THE JOURNAL OF EXPERIMENTAL ZOOLOGY 276:201–208(1996)

Mechanisms of Cell Number Regulation in the Peri-Implantation Mouse Blastocyst

S.V. EVSIKOV, I.N. VAGYNA, AND A.P. SOLOMKO
Department of Biochemical Genetics, Institute of Molecular Biology and Genetics, National Academy of Sciences of the Ukraine, Kiev 252627, Ukraine

ABSTRACT Low viability of manipulated or in vitro cultured embryos is caused primarily by the reduced cell number in the implanting blastocysts. In order to investigate the effect of implantation delay on embryo viability and cell number, mouse blastocysts were transferred into oviducts of day 0 pseudopregnant females. This type of transfer improved embryo survival rates, indicating that embryos retarded by in vitro culture restored their viability during 3 days of delayed implantation. Our results showed that even in the cases when the initial cell count was as low as 28.2 ± 0.7 cells per blastocyst (vs. 60.5 ± 1.4 cells in the control blastocysts, developed in vivo), implantation delay increased this number to 107.2 ± 3.5 cells (control blastocysts had at this stage on average 111.0 ± 3.7 cells). Half-blastocysts, developed from the single blastomeres of the 2-cell embryos or from experimentally produced tetraploids, had around 50 cells after 3 days of implantation delay. This indicates that the start of blastocyst dormancy is triggered during the eighth cell cycle and independent of the absolute cell number or the number of cytokineses. Implantation-delayed blastocysts, developed from the half-embryos with the doubled volume of cytoplasm, had on average 70.5 ± 2.4 cells, suggesting that embryo fall into quiescence is also dependent upon the attainment of a definite nucleo-cytoplasmic ratio. We conclude that blastocyst readiness for implantation is determined by two factors: number of cell cycles and nucleo-cytoplasmic ratio.

If an embryo fails to reach hatched blastocyst stage by the time of "implantation window," this makes implantation impossible; decreased cell number in the implanting blastocyst significantly impairs postimplantation development. These problems arise when cultured in vitro and/or micromanipulated embryos are being transferred into foster mothers. An observation that during the first few days of delayed implantation, before falling into developmental quiescence, blastocysts increase their cell numbers (McLaren, '68; Kaufman et al., '77; Weitlauf et al., '79), suggests a way to restore embryo viability by experimentally extending the preimplantation period. Since the basic work of McLaren and Michie ('56), a commonly used method of mouse blastocysts transfer is a transfer of 3-day-old (day of conception, day 0) blastocysts into the uteri of day 2 pseudopregnant females. Such asynchronous "3→2" transfer is considered superior to synchronous, "3→3" transfer since it provides an extra 24 h, necessary for the retarded embryos to reach implanting blastocyst stage. Moreover, a good survival rate of the half-embryos was reported for the highly asynchronous blastocysts transfers into the oviducts of day 0 pseudopregnant females ("3→0" transfer) (Tsunoda and McLaren, '83). Further studies, however, did not provide unequivocal evidence as to the advantage of this method over conventional, "3→2" transfer (Tsunoda et al., '85).

The preimplantation period may be experimentally extended by the blastocyst transfer into ovariectomized (Bergström, '78) or immature (Papaioannou, '86) females. In these cases of delayed implantation blastocysts are provided with a chance to double their cell number before implantation (Kaufman et al., '77). Highly asynchronous ("3→0") transfer models to some extent the situation of delayed implantation. This study was undertaken in order to investigate the consequences of highly asynchronous transfers on embryo implantation and survival rates. The second aim of this project was to reveal the mechanisms

Received December 20, 1995; revision accepted May 10, 1996.
Address reprint requests to Sergei V. Evsikov, Ph.D., Reproductive Genetics Institute, Illinois Masonic Medical Center, 836 W. Wellington Avenue, Chicago, IL 60657.

of size regulation, operating at the peri-implantation stage. Half-embryos, embryos with the halved nucleo-cytoplasmic ratio and the embryos which developed in suboptimal in vitro conditions, were analyzed before and after implantation delay. Our results indicate that the mechanisms determining the stage of embryo fall into quiescence (considered here as the stage of embryo readiness for implantation), differ markedly from the ones operating at the earlier stages.

MATERIALS AND METHODS

Hybrid B6D2F1 (BALB/c × C57BL/6) females were superovulated by i/p injections, 48 h apart, of 7.5 IU of pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) and were then caged with C57Bl/6 males overnight. Zygotes were flushed from the oviducts 24 h following hCG injection. Cumulus cells were dispersed using hyaluronidase (200 IU/ml) in M2 medium. Embryos were cultured to the morula and blastocyst stages in M16 medium supplemented with 1 mM of L-glutamine and 100 μM of Na_2-EDTA (Whittingham, '71; Abramczuk et al., '77; Chatot et al., '89), under light paraffin oil, at 37°C in the presence of 5% CO_2 in air.

Figure 1 represents the four types of embryos used in this study. Diploid half-embryos (1/2-embryos) were produced by puncturing plasma membrane of one blastomere of the 2-cell embryo. Tetraploid half-embryos were produced by culturing zygotes for 20 h in the presence of 0.5 μg/ml Cytochalasin D. This treatment completely suppressed zygote cleavage, not affecting karyokinesis. At the "2-cell stage" each Cytochalasin D–treated morphologically one-cell embryo contained two diploid nuclei (Latham et al., '92). Microsurgical removal of one nucleus resulted in diploid half-embryos with the doubled amount of cytoplasm (halved nucleo-cytoplasmic ratio); normal postimplantation development of such embryos has already been shown (Barra and Renard, '88). The micromanipulation technique of McGrath and Solter ('83), as modified by Tsunoda et al. ('86), was used. All micromanipulations were performed in glass chambers using KM-2 micromanipulators under Axiovert 35 microscope with DIC optics.

Blastocysts were transferred into either uteri (Hogan et al., '86) or oviducts (Pease et al., '89) of ICR females, mated with vasectomized or fertile ICR males. About 10 embryos were transferred per pseudopregnant, 3–5 per pregnant. In the cases when 1/2-blastocysts (blastocysts developed from single blastomeres of 2-cell embryos) were transferred into pseudopregnant recipients, pregnancies were assured by the transfer of control blastocysts into contralateral oviducts. Recipients were killed on day 16 of gestation, and the number of resorptions and live fetuses was recorded. The number of implantations was determined as the sum of resorptions and live fetuses. Transferred hybrid embryos were distinguished from native albino (ICR) embryos by their eye color. While assessing the proportion of transferred embryos that developed to term, only data from embryo transfers that resulted in pregnancy were used.

In order to investigate the increase in blastocyst cell number, occurring during implantation delay, 3-day-old blastocysts were divided into two groups. Some were used for cell counts, the rest were transferred into oviducts of day 0 pseudopregnant females. Three days later, prior to implantation, blastocysts were flushed from the uteri and cell numbers in such delayed blastocysts were determined.

Cell number in morphologically normal blastocysts was determined using Dyban's technique of embryo fixation (Dyban, '83), followed by staining with Giemsa. Statistical significance of the results was determined by Student's t-test (see Table 2) and χ^2 analysis.

RESULTS

Table 1 presents data on the viability of mouse blastocysts, transferred into foster mothers. When blastocysts were flushed from the uteri of mice on the third day of pregnancy and retransferred into pseudopregnant females, site of embryo transfer ("3→2," uterus vs. "3→0," oviduct) had no effect on embryo survival (Table 1, lines 1 and 2). This result suggests that neither implantation rate nor postimplantation development of the fully viable blastocysts could be improved by highly asynchronous transfers.

Retransfer of in vivo developed embryos served as a reference point for the evaluation of the cultured and micromanipulated embryo transfers. When in vitro developed blastocysts were transferred into oviducts of day 0 pseudopregnant females, they had the same survival rates as in vivo developed blastocysts (Table 1, lines 3a vs. 2).

Half-embryos had the same implantation rate as control embryos transferred into contralateral oviduct: 62 and 74%, respectively. However, overall survival rate was significantly ($P < 0.001$) lower for half-embryos (15%) than for controls (52%). To exclude the possibility that reduced viability of

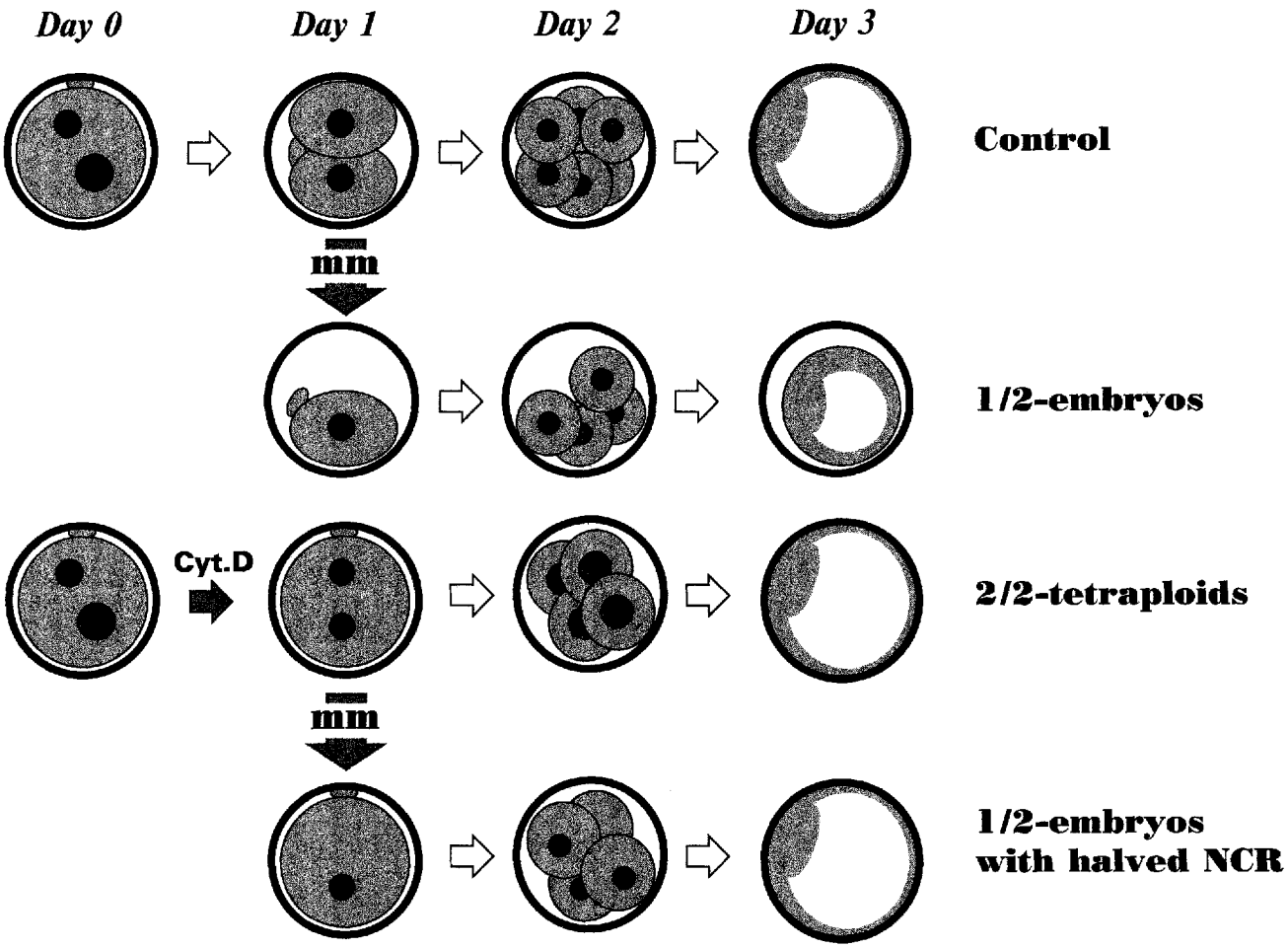

Fig. 1. Embryos used in this study. Cyt.D, embryo culture in the presence of Cytochalasin D. mm, micromanipulations (see Materials and Methods).

the half-embryos was caused by inappropriate uterine response, control blastocysts and half-blastocysts were transferred into pregnant recipients. The results are presented in Table 1, lines 4 and 5. Since the receptive state of the uterus begins a few hours before the native embryos start implantation, asynchronously transferred blastocysts have an advantage over native embryos (McLaren and Michie, '56; Marsk, '77). In vivo developed blastocysts showed better survival rates ($P < 0.05$) when transferred into pregnant recipients (Table 1, line 4 vs. 2). Implantation rate was significantly ($P < 0.01$) decreased when pregnant females were used as recipients of the half-embryos. This was partially compensated by elevated survival rate at the post-implantation stage (29% of live fetuses in the pregnant females, bearing 1/2-embryos vs. 15% in pseudopregnant recipients).

In order to elucidate the mechanisms of blastocyst size regulation that affect embryo viability, we determined cell number in the blastocysts at about the time they would have normally started implantation and 3 days after highly asynchronous transfer, at the actual periimplantation stage. The results are presented in Table 2. In vitro culture conditions significantly retarded development and reduced cell number in 96 h post-hCG-old blastocysts. Zygote culture in the medium without EDTA halved the number of cells in the blastocysts (28.2 ± 0.7 cells per blastocyst vs. 60.5 ± 1.4 in the control, in vivo developed blastocysts). However, following 3 days of passage through the oviducts both in vivo and in vitro cultured blastocysts reached the same 110-cell stage. Embryos that were developing from single blastomeres of the 2-cell embryos had at each developmental stage halved the number of cells of the corresponding intact embryos. On the other hand, it should be noted that following 3 days of delayed implantation half-blastocysts had on average 49.9 ± 2.0

TABLE 1. Viability of the blastocysts transferred into uteri or oviducts of recipient mice[1]

No.	Recipients, site of transfer (Day 2, uterus; Day 0, Right or Left oviduct)		Embryo types, developmental conditions	No. pregnant/no. of recipients (%)	No. of embryos (pregnancies only)		
					Transferred	Implanted (%)	Live on day 16 (%)
1	Pseudopregnant,	uterus	Blastocysts, in vivo	17/18 (94)	171	132 (77)	91 (53)
2	Pseudopregnant,	R, ov.+L. ov.	Blastocysts, in vivo	12/14 (86)	120	80 (67)	57 (48)
3a	Pseudopregant[2]	R. ov.	Blastocysts, in vitro	15/15 (100)	102	75 (74)	53 (52)
3b		L. ov.	1/2-blastocysts, in vitro		111	69 (62)	17 (15)
4	Pregnant	L. ov.	Blastocysts, in vivo	15/22 (68)[3]	47	ND	31 (66)
5	Pregnant	L. ov.	1/2-blastocysts, in vitro	7/14 (50)[3]	35	ND	10 (29)

[1]ND, can not be determined.
[2]Both intact and 1/2-blastocysts were transferred into the same recipient, see Materials and Methods.
[3]Number of pregnant recipients, bearing alien embryos.

TABLE 2. Cell number (mean ± s.e.) in the balstocysts before and 3 days after transfer into the oviducts of pseudopregnant females[1]

Culture conditions, embryo types	NCR at the 2-cell stage	Percent of blastocysts by the 96 h post-hCG	Cell number in the 96 h post-hCG-old blastocysts	Cell number in the 190 h post-hCG-old blastocysts
In vivo	[2N/C] × 2	90 [151/164]	60.5 ± 1.4 [91][a]	111.0 ± 3.7 [18][e]
In vitro	[2N/C] × 2	75 [223/298]	38.7 ± 0.9 [101][b]	109.3 ± 2.5 [58][e]
In vitro, without EDTA	[2N/C] × 2	82 [145/177]	28.2 ± 0.7 [34][c]	107.2 ± 3.5 [35][e]
In vitro, 1/2-embryos	[2N/C]	73 [288/395]	17.8 ± 0.8 [45][d]	49.9 ± 2.0 [40][f]
In vitro, 2/2 tetraploids	[4N/2C]	85 [67/79]	16.8 ± 0.9 [18][d]	51.9 ± 3.8 [17][f]
In vitro, 1/2-embryos with halved NCR	[2N/2C]	27 [64/238]	17.7 ± 1.1 [26][2,d]	70.5 ± 2.4 [43][g]

[1]Number of embryos is given in brackets. NCR, nucleo-cytoplasmic ratio; 2N, diploid nucleus; C, cytoplasm volume in a single blastomere of the 2-cell embryo.
[2]Eighteen morulae and 8 blastocysts among them.
*In each column the groups showing statisticaly significant differences ($P < 0.001$) in their cell numbers are marked wtih differing superscripts.

cells, which is comparable with the number of cells in intact blastocysts on the day of implantation (60.5 ± 1.4).

In order to reveal the factor(s), triggering the state of blastocyst dormancy, tetraploid half-embryos and half-embryos with the halved nucleo-cytoplasmic ratio were produced. The results are presented in Table 2. Figure 2 represents histograms of cell distribution among half-embryos with the halved nucleo-cytoplasmic ratio (Fig. 2B), as compared with the half-blastocysts (Fig. 2A, combined diploids and tetraploids) and intact blastocysts (Fig. 2C, combined in vivo and in vitro cultured blastocysts). These results are discussed below.

DISCUSSION

Investigation of the parameters, characteristic for the implanting mouse blastocyst is complicated by the fact that blastocysts start to implant asynchronously and by the briefness of the peri-implantation stage of each individual blastocyst. The period of embryo readiness for implantation may be extended for more than a week either naturally, in lactating mice, or experimentally, in the uteri of the ovariectomized or immature females (Mantalenakis and Ketchel, '66; McLaren, '68; Weitlauf and Greenwald, '68; Papaioannou, '86). During the state of delayed implantation cellular multiplication, DNA synthesis, and overall metabolic activity are depressed (McLaren, '68; Weitlauf et al., '79; Weitlauf, '85; Mead, '93). Thus, dormant blastocysts may be considered as a synchronized population of the embryos, ready for implantation (Gardner et al., '88).

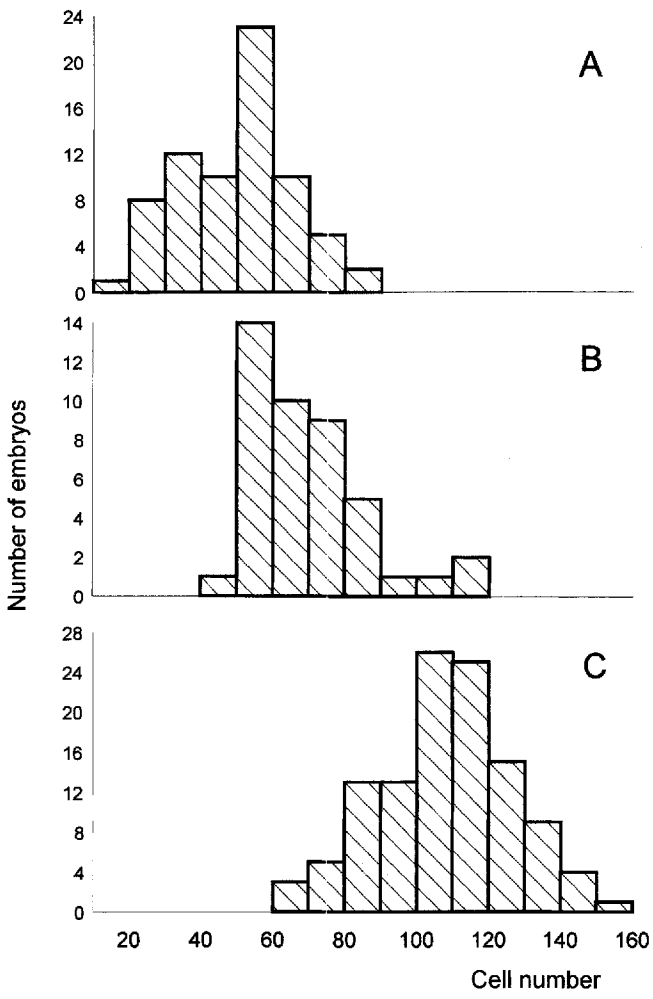

Fig. 2. Cell distribution among 190 h post-hCG-old implantation-delayed blastocysts. **A:** Half-blastocysts. **B:** Half-blastocysts with the doubled amount of cytoplasm. **C:** Control blastocysts.

Blastocyst transfer into oviducts of day 0 pseudopregnant females and their subsequent recovery 3 days later provides 6-day-old blastocysts, synchronized during implantation delay and reactivated in the uteri on the day of recovery. During the state of delayed implantation blastocyst development does not half abruptly: before falling into developmental quiescence blastocysts increase their cell number (Table 2). While using conventional, "3→2" transfer, our attempts to get viable mice from the divided or in vitro cultured embryos were less than satisfactory: about 30% of pregnancies and 28% of live young from the cultured, 8% of live young from the halved embryos (Evsikov et al., '90). The results on highly asynchronous, "3→0" blastocyst transfers (Table 1) support previous findings (Kaufman et al., '77; Tsunoda and McLaren, '83) that viability of initially retarded mouse embryos is partially restored during implantation delay. Our results on embryo transfer into pregnant vs. pseudopregnant recipients (Table 1, lines 3b and 5) showed that low viability of the half-embryos is caused by some endogenous factor, not by inadequate uterine receptivity. It thus may be concluded that elevated cell number in implantation-delayed half-blastocysts is the most probable explanation for the improved survival rate of the half-embryos during highly asynchronous transfers.

Following 3 days of implantation delay blastocysts in which the number of cells was halved by suboptimal in vitro conditions have the same number of cells as control, in vivo developed blastocysts (Table 2). However, it is not the absolute cell number that is a critical factor for the start of a quiescent state. Although implantation delay allows an embryo to increase its cell number, both diploid and tetraploid half-embryos have at the

stage of blastocyst dormancy half the number of cells, characteristic for the control dormant blastocyst (49.9 ± 2.0 and 51.9 ± 3.8 vs. 109.3 ± 2.5 cells, see Table 2). A similar result was obtained by Smith and McLaren ('77) during their investigation of the parameters of the start of blastocyst formation. As discussed in that study, such a result excludes the number of cytokineses, but leaves the number of DNA replication cycles, or the attainment of a definite nucleo-cytoplasmic ratio, as the most probable candidates for the factor(s), triggering the start of implantation. To discriminate between these possibilities we produced half-embryos with the doubled amount of cytoplasm (halved nucleo-cytoplasmic ratio). Histograms presented in Figure 1 show that the majority of delayed blastocysts with the halved nucleo-cytoplasmic ratio had the same number of cells as control half-blastocysts. This indicates that the primary trigger for the state of blastocyst dormancy is not the attainment of a definite nucleo-cytoplasmic ratio, but some other factor, characteristic for the eighth cell cycle. On the other hand, increased number of cells in such blastocysts (70.5 ± 2.4, see Table 2) and the fact that embryo distribution by their cell numbers does not fit normal distribution (Fig. 1B) indicate that the start of the eighth cell cycle is not the only factor determining the state of blastocyst dormancy. We suggest that decreased nucleo-cytoplasmic ratio was the other factor responsible for such a result.

Preimplantation embryos of eutherian mammals depend mostly on the nutrients and energy supplies, stored in the ooplasm. Consequently, total volume of preimplantation embryo is not subjected to significant changes, and each consecutive cleavage halves the amount of cytoplasm in the blastomeres. At the 120-cell stage nucleo-cytoplasmic ratio reaches the level of somatic cells (Surani, cited from Smith and Johnson, '86) and hence the embryo cannot rely any longer on the internal energy and nutrients supplies. In intact blastocysts the eighth cell cycle coincides with the 120-cell stage, i.e., with the depletion of the internal energy sources. This explains why the total cell number in delayed blastocysts does not exceed the range occurring in non-delayed blastocysts immediately before implantation (Copp, '82 and references therein). Producing embryos with the doubled amount of cytoplasm (histogram B, Fig. 1) we circumvented the restrictions imposed by the high nucleo-cytoplasmic ratio and it was possible to obtain a few delayed blastocysts with the majority of their cells in the ninth cell cycle.

We conclude that the state of blastocyst dormancy is started by some factor, characteristic for the eighth cell cycle. Our results suggest that this mechanism is fault-prone, and in normal conditions blastocyst dormancy is assured by the decrease in metabolic activity, occurring around the 120-cell stage, when the nucleo-cytoplasmic ratio reaches the level of somatic cells.

ACKNOWLEDGMENTS

We thank Dr. J.-P. Renard for the helpful comments on the manuscript. This research was made possible in part by grant U47000 from the International Science Foundation.

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