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STUDIES ON DEVELOPMENTAL POTENTIALS OF BISECTED MOUSE EMBRYOS *IN VITRO* AND *IN VIVO*

Akira NAKAGAWA, Yoshiyuki TAKAHASHI and Hiroshi KANAGAWA

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To determine the critical cell numbers needed to form blastocyst stage embroys, bisected mouse embryos of 8–cell stage and compacted morula (approximately 16 cells) containing different numbers of blastomeres were cultured *in vitro*. The percentage of bisected compacted morulae with 7–9 blastomeres that developed to eu-blastocysts was 98.8%, while only 64.2% of the bisected 8–cell embryos with 4 blastomeres developed to eu-blastocysts. The percentage of eu-blastocysts decreased, while that of pseudo-blastocysts and trophectodermal vesicles increased as the number of blastomeres decreased in the bisected embryos of the two stages. Inner cell masses were observed in all the eu-blastocysts and in 87.3% of the pseudo-blastocysts that developed from bisected embryos. The cell number of inner cell mass in the pseudo-blastocysts was significantly less than that of eu-blastocysts ($P<0.01$). The ratios of inner cell mass to total cell number of eu-blastocysts that developed from 4 blastomeres in 8-cell embryos and from 7–9 blastomeres in compacted morulae were similar to those of control embryos, respectively. However, the ratios decreased in proportion to the decrease in the number of blastomeres in the two groups. Five sets of monozygotic twins and 4 singletons of live fetuses were obtained after transfer of the 20 twin sets of eu-blastocysts that developed from the bisected compacted morulae with 7–9 blastomeres. However, no implantation occurred when the pseudo-blastocysts that developed from bisected compacted morulae with 6 or fewer blastomeres were transferred. Weight of fetuses that developed from bisected embryos on Day 18 of pregnancy were significantly lower than those of the control.

Key words: Mouse, embryo, bisection, blastomere, developmental potential

INTRODUCTION

Since the first success in achieving normal development from blastomeres isolated at the 2–cell embryos in the rat by NICHOLAS & HALL (1942), many studies on the

Department of Veterinary Obstetrtrics, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

developmental potentials of the isolated blastomeres from the first few cleaved embryos in mammals have been made. The developmental potentials of mouse blastomeres were reported in 2-cell embryos by TARKOWSKI (1959a, b), MULNARD (1965), HOPPE & WHITTEN (1972) and SHERMAN (1975); in 4- and 8-cell embryos by TARKOWSKI & WROBLEWSKA (1967), KELLY (1975), ROSSANT (1976) and ROSSANT (1977); and in 2-, 4- and 8-cell embryos by FISHER & MACPHERSON (1975) and O'BRIEN et al. (1984). Attempt have also been made to produce a monozygotic twin from bisected mouse morulae (BAUNACK, 1980; NAGASHIMA & OGAWA, 1981; TSUNODA & MCLAREN, 1983; NAGASHIMA et al., 1984). The viability of the bisected mouse morulae was found to be seriously affected by slight damage to the embryos by micromanipulation (NAGASHIMA et al., 1984). However, there are no reports that relate the number of component blastomeres in bisected mouse morulae to its developmental potentials. TARKOWSKI (1959a, b), TARKOWSKI & WROBLEWSKA (1967) and KELLY (1975) reported that blastomeres isolated from embryos until the 8-cell stage are developmentally labile. The morula is regarded as the initiating stage of differentiating blastomeres into inner cell mass (including primitive ectoderm and endoderm; ICM) and trophectoderm (DUCIBELLA, 1977; JOHNOSON et al., 1977; JOHNSON, 1979). In the present study, bisected mouse embryos of 8-cell stage and compacted morulae containing different numbers of blastomeres were cultured *in vitro* to determine if a critical cell number is necessary to form blastocyst stage embryos. Viability of the bisected embryos *in vivo* that developed to eu-blastocysts and pseudo-blastocysts were also studied.

MATERIALS AND METHODS

Collection of embryos

Embryos were obtained from virgin ICR strain mice, 8–10 weeks old. These mice were induced to superovulate using 7.5 IU of PMSG, followed by 10 IU HCG at 48 hr apart. They were then mated with males of the same strain. Flushing was carried out 66 hr and 76 hr post HCG injection in order to obtain 8-cell embryos and compacted morulae, respectively. WHITTINGHAM's medium (WHITTINGHAM, 1971) was used for collection and culture of embryos. The zona pellucida was removed with 0.5% pronase (MINTZ, 1962) and was washed three times with the medium.

Bisection and in vitro culture

Decompaction of compacted morulae was carried out by incubating embryos in Ca^{2+}- Mg^{2+}-free phosphate buffer saline (DULBECCO's modified PBS) for 10–20 min at 37°C in the presence of 5% CO_2 in humidified air.

All the above embryos were mechanically bisected using a fine glass needle (NAGASHIMA & OGAWA, 1981) under an inverted microscope with NOMARSKI interference optics. During bisection, the embryos were placed in a small droplet of the medium covered by paraffin oil. The number of blastomeres in all the bisected embryos was recorded. Each pair of the bisected embryos was then cultured in a small droplet of

medium under paraffin oil in humidified air with 5% CO_2 at 37°C. For the control, unbisected zona-free 8–cell embryos and compacted morulae that had been decompacted were cultured.

Morphological classification of embryos

After 48 hr of *in vitro* culture, all embryos were morphologically classified into the following six types; type 1: eu-blastocyst that contained a distinct ICM and well developed trophectoderm; type 2: pseudo-blastocyst that appeared to contain a small clump of cells (poorly developed ICM) inside the trophectodermal layer; type 3: trophectodermal vesicle containing no cell mass; type 4: non-integrated form with disorganized clusters of cells, of which some were vacuolated; type 5: morula and type 6: degenerated embryo.

This classification was a modification of that by NAGASHIMA et al. (1984).

Antiserum and complement

Anti-mouse-spleen serum was produced by three intravenous injections of ICR mouse spleen cells into a rabbit at a dose of 4×10^8 spleen cells per injection given 10 days apart. Collection of serum was done 7 days after the third injection. In order to inactivate the rabbit complement, the serum was heated at 56°C for 30 min. It was then stored at −40°C.

Fresh guinea pig serum was absorbed with ICR mouse spleen cells and used as a source of complement. The serum was diluted 1: 50 in DULBECCO's modified minimum essential medium (D-MEM) prior to use.

Cytotoxity test

Cytotoxity test of anti-mouse-spleen serum on zona-free blastocysts was carried out. In order to find the optimal dilution rate, zona-free blastocysts were incubated for 30 min in different antisera at a dilution rate of 1: 5 to 1: 10000. Zona-free blastocysts were then washed three times in D-MEM supplemented with 10% fetal calf serum followed by incubation for 30 min in the complement. The extent of lysis was determined visually. To confirm these results, all embryos were treated with 0.4% trypan blue in saline and the extent of dye exclusion was examined.

Total and ICM cell numbers

Eu-blastocysts and pseudo-blastocysts were counted after 48 hr of *in vitro* culture to determine the cell number of ICM. Optimal antiserum dilution (1: 100), which was obtained from the cytotoxity test, was used for the isolation of ICM (SOLTER & KNOWLES, 1975). Cell numbers of isolated ICM were counted in all eu-blastocysts and pseudo-blastocysts that developed from bisected embryos and in all control embryos, whereas total cell numbers were counted in all embryos. Examination of cell number was done according to KAMIGUCHI et al. (1978).

Transfer of bisected and control embryos

After 30 hr of *in vitro* culture, the monozygotic pair of bisected compacted morula with 7–9 blastomeres in which both had developed to eu-blastocysts, was transferred

to one uterine horn of a Day 4 pseudopregnant recipient (day of vaginal plug=Day 1). Similarly, a monozygotic pair of pseudo-blastocysts with 6 or fewer blastomeres was also transferred. In all instances, 2 control embryos of compacted morulae were transferred to the contralateral side of the uterus. The number of implantations, live fetuses and weights of fetuses and placentae were recorded.

RESULTS

Cytotoxity test

When zona-free blastocysts were incubated for 30 min, washed, and then transferred to complement, all the blastocysts lysed trophectoderm in a diltuion of antiserum up to 1 : 100 (figure). Even at a dilution of 1 : 10000, more than 40% of the trophectoderms had lysed. Lysis of ICM was never observed in any of the dilution rates. If, however, isolated ICMs were re-exposed to antiserum and complement, lysis of the ICM occurred in 1 : 100 antiserum dilution. However, only one-fifth of the isolated ICMs lysed when the antiserum dilution was 1 : 1000. It was found that the optimal dilution level was 1 : 100.

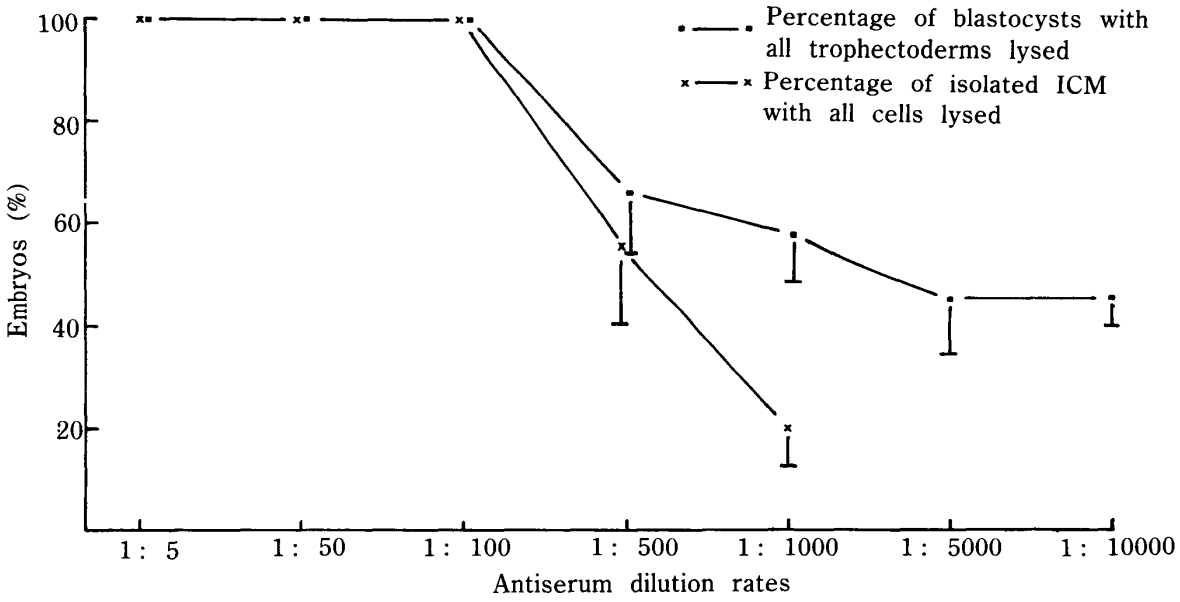

FIGURE *Percentage of zona-free blastocysts with complete lysis of trophectoderms and isolated ICMs with different dilution rates of antiserum. Points represent means with standard deviation bars.*

In vitro development of bisected embryos

After 48 hr of *in vitro* culture, 55.3% and 80.3% of bisected embryos developed to eu-blastocysts in 8–cell embryos and compacted morulae, respectively. In the control group, 96.3% and 96.7% of the zona-free embryos developed to eu-blastocysts (table 1). No significance in the percentage of eu-blastocysts was observed between the two control groups. However, the percentage of eu-blastocysts that developed from bisected embryos in compacted morula was significantly higher than that of 8–cell embryos ($P<0.05$).

In 8–cell embryos, the number of eu-blastocysts that developed from 4 and 3 blastomeres was 64.2% and 42.1%, respectively. However, it was found that 2 blastomeres of 8–cell embryos could not form eu-blastocysts. The percentage of eu-blastocysts that developed from 92 bisected compacted morulae with 7–9 blastomeres was 98.9%. A lower rate of eu-blastocysts developed from 5 and 6 blastomeres (33.3% and 64.7%), and no eu-blastosyst developed from 3 and 4 blastomeres of bisected compacted morulae. In the two stages, the percentage of eu-blastocysts decreased, while that of pseudo-blastocysts and trophectodermal vesicles increased as the number of blastomeres decreased.

The percentage of monozygotic pairs obtained from bisected embryos, in which both had developed to eu-blastocysts, was 36.8% (14/38) and 68.2% (45/66) in 8–cell embryos and compacted morulae, respectively. The percentage of monozygotic pairs of bisected embryos in compacted morulae was significantly higher than that of 8–cell embryos ($P<0.005$).

Total and ICM cell numbers

Total cell numbers and ICM cell numbers of bisected and control embryos after 48 hr of *in vitro* culture in 8–cell embryos and compacted morulae are shown in table 2.

Mean percentage of total cell numbers of eu-blastocysts that developed from half the number of blastocysts of the control embryos in 8–cell embryo and compacted morula was 41.8% and 42.5%, respectively. Each level was less than half the total cell number of eu-blastocysts that developed from control embryos.

In the two groups, the total cell number decreased from eu-blastocysts to trophectodermal vesicles. The total cell number of eu-blastocysts, pseudo-blastocysts and trophectodermal vesicles decreased in proportion to the decrease in the number of blastomeres. ICMs were observed in all eu-blastocysts and in 87.3% (20/23) of the pseudo-blastocysts that developed from bisected embryos. The cell number of ICM in pseudo-blastocysts was 2–4 cells, which was significantly less than that of eu-blastocysts ($P<0.01$).

It was observed that the ratios of ICM cell number to total cell number of eu-blastocysts that developed from 4 blastomeres in 8–cell embryos and from 7–9 blastomeres in compacted morulae similar to those of control embryos, respectively, while those of pseudo-blastocysts were lower (table 3).

TABLE 1 *Morphological classification of control and bisected embryos after 48 hr of in vitro culture in 8-cell stage and compacted morula.*

EMBRYOS	NO. OF BLASTOMERES	NO. OF EMBRYOS CULTURED	MORPHOLOGICAL CLASSIFICATION OF EMBRYOS*					
			Eu-b	Pseu-b	TV	NIF	Morula	Deg
8-cell stage								
Control		54	52 (96.3)	–	–	1 (1.9)	1 (1.9)	–
Bisected		76	42 (55.3)[a]	15 (19.7)	6 (7.9)	9 (11.8)	–	4 (5.3)
	4	53	34 (64.2)	10 (18.9)	2 (3.8)	5 (9.4)	–	2 (3.8)
	3	19	8 (42.1)	4 (21.1)	1 (5.3)	4 (21.1)	–	2 (10.5)
	2	4	–	1 (25.0)	3 (75.0)	–	–	–
Compacted morula								
Control		61	59 (96.7)	–	–	2 (3.3)	–	–
Bisected		132	106 (80.3)[b]	13 (9.8)	4 (3.0)	5 (3.8)	1 (0.8)	3 (2.3)
	9	20	20 (100.0)	–	–	–	–	–
	8	42	41 (97.6)	–	–	1 (2.4)	–	–
	7	30	30 (100.0)	–	–	–	–	–
	6	17	11 (64.7)	4 (23.5)	1 (5.9)	–	–	1 (5.9)
	5	12	4 (33.3)	4 (33.3)	–	2 (16.7)	–	2 (16.7)
	4	7	–	4 (57.1)	2 (28.5)	1 (14.3)	–	–
	3	4	–	1 (25.0)	1 (25.0)	2 (50.0)	–	–

Percentage of embryos enclosed in parentheses.

* : Eu-b=Eu-blastocyst, Pseu-b=Pseudo-blastocyst, TV=Trophectodermal vesicle, NIF=Non-integrated form, Deg=Degenerated embryo

a vs b ($P<0.05$)

TABLE 2 *Total and ICM cell numbers of control and bisected embryos in 8-cell stage and compacted morula after 48 hr of in vitro culture in relation to embryonic morphology and number of blastomeres*

EMBRYOS	NO. OF BLASTOMERES	MORPHOLOGICAL CLASSIFICATION OF EMBRYOS*						
		Eu-blastocyst		Pseudo-blastocyst		TV	NIF	Morula
		Total	ICM	Total	ICM	Total	Total	Total
8–cell stage								
Control		84.5±10.1[a] (26)	23.8±3.4 (26)				21 (1)	11 (1)
Bisected	4	35.3±3.4 (17)	10.4±1.7 (17)	20.8±4.5 (5)	2.4±0.5 (5)	11.5±0.7 (2)	7.6±4.4 (5)	
	3	26.3±2.6 (4)	6.4±0.5 (4)	13.5±2.4 (4)	1.5±1.0 (4)	10 (2)		
	2			12 (1)	0 (1)	8.0±1.0 (3)		
Compacted morula								
Control		128.1±13.9 (30)	31.5±3.0 (29)				21.5±2.1 (2)	
Bisected	9	61.8±2.1 (10)	15.6±1.4 (10)					
	8	54.6±5.2 (21)	13.9±2.2 (20)				14 (1)	
	7	46.6±5.0 (15)	10.7±1.9 (15)					
	6	38.8±2.1 (5)	7.7±1.0 (6)	27.0±1.4 (2)	3.0±1.4 (2)	14 (1)		
	5	30.5±0.7 (2)	5.5±0.7 (2)	22.0±2.8 (2)	3 (2)		12 (1)	
	4			17.5±2.1 (2)	2 (2)	9.5±0.7 (2)	12 (1)	
	3			12 (1)	0 (1)	8 (1)	8.5±0.7 (2)	

Number of embryos examined enclosed in parentheses.

* : TV=Trophectodermal vesicle, NIF=Non-integrated form, Total=Total cell number, ICM=ICM cell number

a : Mean ±S. D.

TABLE 3 *ICM ratio of eu- and pseudo-blastocysts developed from control and bisected in 8-cell stage and compacted morula*

EMBRYOS	TYPE OF BLASTOCYST	NO. OF BLASTOMERES	ICM RATIO*
8-cell stage			
Control	Eu-blastocyst		0.282
Bisected	Eu-blastocyst	4	0.295
		3	0.240
	Pseudo-blastocyst	4	0.106
		3	0.111
		2	0
Compacted morula			
Control	Eu-blastocyst		0.246
Bisected	Eu-blastocyst	9	0.252
		8	0.255
		7	0.230
		6	0.198
		5	0.180
	Pseudo-blastocyst	6	0.111
		5	0.136
		4	0.114
		3	0

* : ICM ratio=mean ICM cell number / mean total cell number

Viability of bisected embryos after transfer

The results of monozygotic pairs of eu-blastocysts and pseudoblastocysts that were transferred to 20 and 6 recipients, respectively, are shown in table 4. The number of implantations and live fetuses after transfer of the eu-blastocysts that developed from bisected embryos was significantly lower as compared to that of control groups ($P<0.05$). Five sets of monozygotic twins and four singletons were obtained from the transfer of 20 pairs of eu-blastocysts. However, implantation did not occur in any of the 6 pairs of pseudo-blastocysts, while in the contralateral side, 1 or 2 live fetuses were obtained. No abnormality was observed in the fetuses from bisected embryos nor in those from control embryos. The weight of fetuses from bisected embryos was significantly less than that of control embryo ($P<0.01$). No significant difference was observed in the placenta.

TABLE 4 *The viability* of bisected and control embryos from compacted morulae after transfer*

TYPE OF EMBRYOS TRANSFERRED	NO. OF ENBRYOS TRANS-FERRED	NO. (%) OF IMPLANTA-TION	NO. (%)** OF LIVE FETUSES	LIVE FETUSES WEIGHT***(g)	PLACENTAE WEIGHT***(g)
I Bisected (eu-blasto.)	40	22 (55.0)[a]	14 (35.0)[c]	1.10±0.14[e]	0.12±0.02
Control	40	31 (77.5)[b]	24 (60.0)[d]	1.23±0.12[f]	0.13±0.01
II Bisected (Pseudo-blasto.)	12	0 (0)	0 (0)	–	–
Control	12	9 (75.0)	7 (58.3)	1.24±0.14	0.13±0.03

* : Necropsy was done on Day 18 of pregnancy to determine viability.
** : No. of live fetuses / No. of embryos transferred
*** : Mean±S. D.
a vs b, c vs d ($P<0.05$), e vs f ($P<0.01$)
c : Five sets (25.0%) of monozygotic twin were obtained.

DISCUSSION

In the present study, zona-free mouse embryos were successfully bisected using a fine glass needle without the use of any holding pipette. *In vitro* development after bisection of mouse 8–cell embryos and morulae has been reported by various researchers. The percentage of bisected embryos that developed to blastocysts was 59% (RODRIGUES, 1981) and 77% (KÖSTER, 1982) in 8–16 cell embryos; and 74–84% (MOUSTAFA & HARN, 1978), 27–54% (RODRIGUES et al., 1980), 78% (TSUNODA & MCLAREN, 1983) and 65% (NAGASHIMA et al., 1984) in morula. The results of the present study were in agreement with these reports.

It has been found that intracellular focal tight junction and gap junction are formed during the compaction stage of the morula (DUCIBELLA & ANDERSON, 1975a, b). The Ca^{2+}–free medium treatment used for decompaction causes disruption of these apical focal tight junctions (DUCIBELLA, 1977), thus minimizing damage that might occur to the blastomeres during the bisection process. Thus, a higher percentage of eu-blastocysts was obtained from compacted morulae.

In the two groups, the total cell number of eu-blastocysts that developed from half the number of blastomeres was less than the expected 50% levels. This was probably due to death or disassociation of the blastomeres occurring during the cultured period (SMITH & MCLAREN, 1977) or to the delay in development caused by the extensive manipulation of the embryos (FERNANDEZ & IZQUIERDO, 1980). Delayed development of bisected embryos was also suggested to be related to the lowered

levels of mitotic indices occurring just after bisection (NAKAGAWA et al., 1985).

As the number of blastomeres decreased in bisected embryos of 8–cell and compacted stage, the percentage of eu-blastocysts decreased, while that of pseudo-blastocysts and trophectodermal vesicles increased. This is in agreement with the results of TARKOWSKI and WROBLEWSKA (1967). In bisected 8–cell embryos, the number of component blastomeres itself does not seem to be a critical factor in the development into eu-blastocysts. The spatial position of the component blastomeres in morula appears to play an important role in prescribing the developmental fate of the blastomeres (HANDYSIDE, 1980; JOHNSON, 1981).

The ratios of ICM to the total cell number of eu-blastocysts that developed from 4 blastomeres in 8–cell embryos and 7–9 blastomeres in compacted morulae were similar to those of control embryos, respectively. However, the ratio as well as the cell number of ICM decreased in proportion to the decrease in the number of blastomeres.

During compaction of the morula, which is regarded as the initiating stage of differentiation of blastomeres (DUCIBELLA, 1977; JOHNSON et al., 1977; JOHNSON, 1979), some blastomeres become completely enclosed by others. It was thought that these enclosed 'inner blastomeres' and the surrounding 'outer blastomeres' differentiate into ICM and trophectoderms in the blastocyst, respectively (TARKOWSKI & WROBLEWSKA, 1967; HILLMAN et al., 1972). The formation of 'inner blastomeres' in bisected compacted morula containing 6 or fewer blastomeres is probably incomplete and results in a poor ICM, thus leading to development of pseudo-blastocysts or trophectodermal vesicles.

Bisected compacted morulae were transferred to study the relationship between the number of blastomeres of bisected embryos in which cell differentiation have occurred and the viability of the embryos *in vivo*. Live fetuses were obtained after transfer of eu-blastocysts, while no implantation occurred when pseudo-blastocysts were transferred. Only the ICM gives rise to the fetus (ROSSANT, 1977). It was thus suggested that the embryos with a lower total cell number and poor ICM gave no implantation. Then, how many blastomeres were needed in the bisected compacted morula to form a normal fetus? From this study, it was found that bisected embryos of 7 or more blastomeres developed to eu-blastocysts, and gave rise to normal fetuses. Thus, minimizing loss of component blastomeres during micromanipulation was considered to be a prerequisite to obtain live fetuses from bisected embryos. It was also suggested that morphological selection of micromanipulated embryos carried out prior to transfer can increase the number of live fetuses (NAGASHIMA et al., 1984). TARKOWSKI (1959a, b) reported that fetuses from half embryos were in the same stage of development as the control on Day 12 of pregnancy and that no difference in birth weight occurred. In contrast, the present study showed that the body weight of live fetuses from bisected embryos on Day 18 of pregnancy was significantly lower than that of the control. Similar findings were reported by TSUNODA & MCLAREN (1983).

It was thought that the cause of the lowered body weight is probably due to the lower number of ICMs present in the bisected embryos. Further research on the developmental and reproductive ability of mice from bisected embryos is needed.

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