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Trophoblastic vesicles as carriers of embryonic cells for mammalian cloning*

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ABSTRACT

Inner cell mass-free blastocysts, also referred to as trophoblastic vesicles (TVs) could be used as carriers to be injected with *in vitro* cultured embryonic cells and to generate clones of animals, providing an alternative method of mammalian cloning. Here we compare three methods of obtaining mouse TVs. Culturing preimplantation embryos in heat shock conditions brings about up to 35% of TVs having up to 47 cells and the efficiency is dependent on the stage of cultured embryos. Live birth was obtained after transfer of ES cell-injected TVs to recipient mice. Culture with radioactive precursor yields up to 55% of TVs, but only at concentrations supplying 37% TVs is their cell number sufficiently high. The effects of PMA treatment are 47% of TVs.

KEY WORDS: mouse, animal biotechnology, trophoblastic vesicles

INTRODUCTION

Mutant mice strains can be generated by producing animals derived from transgenic embryonic stem cells, without the intermediate step of chimaerae formation and their further breeding (Wang et al., 1997). Also, animals originating from a

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given cell line constitute a clone, so that this method can be an alternative way to clone mammals, possibly including livestock species. Mice fully derived from embryonic stem cells can be produced by aggregating the cells with cleaving tetraploid embryos (Nagy et al., 1990) or by injecting the cells into tetraploid blastocysts (Wang et al., 1997), into heat treated blastocysts (Amano et al., 2000), or into traophoblastic vesicles (Modliński et al., 1995, 1996). In the cow, aggregation of embryonic stem-like cells with cleaving tetraploid embryos has already been accomplished, leading to the birth of chimaeric calves (Iwasaki et al., 2000).

Trophoblastic vesicles can be obtained by microsurgically removing the inner cell mass from blastocysts, leaving the polar and mural trophoblast intact (Modliński et al., 1995, 1996). This avoids inner cell mass (ICM)-contamination from the carrier recipient embryo. Microsurgical removal of ICMs is a technically difficult method, however, and it is additionally complicated by instances in which cavity re-expansion after ICM removal does not occur, which prevents subsequent cell injection. Therefore, alternative ways of producing ICM-free blastocysts would be of interest. There are some observations suggesting that ICM-free blastocysts (further referred to as "trophoblastic vesicles") can be obtained by culturing preimplantation embryos at temperatures elevated a few degrees above their physiological requirements (mild chronic heat shock conditions) (Tarkowski, 1970) or in the presence of [^{3}H]thymidine (Ansell and Snow, 1975) and by treating preimplantation embryos with phorbol esters (Ohsugi and Yamamura, 1993).

The aim of this paper was to compare the rate of trophoblastic vesicle production when applying the aforementioned three methods to the embryos of the same strain of mice.

MATERIAL AND METHODS

Isolation of embryos and culture in vitro

Fl (CBA/HxC57BL/10) female mice were induced to ovulate by intraperitoneal injection of 5 i.u. PMSG (Folligon, Intervet, The Netherlands) followed 48 h later by 5 i.u. hCG (Chorulon, Intervet, The Netherlands). After the second injection females were caged with males for mating. Embryos were flushed from the oviducts of mated females 45-50 h post hCG (two-cell embryos), 52-56 h post hCG (early 4-cell embryos), 64-66 h post hCG (late 4-cell embryos) and 68-70 h post hCG (8-cell embryos).

Embryos were cultured in drops of M16 medium (Fulton and Whittingham, 1978) or modified M16, referred to as T6 medium (Quinn et al., 1984), under paraffin oil (Merck, and Corning), in (Falcon or Sterilin) Petri dishes, at 37°C (40°C),

in the atmosphere of 5% CO_2 in the air. Paraffin oil was equilibrated with the medium in the above mentioned conditions for 24 h before starting the culture.

Obtaining ICM-free blastocysts

Embryos were allocated to control or experimental groups and were cultured for 2-3 days until they reached the blastocyst stage.

1. Heat shock conditions: Two-cell embryos, early 4-cell embryos, late 4-cell embryos and 8-cell embryos were cultured at 37ºC (the control) and at 40ºC (experimental group).

2. [^{3}H]methylthymidine (MT) treatment: two-cell embryos were cultured at various concentrations (0.01, 0.025, 0.05 and 0.1 mCi/ml) of [^{3}H]methylthymidine (RRC, Amersham) of the specific activity of 17.6 Ci/mM.

3. Phorbol 12-myristate 13-acetate (PMA) treatment: PMA was dissolved in DMSO and then it was adjusted to the final concentration with the culture medium, just before starting the culture. Two-cell embryos were cultured with various concentrations of PMA (0.5, 0.75 and 1.0 nM/ml) for 24 h. Then they were rinsed of PMA and cultured for next 24-28 h. Some embryos were transferred to the oviducts of recipients immediately after PMA treatment. After the completion of culture, some embryos were transferred to feeder layers to check if ICM cells would develop (Robertson, 1987).

Morphological and karyological analysis

From some blastocysts and trophoblastic vesicles whole mount preparations (Tarkowski and Wróblewska, 1967) were done. Some were stained with Harris haematoxylin; others were air-dried (Tarkowski, 1966) and stained with Giemsa (Gurr).

Development in vivo

PMA-treated embryos were transferred to the oviducts and embryos treated with heat shock or [^{3} H]methylthymidine were transferred to the uteri of pseudopregnant recipients. The recipients were either of outbred Swiss albino strain (own breed) or of BALB/cByJ or CDl strains (Jackson Laboratory, Maine). Females entering spontaneous oestrus were mated to vasectomized Swiss albino or BALB/cByJ males. Oviductal transfers were performed at day 1 and uterine transfers - at day 3.5 of pseudopregnancy (day of the copulation plug = 1).

RESULTS AND DISCUSSION

The rate of trophoblastic vesicle formation in conditions of mild heat shock increased with the age of the embryos (Table 1). Two-cell embryos produced only 2.7% of vesicles, whereas 8-cell embryos produced 35.5% vesicles (Figure 1). The number of normal blastocysts was also higher when later-stage embryos were cultured, with the unexpected exception of late 4-cell stage. To check if the quality of the blastocysts cultured was related to the time spent at elevated temperature, postimplantation development of blastocysts obtained from 4-cell and 8-cell embryos was compared. Those spending more time in heat shock conditions were still viable. In fact, out of 12 blastocysts from 4-cell embryos, 9 young were born (6 males and 3 females) and out of 18 blastocysts developed from 8-cell embryos, 13 young (9 males and 4 females) were born. Trophoblastic vesicles were also able to induce implantation after transfer to recipients. Out of 7 vesicles obtained from early 4-cell embryos and transferred to recipients, 5 induced a strong decidual reaction.

The mean cell number (± SD) of blastocysts developed at 40°C from 2-cell embryos was 27.5 (n=2), from 4-cell embryos it was 39.6 ± 4.2 (n=7), and from 8-cell embryos 47.5 ± 3.5 (n=9). Cell numbers in trophoblastic vesicles obtained from 4- and 8-cell embryos were similar to those of blastocysts.

Fifty two trophoblastic vesicles obtained after chronic mild heat treatment were later injected with D3 ES cells, each with about 15 cells. After 24 h twenty eight vesicles were selected for transfer. After transfer to 3.5 day pseudopregnant recipients two 13-day foetuses (one of them alive) and one normal live birth were obtained.

TABLE 1

Formation of trophoblastic vesicles from embryos cultured in heat shock conditions

Stage of development	°C	Percent of cavitation			No. of embryos
		TVs	blastocysts	total	
2-cell	37	0	31.2	31.2	16
	40	2.7	8.3	11.0	36
Early 4-cell	37	0	68.2	68.2	22
	40	6.2	41.1	47.3	112
Late 4-cell	37	n.d.	n.d.	n.d.	n.d.
	40	18.2	2.3	20.5	44
8-cell	37	0	93.7	93.7	32
	40	35.6	39.4	75.0	104

n.d. - not done

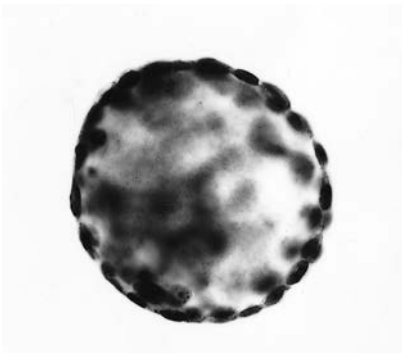

Figure 1. Trophoblastic vesicle obtained after heat-shock treatment of an 8-cell embryo

Figure 2. Trophoblastic vesicle (left) and a blastocyst (right) obtained after treatment of 2-cell embryos with 0.05 Ci/ml of [^{3}H]methylthymidine. Inside the blastocyst 2-3 cells forming the reduced ICM are visible

TABLE 2

Formation of trophoblastic vesicles from 2-cell embryos cultured with MT

MT concentration mCi/ml	Percent of cavitation			No. of embryos
	TVs	blastocysts	total	
0.01	11.1	69.7	80.8	43
0.03	36.4	40.4	76.8	52
0.05	55.5	13.9	69.4	36
0.1	25.6	2.6	28.2	39
Control	0	88.9	88.9	27

Recently, acute heat shock was successfully applied to obtain totally ES cells-derived mice. Recipient blastocysts were maintained for 20 min at 45^{o}C before injecting them with ES cells. Such short term heat shock selectively blocked ICM cells division, leaving trophoblast unaffected (Amano et al., 2000).

The frequency of trophoblastic vesicle formation after culture in the presence of [^{3}H]methylthymidine (MT) is clearly dependent on the concentration of the radioactive precursor (Table 2). The highest percentage of vesicles (55%) was obtained at 0.05 mCi/ml of [^{3}H]methylthymidine (Figure 2). Increasing the concentration to 0.1 mCi/ml reduces the percentage of developing blastocysts and also the number of their ICM cells. In blastocysts developed at a concentration of 0.05 mCi/ml there were only 2-3 ICM cells (Figure 2), whereas about 10 ICM cells were present in blastocysts developed at 0.03 mCi/ml, which is in agreement with the observations of Ansell and Snow (1975).

Although the cell numbers did not differ between blastocysts and trophoblastic vesicles cultured at the same concentrations of [^{3}H]methylthymidine, they both

decreased with increasing concentration. For concentrations 0.01, 0.03, 0.05 and 0.1 mCi/ml, the mean cell numbers (±SD) were 42.4 ± 3.5, 32.1 ± 3.1, 27 ± 2, and 15.5 ± 1.5, respectively.

Both blastocysts and trophoblastic vesicles obtained after MT treatment were able to implant in the uterus. Following a transfer to 4 recipients of 10 blastocysts which developed in the presence of 0.01 mCi/ml MT and 0.03 mCi/ml MT, six and five foetuses, respectively, were obtained between the 12th and 14th day of pregnancy. Only two 13-day foetuses of the first group were alive; the remaining ones were being resorbed. After 10 trophoblastic vesicles that were cultured in 0.05 mCi/ml MT and 0.1 mCi/ml MT were transferred to 4 recipients, five and three decidual reactions were found on day 8.

The frequency of obtaining trophoblastic vesicles with PMA was dependent on the concentration of PMA (Table 3). The highest percentage of vesicles was obtained at 1.0 nM/ml of PMA, which is in agreement with the observations of Ohsugi and Yamamura (1993).

TABLE 3

Formation of trophoblastic vesicles from 2-cell embryos cultured with PMA

PMA nM/ml	Percent of cavitation			No. of embryos
	TVs	blastocysts	total	
0.5	15.3	50.0	65.3	72
0.75	31.7	17.5	49.2	63
1.0	43.6	3.3	46.8	94
Control	0	50.0	50.0	26

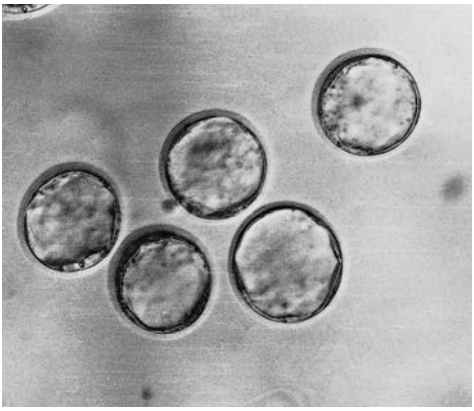

Figure 3. The group of trophoblastic vesicles obtained after treatment of 2-cell embryos with 1.0 mM/ml of PMA

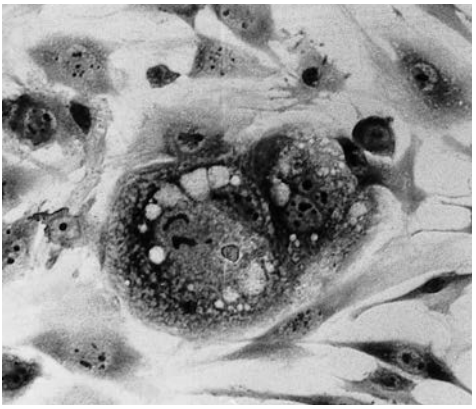

Figure 4. Trophoblastic outgrowth from a vesicle obtained after PMA treatment (1.0 mM/ml) of a 2-cell embryo. No ICM cells are present. Trophoblastic giant cells are visible in the middle

When trophoblastic vesicles obtained after PMA treatment (Figure 3) were seeded on feeder layers, only trophoblast cells proliferated. Soon they formed trophoblast giant cells (Figure 4). It must be added, however, that trophoblastic vesicles adhered poorly to the feeder layer (5 out of 11 adhered, as compared to 6 out of 7 blastocysts). Those blastocysts which retained even the smallest remnants of ICM cells, produced ICM cells *in vitro*. When 9 embryos treated with 0.5 nM/ml PMA, 10 embryos treated with 0.75 nM/ml PMA and 10 embryos treated with 1.0 nM/ml PMA were transferred to the oviducts of recipients, four, four and three implantation sites, respectively, were found on day 6.5. These results confirm those of Ohsugi and Yamamura (1993), who proved that PMA-treated embryos are able to induce implantation. However, only those cultured with low concentration of 0.1 nM/ml PMA can develop normally until day 18 after transfer.

CONCLUSIONS

Culture of 4- and 8-cell embryos at 40°C produced 18-35% of trophoblastic vesicles that were able to induce a decidual reaction. They contained about 39 and 47 cells, respectively. MT treatment at a concentration of 0.03 mCi/ml gave 36% yield of trophoblastic vesicles composed of about 32 cells. At higher concentrations the cell number was too low to be considered, and at lower concentrations the percentage of vesicles fell to 11%. PMA treatment resulted in the formation of up to 43% of trophoblastic vesicles, which proved to be devoid of ICMs. Pilot experiments on injecting ES cells to trophoblastic vesicles showed that, contrary to heat shock- and MT-derived vesicles, the PMA-derived ones could be mechanically unsuitable to survive microsurgery.

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STRESZCZENIE

Pęcherze trofoblastyczne jako biorcy komórek zarodkowych w klonowaniu ssaków

Do blastocyst pozbawionych węzłów zarodkowych (tzw. pęcherzy trofoblastycznych) można wstrzyknąć hodowane *in vitro* komórki zarodkowe i otrzymać z nich klony zwierząt, co jest alternatywną metodą klonowania ssaków. Porównano trzy sposoby otrzymywania pęcherzy trofoblastycznych myszy. Hodowla zarodków przedimplantacyjnych w warunach łagodnego szoku termicznego dostarcza do 35% pęcherzy mających do 47 komórek, a efektywność zależy od stadium hodowanych zarodków. Hodowla z radioaktywnym prekursorem skutkuje powstaniem 55% pęcherzy, ale tylko w stężeniach, w których powstaje 37% pęcherzy, ich liczba komórek jest dostateczna. Skutkiem traktowania estrem forbolu (PMA) powstaje 47% pęcherzy.