Roux's Arch Dev Biol (1994) 203:199–204

Roux's Archives
a century in
Developmental
Biology

Role of ooplasmic segregation in mammalian development

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Received: July 19 / Accepted: July 27, 1993

Abstract. A new micromanipulation technique permitted the scrambling of the zygote cytoplasm. Such interference had no effect on preimplantation development, and when zygotes with scrambled cytoplasm were transfered to the pseudopregnant females, normal and fertile mice were born. This demonstrates that no morphogenetic factors are prelocalized in the egg cytoplasm. Cleavage characteristics of mouse embryos provide the evidence that zygote cytoplasm does not define any determinate type of cleavage. We conclude that the mechanism of ooplasmic segregation is not used in the mouse (and presumably mammalian) development. It is suggested that the turning point in the evolution of mammalian embryogenesis was the transition to the intrauterine development, that started the process leading among other changes, to the loss of the ooplasmic morphogenetic determinants.

Key words: Cell differentiation – Cytoplasm – Micromanipulation – Mouse embryo – Evolution

Introduction

The first cleavage divisions and initial morphogenetic transformations occur in the absence of embryonic genome expression, during the period of maternal control of development. It seems well recognized that ooplasm plays a key role in directing these first developmental stages; specifically, the start of different genetic programs in different blastomeres is initiated by segregation of morphogenetic determinants, prelocalized in the egg cytoplasm during oogenesis (His 1874, cited in Moore 1987; Neyfakh 1961; Davidson and Britten 1971; Raff 1977; Holliday 1989, 1990; Dworkin and Dworkin-Rastl 1990; Gurdon 1992). Although the nature of these determinants remains unclear, their existence was verified for most species investigated. Experiments including egg cytoplasm perturbations as well as investigations of the developmental potential of the early cleavage stage blastomeres and chimeric embryos showed that initial cytoplasmic localization plays an essential, and in some instances, primary role in cell lineage specification (reviews by Slack 1983; Brachet 1985; Moore 1987; Davidson 1991).

However, there is some evidence indicating that, in mammals, these morphogenetic determinants either are not prelocalized in the oocyte or have no role in embryo development. Observations of the zygote cytoplasm uniformity are supported by apparently normal development of single 2–8-cell stage blastomeres (Moore et al. 1968; review by Fehilly and Willadsen 1986), chimeric embryos (reviews by Gardner and Rossant 1976; McLaren 1976) and by embryos that had undergone centrifugation (used for pronuclear visualization when producing transgenic farm animals, also: Mulnard 1970; Tellez et al. 1988), or cytoplasm removal or its addition (Petzoldt and Muggleton-Harris 1987; Evsikov et al. 1990). Contradicting the data obtained for other animal groups, these results demonstrate that blastomere recombinations or cytoplasmic perturbations have no effect on embryo development. Thus, it was proposed that position of a blastomere in the morula (conditional cell lineage specification) is the major factor responsible for the initial steps of cell determination (Tarkowski and Wroblewska 1967). Moreover, there are good reasons to believe that in mammals inductive cell interactions are the *only* factor responsible for cell specification (Wilson and Stern 1975; Raff and Kaufman 1983; Davidson 1991).

Since the distinction between regulative and mosaic embryos has lost its significance (Melton 1991), the question discussed here concerns only the existence and the functional significance of ooplasmic determinants, but not the regulatory abilities of the early mouse embryo. These abilities, although being very high, are not exceptional and were also demonstrated by embryos, relying on mechanisms of autonomous cell lineage specification. Indeed, some observations suggest that ooplasmic segregation plays some role in mammalian development.

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These include the hypotheses on compartmentalization in the ooplasm of the posttranslational activity (Van Blerkom 1985), on the nonuniformity of zygote cytoplasm (reviewed by Mulnard 1970), on the unequal distribution of some cytoplasmic factors, responsible for the unsynchronous cleavage of the blastomeres (Bennett 1982; Spindle 1982), and on the "rotational" type of cleavage of the mammalian embryo (Gulyas 1975). It is therefore generally recognized that the existence of morphogenetic determinants in zygote cytoplasm remains open for discussions (Slack 1983; Tellez et al. 1988; Davidson 1990). Our investigation was undertaken in order to elucidate this problem.

Materials and methods

Embryos of the C57Bl/6J strain were used throughout this study. Embryos were collected from naturally ovulating 8–15 week old females. Five females were caged with a male overnight. One-cell embryos were flushed from the ampulla region of the oviducts and cultured on plastic Petri dishes in droplets of Whitten medium (Whitten 1971) supplemented with 100 μm ethylenediaminetetraacetic acid (EDTA; Abramczuk et al. 1977), under light paraffin oil, at 37° C in the presence of 7.5% CO_2 in air.

To eliminate any "external" pressure on the cleaving egg, the embryos used for morphological observations were denuded of the zona pellucida with 0.5% pronase; such zygotes successfully reached morula and blastocyst stages in vitro.

In a preliminary series of experiments, oil droplets injected beneath the plasma membrane were used as markers of the cortical regions of the zygote's cytoplasm.

Before cytoplasm scrambling, 2-pronuclei stage zygotes were cultured in the presence of cytochalasin B (7.5 μg/ml) and colcemid (0.1 μg/ml) for 40 min. KM-2 micromanipulators and Amplival microscope were used for micromanipulations. A needle with a piezocrystal pressed to its side was used for cytoplasm scrambling. When 25–30 V, 80–90 kHz voltage was applied to the piezoelement, the tip of the needle started to vibrate. The frequency and the voltage were adjusted to make the amplitude of the tip rotations 2–3 microns. The needle was inserted into the zygote, the voltage on the piezoelement was applied, and the egg cytoplasm was completely scrambled in 10–20 sec (Fig. 1). Cytoskeletal inhibitors effectively prevented puncturing of the plasma membrane or the nuclear membranes of pronuclei. The scrambling was assumed to be complete when cytoplasmic granules and pronuclei had rotated a few times around the cytoplasm and had changed their position according to the egg centre.

Micromanipulated and control zygotes were cultured for 3 days; by 90 h after ovulation, the cell number in morphologically normal morulae and blastocysts was determined using Dyban's technique of embryo fixation (Dyban 1983) and staining with Giemsa stain.

For scanning electron microscopy, embryos without zona pellucida were fixed according to Holy et al. (1991). Embryos were examined in a ISI-SS40 microscope under operating voltage 20 kV.

Two-cell embryos were transferred to ampullae of day 1 pseudopregnant ICR females.

Results and discussion

Classically, it is the invariant cleavage pattern that is a device by which cytoplasmic informational determinants are segregated to the appropriate founder cells (Davidson 1989; Horvitz and Herskowitz 1992). Some

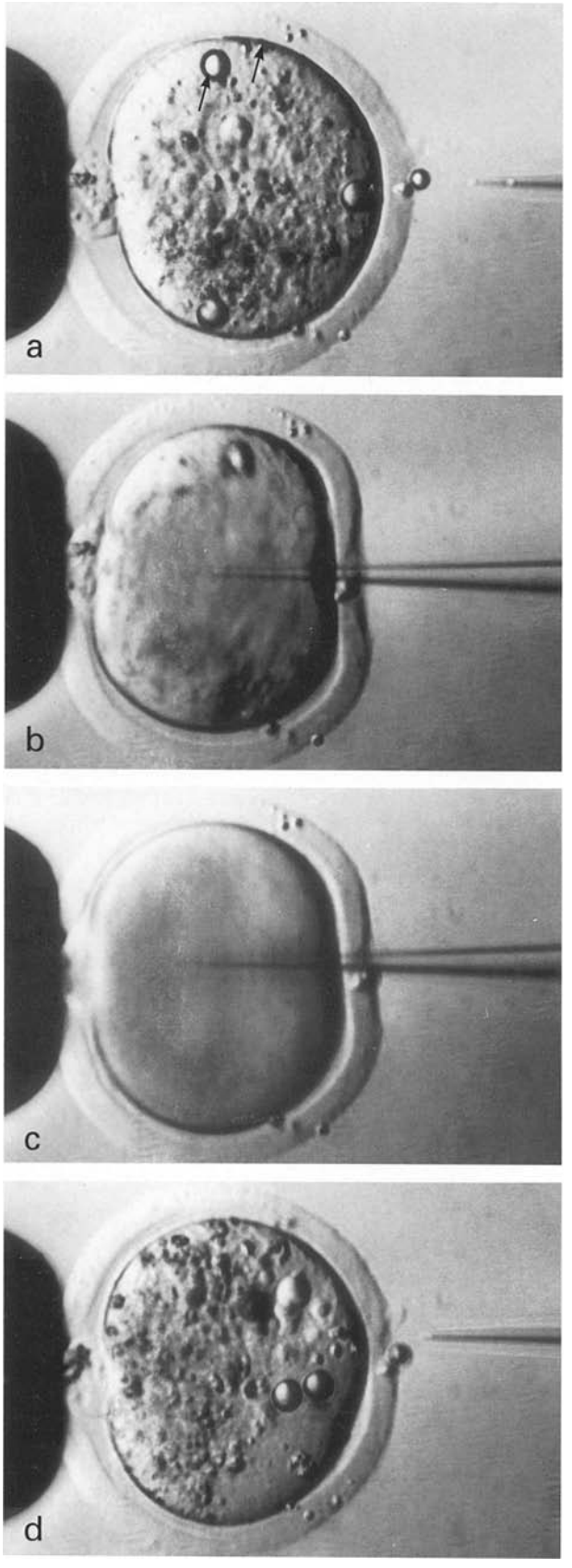

Fig. 1a. Oil droplets *(arrows)*, injected beneath the plasma membrane mark the peripheral regions of the cytoplasm, (**b**, **c**) Scrambling of the zygote cytoplasm, **d** Zygote immediately after cytoplasm scrambling. × 500

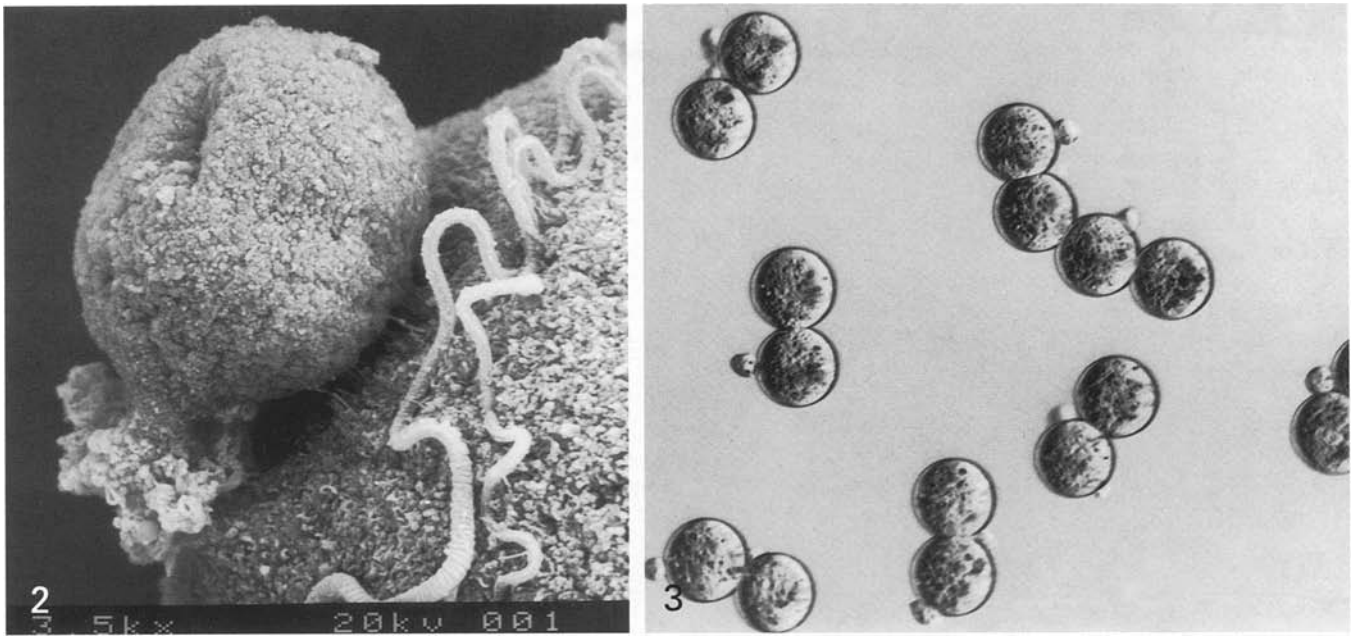

Fig. 2. Second polar body remains connected with a zygote by a cytoplasmic bridge, "midbody" (left)

Fig. 3. First cleavage furrow passes irrespectively of the oocyte pole of axial symmetry, marked by the second polar body. ×200

Table 1. Effect of cytochalasin B (CB), and colcemid (COL) treatment and cytoplasm scrambling on the rate of embryo development and on the start of cavitation of C57Bl/6J mouse embryos

Embryo stage at the start of culture	Embryo age at fixation (h after ovulation)	Percentage of embryos reaching blastocyst stage	Mean cell number in	
			Morulae and blastocysts pooled	Nascent blastocysts[a] among these
1-cell	87	60±4	22.4±0.8 [97]	25 ±1 [25]
1-cell, 2 h treatment with CB and COL	87	60±4	23.8±0.5 [148]	25 ±1 [42]
1-cell[b]	90	72±2	26.1±0.4 [326]	28.2±0.2 [198]
1-cell with scrambled cytoplasm	90	71±4	28 ±1 [64]	28 ±2 [24]

The number of embryos is given in parentheses after the mean ±SE

[a] Stage at the beginning of cavitation. Note that the other blastocysts included in the pooled sample (previous column) had progressed beyond this stage

[b] From Evsikov et al. (1990)

kind of definite cleavage pattern has been described for most studied species, and for all of them, autonomous specification of some cell lineages does exist. Since the second polar body remains connected with the zygote via cytoplasmic bridge, "midbody" (Gulyas 1986; Fig. 2), it can be used as a marker of the oocyte's pole of symmetry. Position of the second polar body on the surface of the 2-cell embryos demonstrates that the cleavage furrow passes irrespectively of the (presumed) axis of zygote symmetry (Fig. 3). So, the first cleavage may be called "meridional" (Graham and Deussen 1978) only as a reference point for the subsequent cleavages. Following the second and the third cleavages, blastomeres do not form any definite spatial structures (e.g., Graham and Lehtonen 1979; Talansky and Gordon 1988). Thus, the crosswise position of the blastomeres is not a consequence of the embryo cleavage pattern, but an "epigenetic" factor, i.e., interaction with the zona pellucida, is responsible for it. Irrespective of the cleavage planes, the blastomeres are compressed against each other by the zona pellucida, until they occupy a minimal volume (Gulyas 1975; Graham and Deussen 1978).

Mouse zygotes do not reveal any determinate type of cleavage. As was shown already in 1884, hypothetical morphogenetic determinants would eventually be in a proper place irrespectively of the cleavage type (Pflüger

1884, cited in Moore 1987). Normal development following experimental shifts in embryo cleavage planes, as well as the absence of a precise cleavage pattern do not necessarily exclude the existence of these determinants.

Zygote treatment with cytoskeletal inhibitors does not aggravate embryo development (Table 1). This result does not necessarily contradict the existence of morphogenetic determinants: microtubule depolymerization without further perturbations of the cytoplasm would not change the general structure of the gradients. It was reported that droplets of silicone fluid injected into the peripheral area of a 1/2-blastomere had been found exclusively in the trophoblast (Wilson et al. 1972; Graham and Deussen 1978). Taken together with the observation that mouse zygote cytoplasm seems to be passively segregated between the blastomeres (Karasiewicz and Modlinski 1985), this may imply the existence of the "trophectoderm-inducing determinants" in the egg cortex. To visualise the extent of cytoplasmic disturbance, oil droplets were used to mark the egg cortex (Fig. 1 a). After cytoplasm scrambling, all of them had changed their positions (Fig. 1 d), the tiniest droplets (about 2 μm in diameter) were lost in the cytoplasm. The possibility exists that the subplasmalemmal regions of the cytoplasm have not been disturbed, or that asymmetric distribution of some membrane proteins will play a role in differentiation (De Loof et al. 1992). Nevertheless, it may be safely concluded that any gradients set during oogenesis or by fertilization have been destroyed by the scrambling procedure.

Following cytoplasm scrambling, 119 out of 136 zygotes (88%) developed in vitro to the morula and blastocyst stages. Neither cleavage rate, nor that of morphogenesis, or the stage of the start of cavitation were affected by cytoplasm scrambling (Table 1). Normal preimplantation development of such zygotes proves that the first steps of cell differentiation do not involve any factors of autonomous cell specification. Twelve embryos, developed after cytoplasm scrambling, were transferred to a pseudopregnant female, and 3 normal, fertile males were born.

These results present an experimental proof for the idea that the pattern of embryo body is set up exclusively by cell interactions rather than by segregation of cytoplasmic determinants. Thus, our results confirm the observations that cytoplasm stratification starts relatively late in development, according to the position occupied by a blastomere in the morula; it is only at this stage that an embryo acquires the first (radial) axis (reviewed in: Johnson et al. 1986; Ziomek 1987; Fleming and Johnson 1988; Gardner 1989; Maro et al. 1990; Gueth-Hallonet and Maro 1992). Accumulating evidences show that this conclusion may be extended for the other eutherian mammals (Pedersen 1988; Iritani 1988).

The absence of any kind of "prelocalization" in the egg leading to a truly regulative type of development gives an obvious advantage to an embryo dealing with an everchanging environment. Therefore, it is not surprising that mammals are not unique in their developmental strategy: teleost fishes and sauropsids also seem to rely mostly on the mechanisms of conditional cell specification (Cooke and Webber 1983; Kimmel et al. 1990, 1991; Eyal-Giladi 1991, 1993). In our opinion, the loss of ooplasmic morphogenetic determinants was initiated in mammals by the transition to intrauterine development. The evidence leading to this conclusion has been presented elsewhere (Evsikov 1992). Briefly, while considering the pattern of evolutionary changes leading to this type of development, demonstrated by preimplantation mammalian embryos, two salient features of the evolution of ontogenies [phylembryogenesis according to Severtsov (1922)] should be taken into account. First of all, evolution leads to the maximization of the autonomy of development, its independence from the everchanging environmental factors (Schmalhausen 1949). Secondly, parents should be considered as one of the factors, increasing the chances of embryo survival. In mammals, the input of this factor reaches a culmination: mother provides an environment which is no longer alien to the embryo (Darlington 1971); thus, the transition to intrauterine development marks the moment when ontogeny escapes the pressure of the natural selection, directed at the maximization of developmental speed. The necessity for preformed regulatory factors, which permit the high speed of initial stages of development (Davidson 1989, 1990) disappears, the regulatory role of zygote cytoplasm diminishes, leaving genomic activity and inductive cell interactions as the only mechanisms controlling the first developmental stages (Woodland 1982). This would explain the exceptionally high regulative capacities of the mammalian embryos and, also, the rather limited role of zygote cytoplasm in directing preimplantation development.

Marsupials seem to rely in part on the mechanisms of autonomous cell specification (Selwood 1986), thus presenting an intermediate step in mammalian phylembryogenesis. On the other hand, the hypothesis suggests that in the ovoviviparous species, the transition to intrauterine development led to changes in the mode of early development similar to those which had taken place in mammals. So far, the discovery that embryogenesis of marsupial frogs resembles that of mammals, but not other amphibia (reviewed in: del Pino 1989) seems to be in good agreement with the hypothesis.

Acknowledgments. Scanning electron microscopy was performed by A.V. Evsikov.

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