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The role of the nucleocytoplasmic ratio in development regulation of the early mouse embryo

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Summary

The hypothesis suggesting that the blastocoele is able to form only at a definite nucleocytoplasmic ratio was tested. We compared the development of preimplantation mouse embryos under different conditions. The results demonstrated that the start of cavitation is not dependent on the number of cell divisions. Thus, a definite nucleocytoplasmic ratio is not required for blastocoele formation to start. Our studies on embryos with microsurgically altered cytoplasm content provided evidence for the following biological clock mechanism: a change in the cell program of morphogenesis needs definite concentration of the products of a previous genetic program.

Key words: nucleocytoplasmic ratio, blastocoele formation, biological clock, microsurgery, mouse embryo.

Introduction

Various epigenetic factors presumed to be involved in a biological clock have been tested. It has been shown that none of them determine the morphogenesis of the preimplantation embryo. Thus, it has been reported that blastocoele formation is independent of the time elapsed since fertilization, cell number, cytokinesis (Smith and McLaren, 1977; Surani *et al.* 1980), number of DNA cycles of replication (Alexandre, 1979; Dean and Rossant, 1984) and cell–cell interactions (Surani *et al.* 1980; Prather and First, 1986).

At the early stages of embryo development, cytoplasm content per diploid genome is halved with each consecutive cleavage. Consequently, the nucleocytoplasmic ratio increases with each replication round and subsequent cytokinesis. This fact underlies the hypothesis that the biological clock mechanism may be in some way related to the nucleocytoplasmic ratio (at least until the ratio attains the level of somatic cells). It has been suggested that the egg cytoplasm possesses regulatory factors that are titrated during cleavage, thereby making possible a switchover to the next cell program upon attainment of a definite nucleocytoplasmic ratio. This type of biological clock mechanism operates in fishes and amphibia at early developmental stages (Rott and Sheveleva, 1968; Chulitskaia, 1970; Newport and Kirschner, 1982).

There is evidence indicating that the nucleocytoplasmic ratio may be of importance in mammalian preimplantation development. It has been observed that a decrease in the nucleocytoplasmic ratio produced by experimental manipulation is deleterious for early mouse development: haploids (Kaufman and Sachs, 1976; Modlinski, 1980) and embryos resulting from the fusion of 8-cell nuclei with two enucleated blastomeres of the 2-cell embryo (Howlett *et al.* 1987) rarely develop even beyond the first stage of morphogenesis. 'Normalization' of the nucleocytoplasmic ratio (by decrease in cytoplasm content in haploids, transfer of the 8-cell stage nucleus to the enucleated blastometre of the 2-cell embryo) allowed successful preimplantation development of most haploid embryos (McGrath and Solter, 1983) and some of the reconstructed embryos developed to term (Howlett *et al.* 1987; Kono and Tsunoda, 1989). These results, however, are not very informative about the role of the nucleocytoplasmic ratio in regulating the development of intact embryos. The observation that manipulated haploids start to form blastocoeles a division later than the controls (Witkowska, 1973; Kaufman and Sachs, 1976) seems to need additional confirmation.

The role of the nucleocytoplasmic ratio in the development of mammals has been studied on embryos using inhibitors of polyamine synthesis and DNA replication (Alexandre, 1979; Bolton *et al.* 1984; Dean and Rossant, 1984; Smith and Johnson, 1985). From these studies, it was concluded that morphogenesis is independent of the number of DNA replication cycles and the nucleocytoplasmic ratio. In interpreting these experiments, it is important to note that the nucleocytoplasmic ratio has been altered by bringing about changes in the embryonic genome that might have affected the developmental program. Experimental increase of the nucleocytoplasmic ratio by microsurgical removal of some part of the zygote cytoplasm was found to be deleterious for embryo development (Barton and Surani, 1983; Petzoldt and Muggleton-Harris, 1987).

Although Petzoldt and Muggleton-Harris (1987) have not considered the role of the nucleocytoplasmic ratio in the triggering of morphogenetic events, they have noted that stage-specific gene expression is independent of the nucleocytoplasmic ratio.

We were interested in seeing how experimental alteration of the nucleocytoplasmic ratio might affect the process of blastocoele formation. We began with the idea that meaningful results would be obtained in developmental studies of embryos with the nucleocytoplasmic ratio altered by changes in cytoplasm content and not ploidy. The conclusions were based on *in vitro* data. To prove the applicability of morphological criteria to embryo viability estimates, morulae and blastocysts, developed *in vitro*, were transferred to pseudopregnant mice.

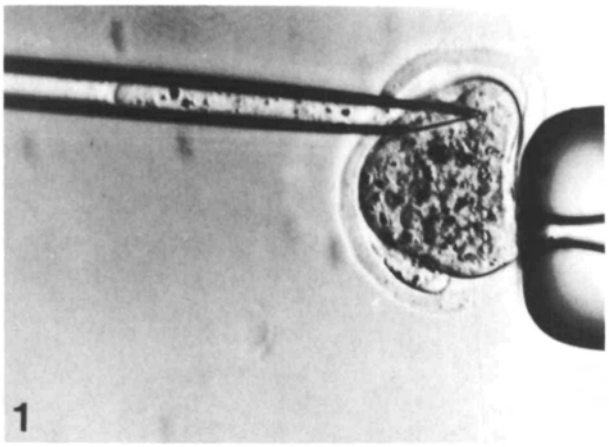

Fig. 1. Microsurgical removal of a part of the zygote cytoplasm.

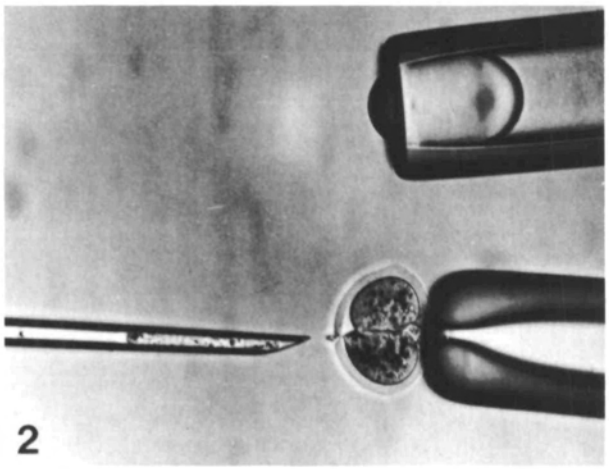

Fig. 2. Production of the 1/2-blastomere with double cytoplasm content. Enucleation of the blastomere.

Materials and methods

Removal and cultivation of embryos

Mice of the C57Bl/6J strain were maintained on a 16:8 h light:dark schedule. Each male was kept with five females overnight. The zygotes at the 2-pronucleus stage were removed at 10.00–13.00 h. To remove cumulus cells, the zygotes were treated with Hepes-buffered hyaluronidase (200 i.u. ml^{-1}) in Whitten's medium (Whitten, 1971); the 2-cell embryos were removed from the oviducts of mice on day 2 of pregnancy. The embryos were cultivated to the morula and blastocyst stages. Cultivation was carried out in plastic Petri dishes in droplets of Whitten medium with a 100 μM Na_2EDTA supplement (Abramczuk *et al.* 1977). The procedure was done under paraffin oil at 37°C in an atmosphere of 7% CO_2 in air.

The number of cells in morphologically normal morulae and blastocysts was estimated by the technique of Tarkowski (Tarkowski, 1966). Student's *t*-test was used to determine the significance of the differences in the cell division rate and the stage at start of cavitation between the embryos.

The cultured morulae and blastocysts were transferred to the right uterine horn of BALB/c females on day 2 of pseudopregnancy.

Cytoplasmic micromanipulations

A part of the cytoplasm was removed in glass chambers using micromanipulators KM-2 under an Amplival microscope (Fig. 1). The microscope was fitted with a system providing stereoimage of the embryo (the optic was designed by Dr Gruzdev). The external diameter of the micropipette for embryo fixation was 100 μm, the internal diameter of the needles for the removal of the karyoplasts and cytoplasm was 15–20 μm. The method for cytoplasm removal was as described for embryo enucleation (McGrath and Solter, 1983). Estimates of cytoplasm content were based on microphotographs. The cytoplasm content withdrawn was 20–50% of the total, 35% on the average.

Single blastomeres of the 2-cell embryo (1/2-blastomeres) were produced by puncturing the plasma membrane of the second blastomere. In some instances, the remains of a destroyed blastomere were extracted out of the perivitelline space. The extraction had no effect on subsequent development. In the experiments where the zonae pellucidae were removed by enzymic treatment and the cells were separated in a calcium- and magnesium-free medium (Hogan *et al.* 1986), the 1/2-blastomeres developed faster for some unknown reason.

Cytoplasm content was doubled as follows. A blastomere was enucleated according to McGrath and Solter (1983). To mediate fusion, we used a suspension of inactivated Sendai virus stored in a tube 'plugged' with an oil droplet (Fig. 2, top); the suspension was aspirated into the enucleation pipette, and then it was injected into the perivitelline space. Two hours after cultivation, the fused blastomeres were transferred to a separate droplet. Inactivated Sendai virus was injected repeatedly into some embryos with unfused blastomeres, but the virus rarely mediated fusion in these cases.

Sendai virus was propagated and inactivated by exposure to UV-rays according to Harris and Watkins (1965) and Giles and Ruddle (1975).

Results and Discussion

Table 1 presents the results obtained from the cultivation and transfer of C57Bl/6J embryos. The results show that the *in vitro* development conditions support the viability of the cultured embryos.

Table 1. *Development of C57Bl/6J mouse embryos cultured at the preimplantation stage*

Embryo stage at start of culture	Embryo development		
	in vitro, morulae and blastocysts (%)	*In vivo*, after transfer of cultured morulae and blastocysts to pseudo-pregnant BALB/c females (%)	
		Pregnancies	Pups born
1-cell	90	31	28
	1171	16	30 (+5 dead)
	1306	52	109
2-cell	91	91	36
	206	10	24
	227	11	67
1/2-blastomere*	91	28	8
	600	5	6 (+13 dead)
	662	18	79

* 15–20 embryos transferred to each recipient.

The cell counts of embryos at day 3 of development are given in Table 2. The mean cell numbers counted for all the embryos of a particular type provide a measure of cleavage rate. Cell number for 3-day-old embryos cultured *in vitro* from the 1-cell stage is 20% less than that for their counterparts that developed *in vivo*. This means that the cleavage rate decreases in the culture (also Bavister, 1988, review). The results summarized in Table 1 and 2 lead one to accept the hypothesis that decrease in cell number is deleterious for the development of postimplantation embryos.

There is no obvious dependence of morphogenetic events on the number of cell divisions (rounds of DNA replication, the nucleocytoplasmic ratio). A biological clock marking time by cell division may be responsible for triggering cavitation (Smith and McLaren, 1977). If so, the developmental conditions would not affect the number of cells in the embryos starting to form blastocoeles. Retardation of development *in vitro* would affect the time of the appearance of the nascent blastocysts and not their cell number. Table 2 shows that this is not the fact, the cell number in the nascent blastocysts significantly decreases in the course of *in vitro* development ($P>0.999$). A decrease in the cell number of the nascent blastocysts developing in culture is not due to increase in cell death. There was no instance when the number of picnotic nuclei exceeded 0.2 per embryo.

Embryo development related to decrease in cytoplasm content

After microsurgical removal of some amount of the cytoplasm (Fig. 1), 93% (236 of 253) of the zygotes developed to morphologically normal morulae and blastocysts. The cell number of the morulae and blastocysts is given in Table 2.

Before and during microsurgery, the embryos were treated with cytochalasin B and colcemid. We tested whether or not exposure to these two cytoskeletal inhibitors for 2 hours affected embryo development. Assurance was obtained that the two inhibitors had no

Table 2. *Development of C57Bl/6J mouse embryos 90 h after ovulation*

Embryo types, developmental conditions	Mean cell number		Blastocysts, as % of total embryo number (morphogenesis rate)
	Morulae and blastocysts (cleavage rate)	Nascent blastocysts (stage at start of cavitation)	
*In vivo**	32.0±0.5 [211]	33.6±0.5 [116]	65±3
In vitro:			
2-cell	29.8±0.7 [127]	30.0±0.5 [78]	71±4
1-cell	26.1±0.4 [326]	28.2±0.2 [198]	72±2
1-cell with decreased cytoplasm content	21.1±0.6 [131]	23.0±0.6 [72]	59±4
1/2-blastomere	14.8±0.4 [157]	15.7±0.4 [95]	69±4
1/2-blastomere with double cytoplasm content**	21±1 [65]	26±2 [30]	52±5

The number of embryos is given in parentheses after the mean±S.E.
* 80 h after ovulation.
** 90–105 h after ovulation.

influence on cleavage rate and the time when the embryo starts to cavitate. This is in agreement with the results reported for the differentiation of the 2-cell mouse embryos (Siracusa *et al.* 1980). Drilling the zona pellucida also did not affect the process of blastocyst formation (see also Malter and Cohen, 1989).

The cleavage rate of the embryos with cytoplasm content reduced by a third was significantly lower than in the control embryos ($P>0.999$). Less drastic reduction of the fertilized egg volume has similar effects (Opas, 1977). It should be noted that, when the cytoplasm is removed according to McGrath and Solter (1983), the plasma membrane does not sustain a puncture. At the time when 35% of cytoplasm content is aspirated into the pipette, the surface area of the formed structure is 2.3-fold greater than that of the zygote. This means that microsurgery resulted in zygotes with cytoplasm content reduced by a third and a plasma membrane stretched out 2.3-fold (the membrane expands 1.2-fold during the first cleavage division, Lehtonen, 1980).

Our next strategy was *reductio ad absurdum*. Having deliberately increased the nucleocytoplasmic ratio to maximum, we left only the zygote karyoplasts for cultivation. Twenty four hours later, some of the karyoplasts were 2-cell. Their further development was, however, arrested. These experiments illustrate well that a major function of the cytoplasm is maintenance of nucleus viability.

Development of embryos with double cytoplasm content

A single blastomere of the 2-cell mouse embryo is capable of development to a normal mouse (Tarkowski, 1959; Tsunoda and McLaren, 1983; also Table 1). Therefore, a 1/2 blastomere may be regarded as a viable embryo. We compared its development with that of the 1/2-blastomere with double cytoplasm content. The data presented in Table 2 are in compliance with previous observations indicating that the preimplantation development of a blastomere is not affected by its single state (Smith and McLaren, 1977; O'Brien *et al.* 1984; Rands, 1985).

Of 239 embryos operated upon, the blastomeres fused in 138, and of these, 113 (82%) developed to morulae and blastocysts. By 90 h after ovulation only 6% of the embryos started to form blastocoeles, but the cell number of the morulae and blastocysts by this time was the same as in the controls developing from the 1/2-blastomeres (results are not presented). This indicates that cell division rate did not change after the nucleocytoplasmic ratio was decreased.

The 1/2-blastomeres served as controls during this experiment, although we were aware of the fact that the nucleocytoplasmic ratio of the blastomere with double cytoplasm content and that of the zygotes was about the same. The timing of blastocoele formation gave no inclination as to whether the age of embryos or the nucleocytoplasmic ratio played the crucial role in the start of cavitation: 70% of the experimental embryos reached the blastocyst stage by 60–65 h of cultivation,

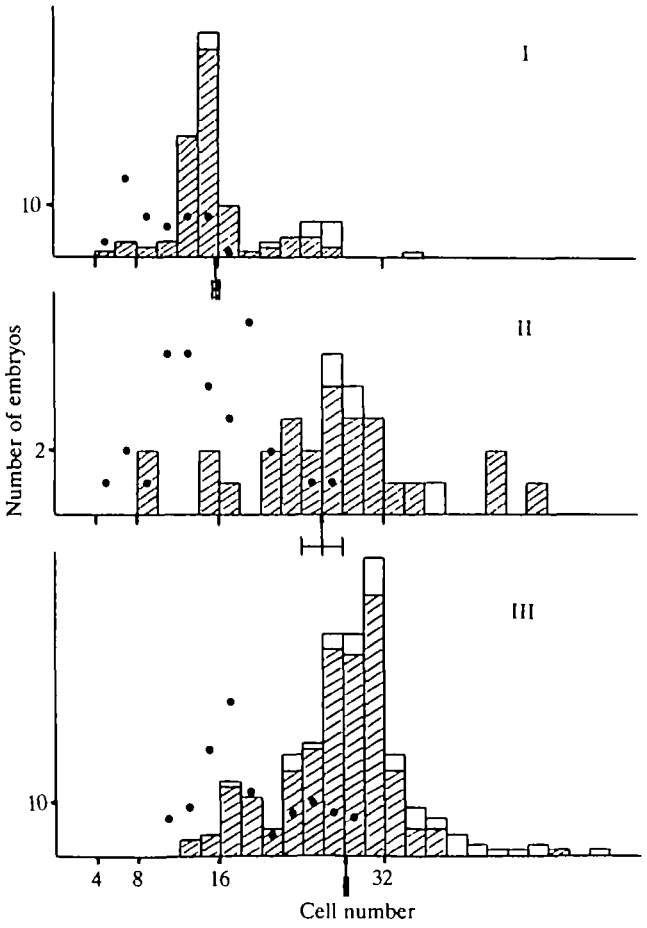

Fig. 3. Cell distribution among morulae (●), nascent blastocysts (■), and blastocysts (□) developed *in vitro* from: I – 1/2-blastomeres; II – 1/2-blastomeres with double cytoplasm content; III – 2-pronucleus stage zygotes. Arrows indicate mean cell number in the nascent blastocysts.

the 1/2-blastomeres reached the stage by 50 h, and the embryos developing from zygotes reached it by 75 h. But the stages at which embryos developing from blastomeres with double cytoplasm content started cavitation were similar to those kept in culture from the 1-cell stage (Table 2). These results are depicted in the histogram (Fig. 3).

Conclusion

The present results suggest that morphogenesis of mammalian preimplantation embryos depends on the absolute time elapsed since the beginning of development as well as on the nucleocytoplasmic ratio.

Table 1 shows clearly that *in vivo* developmental conditions cannot be changed without decreasing the embryo viability. The embryos react to the suboptimal conditions very specifically: the most evident changes are observed in the embryo cleavage rate (Table 2). The possible explanation might be like this. Autonomous cortical activity is evidence of a cytoplasmic clock (Waksmundzka *et al.* 1984) influencing cytokinesis. On the other hand, transcription and translation

triggering cavitation terminate just a few hours before the blastocoele starts to form (Kidder and McLachlin, 1985). Hence, cavitation is more dependent on the nucleus than on any type of cytoplasmic activity. If we suppose that cytoplasm acts as a buffer between the environment and the nucleus, it would not be difficult to explain why the processes controlled by the nucleus would be relatively independent of the developmental conditions.

The developing embryos start to cavitate at a much smaller cell number *in vitro* than *in vivo*. This suggests that neither the number of DNA replication rounds nor the nucleocytoplasmic ratio can be considered as the triggers of blastocoele formation. Thus, it appears unlikely that some regulatory factors in the egg cytoplasm, when reaching an appropriate titer during the cleavage of the embryo, may switch over the consecutive cell program. What may be true for fishes and amphibia (Rott and Sheveleva, 1968; Chulitskaia, 1970; Newport and Kirschner, 1982), does not necessarily concern mammals for the reason that they differ markedly in the role and function of the cytoplasm during the first stages of development (Tarkowski, 1982; Santiago and Marzluff, 1989).

The embryo development data obtained by altering the nucleocytoplasmic ratio may be interpreted in terms of the regulatory role of the cytoplasm. The embryos with decreased (or increased) cytoplasm content would reach the nucleocytoplasmic ratio corresponding to the cavitation start and, hence, would start to cavitate after a smaller (or a greater) number of cell divisions. The microsurgery data do not contradict this hypothesis (Table 2). An explanation appearing to comply better with the current data is offered. When cytoplasm content was decreased by a third, embryo morphogenesis was slightly retarded, while cleavage rate was decreased by almost 30% (Table 2). This was not unexpected: the normal developmental conditions upset by cultivation were further disrupted by removal of a third of the cytoplasm. The decrease in the cell number of the nascent blastocysts was probably only an indication that the number of cells was small in the embryo when the cavitation started. While not significantly affecting the time of the appearance of the nascent blastocysts, a decrease in cytoplasm content decreased the cleavage rate. The pattern was reversed for the embryos with double cytoplasm content. A decrease in the nucleocytoplasmic ratio had no effect on cleavage rate, but it delayed the formation of blastocoeles. This provides additional support for the hypothesis of the independence of the processes regulating cell division and blastocoele formation.

In an attempt to explain the results, a hypothetical mechanism for the biological clock is offered. After the beginning of the transcription of the first set of genes, each consecutive program beyond the "point of no return" (Johnson, 1981) can be triggered only after the occurrence of a certain morphogenetic event, for example, after a definite concentration of proteins, the products of a previous program, is reached. With a decreased cytoplasm content, the accumulation of the products of posttranscriptional biosynthesis would be inhibited, but, concomitantly, the amount of each protein needed for reaching a definite concentration in cytoplasm would be reduced. Theoretically, morphogenetic processes can accelerate and decelerate. To illustrate, the formation of a male pronucleus is accelerated when the content of the oocyte cytoplasm is decreased (Borsuk and Manka, 1988). Some retardation in the formation of a blastocoele indicates that a decrease in volume is no compensation for the harmful consequences of the removal of a part of a vital supplies. And, in reverse, after an increase in cytoplasm content, the limiting factor would obviously be mRNA; the time for the production of the required amount of transcripts would increase. The formation of blastocoele would be delayed.

We have to admit that an unambiguous interpretation of the results of experimental alteration of the nucleocytoplasmic ratio is quite difficult (Johnson, 1981). Hence, it should be reemphasized that the first proof that the achievement of the definite nucleocytoplasmic ratio is not the prerequisite for the blastocoele formation to start is manifested as follows: with each consecutive deterioration of developmental conditions (*in vivo*→*in vitro* from the 2-cell stage→egg *in vitro*→egg with decreased cytoplasm content *in vitro*), the blastocoeles start to form at decreasing numbers of cell divisions.

In conclusion, whatever the interpretation may be, the present results with the development of blastomeres with double cytoplasm content are relevant to discussion of those obtained with the cloning of mammals. The delay in the triggering of the cavitation of nuclei transferred to egg cytoplasm has been adduced as evidence for genomic reprogramming (Surani *et al.* 1986; Stice and Robl, 1988). However, when we doubled the amount of cytoplasm in the blastomere of the 2-cell embryo, the embryos started to form cavities with a delay of 12 h, and there was no reason for implying genomic reprogramming then. Evidence for alteration of the developmental program is to be sought at another level. Search at the molecular level is underway in an effort to bring previous and new evidence together (Surani *et al.* 1986; Howlett *et al.* 1987; Prather and First, 1988; Larionov *et al.* 1988).

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