonic knot

The marked ability of disaggregated mouse embryos (even blastocysts) to reaggregate and form regular blastocysts was also taken as an argument for developmental lability of blastomeres (*Stern*, 1972).

The type of experiments described above have been reviewed in detail by *Herbert* and *Graham* (1974).

Another line of evidence that theory A might be correct was derived from observations of coincidence; there was a marked increase of uridine incorporated into RNA in the stage when blastomeres begin to acquire an inside position. But note that in the mouse, all major classes of RNA are synthesized from the four-cell stage on, and on the other hand, the problem of interference of intracellular pools of precursors has not yet been solved (reviewed by *Graham*, 1973; *Church* and *Schultz*, 1974).

2. Theory B: Determination Depending on Polar Bilateral Organization of the Egg (Morula) (Fig. 1B_1, B_2)

According to this theory, determination does not depend on the inside or outside position of blastomeres but on morphogenetic factors restricted to a localized area in the egg or morula. In the most elaborate version of this theory (Fig. 1B_2), these factors are presumed to be present in a certain area of cytoplasm of the uncleaved egg; during cleavage, segrega-

tion occurs, and those blastomeres which receive part of this material will be determined to differentiate into a certain direction: e.g. if the factors are embryonic knot-determining, the progeny of these blastomeres will develop into embryonic knot (part of them may, in addition, also form trophoblast).

It is also possible to formulate a more general version of this theory which does not include polar organization of the uncleaved egg but only postulates that, during cleavage, the embryo acquires polarity due to unknown processes (governed e.g., by locally acting exogenic factors (Fig. $1B_1$).

In each case, according to this theory, the primary arrangement of presumptive trophoblast and embryonic knot cells in a cleavage stage embryo would exhibit polarity, depending on the eccentric localization of the determining factors. This is in clear contrast to the radial symmetry suggested by the inside-outside model (theory A, Fig. 1A).

Observations supporting theory B have been made in the course of experimentation and of morphologic and histochemical investigations in several species.

a) Experiments

One of the blastomeres of the rabbit two-cell embryo or three of the blastomeres of the four-cell stage were destroyed by pricking them with a needle, and the developmental potential of the surviving blastomere was followed (*Seidel*, 1952, 1956, 1960). The result, relevant to the problem discussed here, was that not all of these surviving blastomeres formed regular blastocysts: in a certain proportion of them (about 1:2 in case of experimentation in the two-cell stage) the embryonic knot was lacking and only trophoblast (and sometimes also entoderm) developed.

This experiment demonstrates limits of regulative capacities of the rabbit embryo. Referring also to comparative aspects based on numerous data from lower animals, *Seidel* formulated the theory that, in the mammalian egg cytoplasm, a specialized area exists (*Plasmatischer Faktorenbereich*, plasmatic field of factors), which is organized like a formative center (*Bildungszentrum*). Whereas all blastomeres primarily have the potential to form trophoblast, only those of them which receive, during cleavage divisions, part of the cytoplasmic field of factors will be able to differentiate, in addition, an embryonic knot (which means they have all the information available to form a whole blastocyst). The result of the deletion experiment (blastocyst or trophoblastic vesicle) would depend on whether the surviving blastomere by chance was one of those which do possess the factors, or one of those which do not (Fig. 4).

Comparable experiments in the mouse seemed to show the same trend, and at first the same interpretation has been given (*Tarkowski*, 1959, 1961). Later on, the results of studies of the developmental capacities of all blastomeres of four to eight cell embryos were given an interpretation in favor of the inside-outside model (theory A) (*Tarkowski* and *Wróblewska*, 1967) (see general discussion).

Labeling certain parts of cytoplasm by injecting silicone oil droplets revealed in the mouse egg, that there is no important spatial disturbance of the cytoplasmic pattern of the egg during cleavage: the cortical region of the egg being converted to the outer cells of the

morula. (Nevertheless, fusion experiments done with these labeled embryos show that these cortical regions also can be forced to become part of the embryonic knot) (*Wilson* et al., 1972; *Stern* and *Wilson*, 1972).

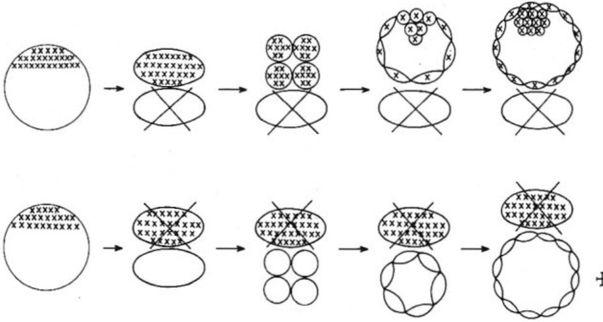

Fig. 4. Seidel's experiment in rabbit. One blastomere of the 2-cell stage is being destroyed (marked by large cross). The surviving blastomere will develop (after transfer of whole embryo into foster mother) into either a regular blastocyst with embryonic knot (*above*), or a trophoblastic vesicle without embryonic knot (*below*). Result is thought to reflect that the surviving blastomere either contained or lacked material of a cytoplasmic field of factors (*Bildungszentrum*) present in a restricted area of egg cytoplasm which provides information for determination of embryonic knot. Formation of trophoblast, on the contrary, is assumed to be a general ability common to all blastomeres (with or without *Bildungszentrum*)

The assumption that the cytoplasmic field of factors determines embryonic knot rather than trophoblast is not necessarily an integral part of theory A. The available experimental results do not completely rule out other interpretations like: Both embryonic knot – determining as well as trophoblast – determining fields of factors might exist at opposite poles of the egg. Or there might be only factors providing the information for determination of trophoblast; these factors might be organized like a field, and the embryonic knot be formed at the opposite pole. Available information does not yet allow one to decide which of these possibilities is the correct one. Because there is more evidence for the trophoblast than for the embryonic knot to undergo real differentiation during these early stages (see p. 61), we like to reillustrate *Seidel*'s experiment assuming that the cytoplasmic factors are trophoblast-determining (Fig. 5). Embryos lacking trophoblast (i.e., pure embryonic knots) (Fig. 5 I) were not found by *Seidel* (1960, 1969), or rarely found by *Tarkowski* and *Wróblewska* (1967). Apart from other possibilities, this could still be explained assuming that the field of factors is trophoblast-determining but extends over a rather large area so that even blastomeres of the opposite pole would still be able to form trophoblast (in addition to embryonic knot) (Fig. 6).

b) Morphologic and Histochemical Data

In a large series of papers *Dalcq* and *Mulnard* (*Dalcq*, 1951, 1954, 1955, 1962a, b, c, 1966; *Mulnard*, 1955, 1965; *Mulnard* and *Dalcq*, 1955) presented a number of data on histochem-

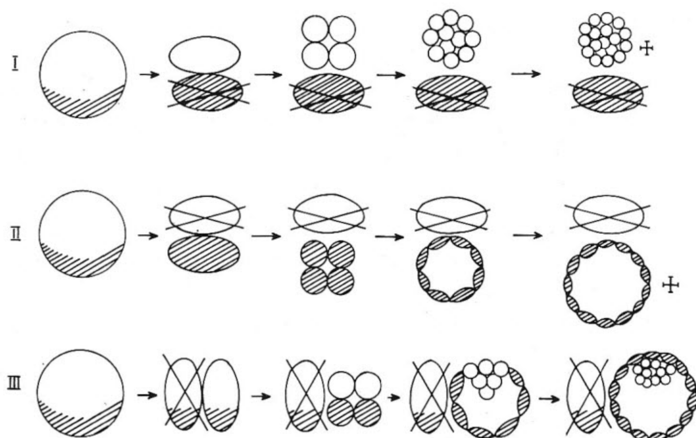

Fig. 5. Seidel's experiment in rabbit (cf. Fig. 4) redrawn assuming that the cytoplasmic field of factors is trophoblast-determining. First cleavage furrow can lie in different planes and either restrict this field to one of two blastomeres (I, II) or devide it (III). For descriptive purposes, the situation is oversimplified in this diagram illustrating mosaic-type reactions: Half-embryos which consist only of "trophoblast-factor cytoplasm" form only trophoblast (II), half-embryos without it form only embryonic knot (I), half-embryos with both types of cytoplasm form both types of cells, i.e. a whole blastocyst (III)

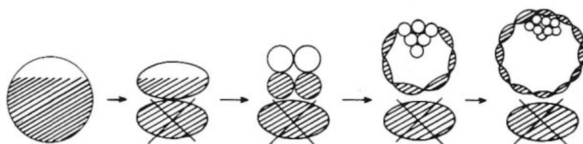

Fig. 6. Same as in Fig. 5 I, but assuming that the area which the trophoblast-determining cytoplasmic field of factors takes in the egg is larger. This could explain why pure embryonic knots (cf. Fig. 5 I) are never (or rarely) seen in the experiments. Another possible explanation for the same phenomenon is provided by theory described in Fig. 4 (legend)

ical differences between blastomeres, and correlated them with differences between trophoblast and embryonic knot seen in blastocysts. Most of this work was done in the rat, including a number of investigations in the mouse. The uncleaved egg was described to exhibit a plane of bilateral symmetry (most obvious in the rat) forming a certain angle with the animal-vegetal axis, the cytoplasm of the so-called dorsal side being especially rich in RNA (basophilic region, cf. *Jones-Seaton*, 1950; see also *De Geeter*, 1954) and exhibiting a characteristic diffuse type of acid phosphatase reaction (*Mulnard*, 1955, 1965). In cleavage stages, these histochemical characteristics were found to be restricted to certain blastomeres that finally will form the embryonic knot after becoming enveloped by the presumptive trophoblast cells (cf. e.g. *Mulnard*, 1966, Fig. 2). As a submicroscopic equivalent of the basophilic region, *Krauskopf* (1968) described in the rabbit egg an area rich in polyribosomes and poor in other organelles. There is no report on comparable observations by other electron microscopists.

The conclusions drawn from the above-mentioned investigations are not widely accepted today (cf. *Tarkowski* and *Wróblewska*, 1967; *Herbert* and *Graham*, 1974). Reinvestigations of the phosphatase distribution using azo-dye methods instead of Gomori-type reactions failed to confirm differences of enzyme reaction between blastomeres in the rat (*Rodé* et al., 1968) or mouse (*Denker*, unpublished), although in the hamster they did show the more intense reaction of the embryonic knot (*Ishida*, 1972).

If there are histochemical differences between blastomeres, it is most relevant for the discussion of theories A and B whether the arrangement is similar to Figure 1 A IIIb or Figure 1 B III. Diagrams showing the latter type of blastomere arrangement can often be found in *Dalcq*'s and *Mulnard*'s publications (e.g. *Mulnard*, 1966, Fig. 2), but unfortunately, photographs documenting this unequivocally are rare because of technical difficulties with photography of whole mounts.

Table 1. List of references showing polar arrangement of two groups of blastomeres in eutherian mammals.

Species	References
Rabbit	*van Beneden* (1880) Pl. IV Fig. 1-3, 7
Rabbit	*Assheton* (1895) Pl. 14 Fig. 18, 20; Pl.15 Fig. 22
Bat	*Duval* (1895) Pl. III Fig. 9, 14, 15, 17, 18, 20, 21; Fig. IX p. 140; Fig. X p. 151
Bat	*van Beneden* (1899) Fig. 1 p. 310
Bat	*van Beneden* (1911) Pl. II Fig. 25, 29; Pl. III Fig. 37, 40
Bat	*van Beneden* and *Julin* (1880) Pl. XXIII Fig. 5, 6
Bat	*Wimsatt* (1944) Fig. 23 p. 404/405
Mole	*Heape* (1886) Pl. XI Fig. 20
Sheep	*Assheton* (1898/99) Pl. 15 Fig. 7, 8; Pl. 18; Text-Fig. p. 222
Goat	*Amoroso* et al. (1942) Text-Fig. 4 p. 388; Text-Fig. 5 p. 390
Pig	*Heuser* and *Streeter* (1929) Fig. 5 p. 16; Fig. 6 p. 17; Pl. 12
Rat, mouse	*Skalko* (1971) Fig. 15-1 d p. 242
	Dalcq; Mulnard (various papers, see list of references)

In the older morphologic literature, illustrations (again mostly drawings) that show such a polar arrangement of blastomeres can be found more often (Table 1). They are mostly based on purely morphologic criteria, like cell size. This often seems to include a lot of interpretation, especially when blastomeres of intermediate size exist but are nevertheless put in one or the other of the two categories of cells (*Heuser* and *Streeter*, 1929; cf. their Fig. 4, p. 14, with Figs. 5 and 6, pp. 16 and 17).

Recently, *Denker* (1970, 1971b, 1972) described a similar polar arrangement of two distinct types of blastomeres in rabbit embryos. After formol-alcohol-acetic acid fixation, which was thought to either chemically modify or extract certain fractions of proteins

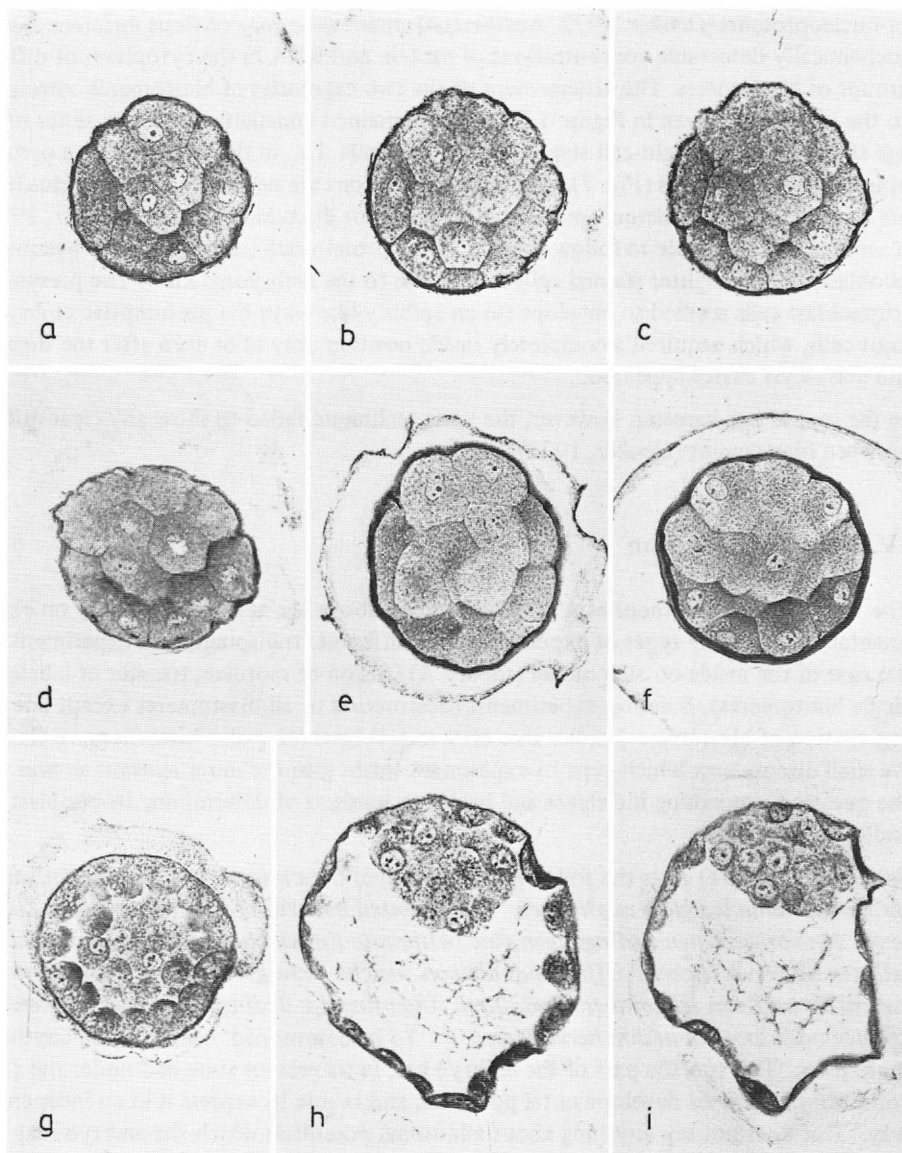

Fig. 7. Aspects of polar arrangement of two groups of blastomeres in the rabbit. Embryos were fixed with formol-alcohol-acetic acid, paraffin sections were taken, proteins were stained with Hg-bromphenol blue. x 260. a-c: 54 h p.c., three sections from same embryo; d: 54 h p.c.; e, f: 63 h p.c.; g: 76 h p.c., blastocyst cavity just appearing (cleft); h, i: 80 h p.c., early blastocyst.

Two categories of blastomeres can be distinguished by intensity of cytoplasmic staining: 1. Lighter stained, not polarized cells; in early blastocyst, light cells form embryonic knot. 2. Darker stained cells which often show very obvious maximum of stain uptake in parts of cytoplasm directed toward center of egg. When cavitation begins, they can be identified as prospective trophoblast cells (g). Trophoblast of early blastocysts also stains more intensely than embryonic knot (h, i). Note that in cleavage stages trophoblast-type cells form a single-layered cap which surrounds other blastomeres only incompletely (polarity!), but area taken in different eggs is different (a-f)

or nucleoproteins (*Denker*, 1972, Aussprache), there were very obvious differences in histochemically detectable concentrations of protein and RNA in the cytoplasm of different groups of blastomeres. The arrangement of the two categories of blastomeres corresponded to the illustration given in Figure 1 B III, and remained constant over a long series of cleavage stages, from the eight-cell stage to the late morula, i.e., in this species over a period of at least three cell cycles (Fig. 7). Although precautions are necessary when conclusions are to be drawn from histochemical observations (for discussion cf., e.g., *Denker*, 1970), it seemed to be possible to follow the fate of the protein-rich (darker) cells to become trophoblast, and the lighter stained cells to give rise to the embryonic knot. The presumptive trophoblast cells seemed to envelope (in an epiboly-like way) the presumptive embryonic knot cells, which acquired a completely inside position only at or even after the time when the blastocyst cavity appeared.

In the mouse and hamster, however, the same technique failed to show any clear differences between blastomeres (*Denker*, 1972).

IV. General Discussion

The two controversial theories A and B described above are both based mainly on experimental data, but the types of experiments are different: transplantation experiments in the case of the inside-outside model (theory A) (fusion of morulae; transfer of labeled single blastomeres), isolation experiments (destruction of all blastomeres except one; disaggregation of blastomeres) in the case of theory B (including also histochemical findings). We shall discuss now which type of experiment might give the more relevant answer to the question concerning the stages and basic mechanisms of determining trophoblast and embryonic knot.

Seidel (1953, p. 91) gives the following *definition of determination: 'Für eine Aufgabe determiniert' kann lediglich ausdrücken: 'Der Keimteil besitzt in seinem derzeitigen Zustand unter den vorhandenen Bedingungen eine bestimmte Entwicklungsbefähigung und kann sie selbständig verwirklichen.' Offen muß bleiben, welche nicht genannten Fähigkeiten noch außerdem im Keim schlummern und durch Änderung der Bedingungen erweckt werden können oder spontan in ihm hervortreten.* (" 'To be determined' cannot mean anything more than: 'This specific part of the embryo has, in its present state and under the given conditions, a defined developmental potential, and is able to express it in an independent way.' This does not say anything about additional potentials which the embryo may have and which could manifest themselves either when conditions are changed, or even spontaneously." Translation by author).

Spemann (1936, p. 23) similarly uses the ability of cells to differentiate independently as the most important criterium: *Wenn ein Keimteil die Ursachen einer bestimmt gerichteten Weiterentwicklung in sich selbst trägt, so kann man sagen, daß er zu seinem Schicksal bestimmt, 'determiniert', ist. Jedenfalls kann man mit Lillie (1929) den Begriff der Determination so fassen, daß man die Selbstdifferenzierungsfähigkeit zu seinem Kriterium macht.* ("Whenever a certain part of an embryo carries in itself the causes for its development into a certain direction, it is possible to say that this part is 'determined' for its fate. At least it is possible to define the term determination that way, using the ability for self-differentiation as a criterium, according to *Lillie* (1929)." Translation by author).

It is not necessary to discuss here the concept of "self-determination" which itself can be criticized for being, in its strict form, too narrow for most systems. The important point is that the ability to differentiate after isolation, which can be proved experimentally, is being used as a criterium. If this definition of determination is accepted, it follows that isolation experiments should give better information about the determined or nondetermined status of a cell than transplantation experiments can do. In the latter type of experiments, the cells are brought under the influence of different parts of the embryo (or other tissues), and the original state of the transplant and the conditions are drastically changed. *Spemann*, who has done a lot of transplantation experiments, comments (1936, p. 31): *"So wird Transplantation im neuen Gewebsverband nur dann sichere Auskunft geben können, wenn Selbstdifferenzierung stattfindet, wenn also die Determination des Implantats genügend befestigt ist, um sich auch gegen einen etwaigen Einfluß der Umgebung durchzusetzen. Der erste Eintritt der Determination wird sich dagegen nur bei völliger Isolierung erkennen lassen."* (Transplantation . . . "will give a clear answer only in case of self-differentiation, i.e., when determination of the implant is stabile enough to dominate over possible influences of the surrounding tissues. The very beginning of determination can only be recognized in case of complete isolation." Translation by author).

According to this, the results of the described isolation experiments which favor theory B would seem more relevant for the discussion of mechanisms involved in determination of trophoblast and embryonic knot. The transplantation experiments described on pp. 62 ff., on the other hand, demonstrate the high regulative capacity of these early embryonic states. When, for example, all blastomeres of a morula, even those which normally would have developed into trophoblast, can be forced to form part of the embryonic knot (p. 63, Fig. 3), this shows in an impressive way the flexibility of the system and that it does not exhibit features of a mosaic. But is seems questionable if this type of experiment can uncover whether cells are already *inclined* (but not irreversibly switched yet) to form trophoblast, which isolation experiments possibly do reveal.

Often a different definition of cell determination is being used, e.g. by *Herbert* and *Graham* (1974): "Cell determination is the process by which the developmental potential of a cell becomes limited during embryogenesis." Transplantation experiments can certainly uncover limited developmental potentials. This is definitely the case when the limitation became irreversible. But, we feel that this is a secondary process following the establishment of an inclination of cells to develop into one or the other direction.

Isolation experiments are also certainly problematic because they do change the state and conditions of cells, both due to the isolation procedure (e.g. pronase and EDTA-treatment, *Tarkowski* and *Wróblewska*, 1967) and to in vitro culture conditions. If part of the embryo is being destroyed but not removed (*Seidel*, 1960), it might influence the results of the experiment: when, in the amphibian embryo, one blastomere of a two-cell stage was destroyed but not removed, the surviving blastomere produced only a half embryo (*W. Roux*); on the contrary, when both blastomeres were completely separated, they both regulated and each one formed a whole twin embryo (*Spemann*, 1936, p. 11 ff.). It is therefore interesting that in *Tarkowski*'s and *Wróblewska*'s experiments (1967), the completely isolated blastomeres did develop into different forms of vesicles (blastocysts, "false blastocysts," trophoblastic vesicles), and some of them even formed only uncavitated masses of cells. The authors felt, however, that these differences might be due to differences in culture conditions. They concluded that: 1. At least in some eggs, all blastomeres have the potential to form vesicular

structures (this view also being part of *Seidel's* theory, see p. 65). 2. Incidence of differentiation into real blastocysts decreases from one-quarter blastomeres to one-eighth forms. *Tarkowski* and *Wróblewska* feel that most probably this is due to the fact that with advancing stage of development of the blastomeres at the time of their separation, the number of cells attained by them at the time of cavitation decreases. Consequently, the probability that cells become enveloped decreases. The authors imagine that up to the eight-cell stage, all blastomeres possess the ability to differentiate into trophoblastic direction, and development into embryonic knot cells is being triggered by an inside position (theory A).

Data presented by *Moore* et al. (1968) (rabbit) unfortunately do not contribute to the discussion of these two theories because an account of purely trophoblastic structures is not included.

As a different kind of approach, morphologic and histochemic analyses give additional information. By fixing the embryos it is intended to preserve certain characteristics of the momentary state of blastomeres without changing them by initiating regulatory processes as it probably happens in both transplantation and isolation experiments. The polar arrangement of different types of blastomeres as seen in the rabbit morula seems to form an argument for theory B (see p. 68 f.). This arrangement also provides important aspects for the interpretation of fusion experiments: if it is correct, as suggested by the histochemical findings, that only some but not all of the peripheral cells are determined to form trophoblast, then the fact that a proportion of labeled outside cells become included in the embryonic knot of the chimera (p. 63 f.) could reflect simply the yet undetermined state of some of the outside cells. This finding would then not contradict theory B anymore.

Theory B, in its elaborate form, implies that a specific area of the egg cytoplasm exerts a regulatory effect on gene activity. Influences of the egg cytoplasm on nuclei are in fact known from experiments in which egg nuclei were replaced by somatic cell nuclei (*Gurdon*, 1962; *Gurdon* and *Woodland*, 1969). There is some first evidence for the same phenomenon in the mouse egg (*Bernstein* and *Mukherjee*, 1972). Localization of factors of this type in specific areas of egg cytoplasm has been demonstrated in certain species (insect egg: *Seidel*, 1936).

A promising different approach is to study cell strains derived from preimplantation embryos, although there are certainly numerous differences between cultured cells and the original cells of the embryo. Trophoblast-resembling cells developed in vitro when isolated blastomeres of a stage as early as the four-cell stage were used (*Edwards*, 1964; *Cole* and *Paul*, 1965; *Cole* et al., 1965, 1966). It is hoped that additional information will come from investigations of teratomas derived from ectopically transplanted embryos or from unfertilized eggs in the ovary (*Evans*, 1972; *Damjanov* and *Solter*, 1974).

Finally, some comparative aspects shall be mentioned. In marsupials, cells are being determined to become embryonic disc (formative cells) without ever having been enclosed by other cells (Fig. 8) (*Hill*, 1911; *Hartman*, 1919). The same seems to hold true for at least one eutherian mammal: Hemicentetes (Tenrecoidea) (*Goetz*, 1937, 1939; *Bluntschli*, 1938) (Fig. 8). The inside-outside model (theory A) cannot be applied to these embryos because inside cells do not exist in these species before formation of the embryonic knot/disc. Probably the polarity of the blastocyst (embryonic disc, trophoblast) here results from a polarity of the uncleaved egg.

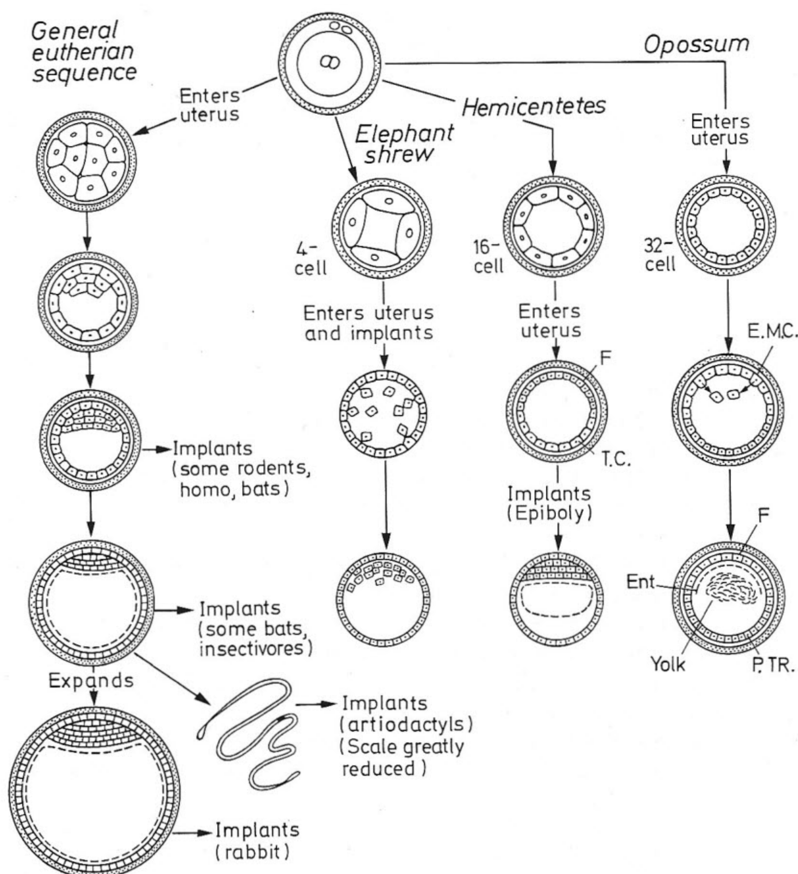

Fig. 8. Comparative aspects. In opossum and in Hemicentetes, cells being determined to form embryonic disc without having been in inside position. *E.M.C.:* entodermal mother cell; *Ent:* entoderm; *F:* formative cells; *P. TR.:* primitive trophoblast; *T.C.:* trophoblast cells. (For data on opossum and Hemicentetes, see *Hill*, 1911; *Hartman*, 1919; *Goetz*, 1937, 1939; *Bluntschli*, 1938) (from *Wimsatt*, 1975, by courtesy of author and of editor, Biology of Reproduction; slightly modified)

In conclusion, it appears well-established that the mammalian cleavage stage embryo possesses vast regulative capacities as impressively demonstrated by transplantation (fusion) experiments. The inside or outside position of blastomeres does influence their fate and can become decisive for their determination to form either trophoblast or embryonic knot. On the other hand, the egg does exhibit polarity, and blastomeres seem to be unequal, independent of their inside or outside position, as shown by their histochemical properties as well as their inclination to form only trophoblast or both trophoblast and embryonic knot. It is probable that this type of "preformation" is, in the beginning, weak and changeable, and can easily escape the experimentalist (cryptic preformation, *Graham*, 1971). The question remains open which type of experiment might be the most suitable to reveal physiologic in vivo mechanisms rather than in vitro regulations.

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Note added in Proof

After this manuscript was finished, a number of publications appeared giving additional information and supporting either theory A or B respectively. *Avendano* et al. (1975) found, in a seven-cell human embryo, two differently staining groups of blastomeres the arrangement of which resembles the polar grouping of presumptive trophoblast and embronic knot cells seen in the rabbit (see p. 70 and Fig. 1 B_2). This may support theory B. An excellent review of studies of early cell determination and differentiation using the experimental teratoma model is given by *Damjanov* and *Solter* (1974). These and additional relevant references are listed below.

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