E-cadherin Localization in Mouse Embryos Cultured in an Oviductal Environment

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Abstract: *One-cell mouse embryos were cultured in the presence or absence of oviductal tissue in order to investigate the role of oviductal tissue on the developmental competence and morphology of mouse embryos cultured in vitro. To synchronize embryo development, one-cell embryos were treated with nocodazole, an inhibitor of tubulin polymerization. The morphology of 4-cell stage embryos that subsequently developed in the absence of oviduct (phosphate-free medium) differed markedly from that of 4-cell stage embryos developed in an oviductal environment. Four-cell embryos that developed without oviductal tissue had spherical blastomeres whereas embryos developed in co-culture had flattened blastomeres and appeared to have undergone premature compaction. Embryos with flattened blastomeres exhibited E-cadherin localization to regions of cell-to-cell contact.*

Key words: *Mouse embryo, Oviduct, Phosphate-free medium, Compaction, E-cadherin*

In many mammalian species, embryo development in vitro is usually blocked at certain specific stages. Although the mechanism of this developmental arrest is not well understood, it can be overcome under certain specific conditions. We recently reported that mouse one-cell embryos co-cultured with oviduct develop normally to the blastocyst stage [1]. The developmentally competent four-cell embryos exhibited cell flattening or a prematurely compacted state [1]. Compaction of the mouse embryo, which normally takes place at the 8-cell stage, marks the beginning of differentiation during preimplantation development. This compaction process is important for the subsequent differentiation of 8-cell blastomeres into two cell types that eventually result in the formation of the trophectoderm and inner cell mass of the blastocyst [2–9]. During compaction, each blastomere becomes flattened and more tightly attached to adjacent cells due to the cell adhesion molecule E-cadherin [10, 11]. Although E-cadherin is phosphorylated just before compaction [12], the phosphorylation of E-cadherin does not occur in prematurely compacted four-cell embryos [13].

It has been reported in the mouse that activation of protein kinase C (PKC) directly or indirectly triggers premature compaction in four-cell stage embryos through effects on the cell adhesion molecule E-cadherin [14]. On the other hand, it is has also been demonstrated that serine/threonine kinase inhibitor 6-dimethylaminopurine (6-DMAP) induced cell flattening through effects on E-cadherin [13].

In the present study, the developmental competence of one-cell mouse embryos cultured with oviductal tissue or those cultured in phosphate-free medium was examined. In addition, the localization of E-cadherin of four-cell embryos developed in these two environments was examined.

Materials and Methods

Culture media

The media used were modified Whitten's medium (m-WM) [1] for embryos co-cultured with oviductal tissue and phosphate-free m-WM (P(−)m-WM) for embryos cultured without oviductal tissue, both containing 3 mg/ml polyvinylpyrrolidone (PVP; K-30, Nacalai tesque, Kyoto, Japan). P(−)m-WM was used for a control culture without an oviductal influence because 1-cell embryos cultured in m-WM can not overcome the 2-cell block *in vitro*. To make P(−)m-WM, KH_2PO_4 was replaced with KCl to avoid modifying the osmotic conditions. Oviducts for co-culture were flushed with m-MW containing 3 mg/ml PVP at the time of embryo collection and the isthmic and fimbrial regions of oviducts were removed. The ampullae were dissected

Received: February 7, 2001
Accepted: March 5, 2001
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longitudinally with micro-scissors and washed two times with m-MW containing PVP and then transferred to 100 μl of culture medium (m-WM containing 3 mg/ml PVP, 1 ampulla/100 μl).

Recovery and culture of embryos

Five- to eight-week-old randomly bred ICR mice were superovulated with intraperitoneal injections of 5-10 IU eCG (Teikoku Hormone M.F.G. Co., Ltd, Japan) followed in 48 h by 5-10 IU hCG (Sankyo Zoki Co., Ltd, Japan). Each female was mated overnight with an ICR male on the day of hCG injection. Twenty-four h after the hCG injection, successfully mated female mice were killed by ether anaesthesia and 1-cell embryos were collected by flushing them from the oviduct. One-cell embryos were stored in m-WM before treatment with nocodazole (Sigma Chemical Co., St. Louis, MO, USA). To synchronize the developmental stage at the 2-cell stage, embryos were cultured in m-WM containing 0.5 μg/ml nocodazole from 27 to 33 h after hCG. After release from nocodazole, 1-cell embryos cleaved to the 2-cell stage within 1.5 h. Synchronized embryos were cultured with oviductal tissue [1] in m-WM or cultured in P(−)m-WM and then used for examination of cell flattening and for immunocytological staining.

Examination of cell flattening in co-culture

To examine the effects of oviductal tissue on cell flattening, synchronized embryos were co-cultured with oviductal tissue for 34 h after release from nocodazole or cultured in P(−)m-WM (without oviductal tissue). The degree of blastomere flattening within each embryo was scored after the end of these culture conditions under a stereo microscope according to the criteria of Aghion *et al.* (1994). Three different categories were utilized: (i) when all blastomeres were rounded and clearly distinguishable, the embryo was considered to be non-flattened (Fig. 1a) and was given a score of zero; (ii) when individual blastomeres were not rounded and became flattened, the embryo was scored as flattened (Fig. 1b) and given a score of one; (iii) when individual blastomeres were indistinguishable, the embryo was scored as prematurely compacted (completely flattened; Fig. 1c) and given a score of two.

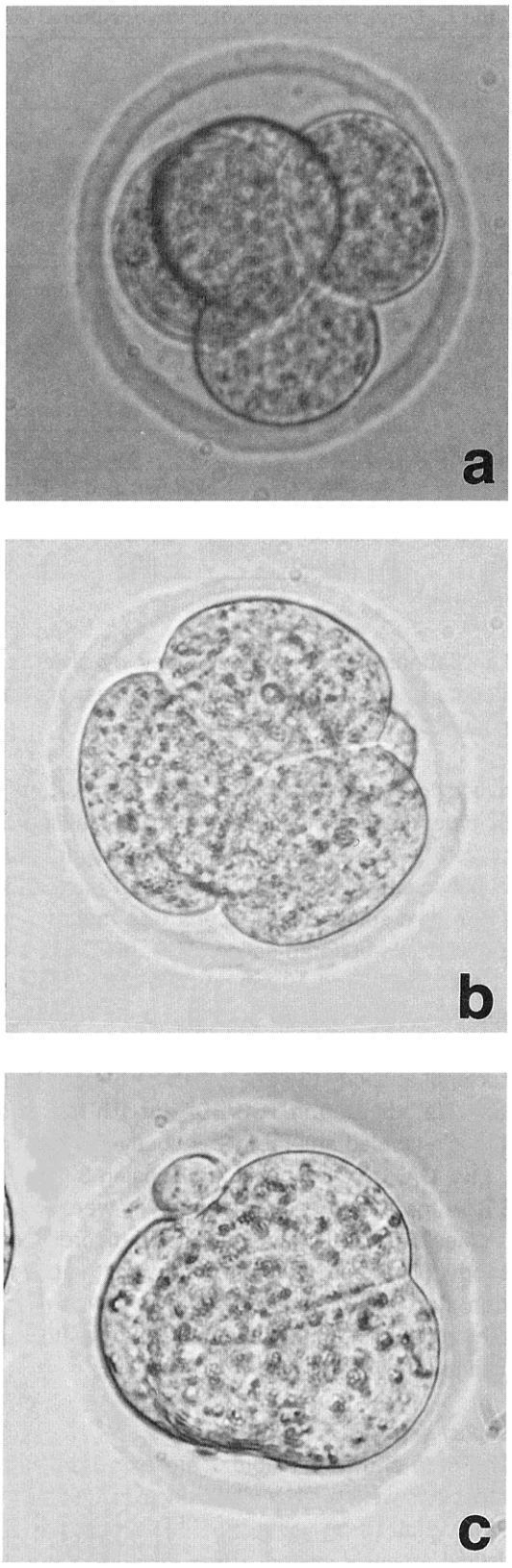

Fig. 1. Morphology of 4-cell embryos cultured in each environment for 34 h after release from nocodazole. A non-flattened embryo containing rounded and clearly distinguishable blastomeres (a) a embryo containing flattened blastomeres (b) a compacted embryo containing indistinguishable blastomeres (c).

Table 1. Development of 2-cell embryos cultured with or without phosphate after nocodazole treatment

Culture conditions	No. of embryos cultured	No. (%) of embryos developed 34 h after cleaving to 2-cell stage					No. (%) of blastocysts developed 82 h after cleaving to 2-cell stage
		2-cell	3-cell	4-cell	8-cell	Morula	
*P(−)N(−)	52	2 (3.8)	0 (0.0)	23 (44.2)	15 (28.8)	11 (21.1)	37 (71.2)[a]
P(−)N(+)	71	5 (7.0)	3 (4.2)	61 (84.7)	1 (1.4)	0 (0.0)	44 (62.0)[a]
P(+)N(+)	67	32 (47.8)	13 (19.4)	21 (31.3)	0 (0.0)	0 (0.0)	10 (14.9)[b]
P(+)N(+) co-culture	120	16 (13.3)	9 (7.5)	95 (79.2)	0 (0.0)	0 (0.0)	87 (72.5)[a]

*Developmental stages of embryos cultured without nocodazole were determined at 67 and 115 h after hCG. [a,b]; Different superscripts differ significantly ($P<0.05$).

Table 2. Flatterning index of 4-cell embryos cultured with or without an oviductal environment

Culture conditions	No. (%) of embryos after culture			Flattening index (No. of embryos)
	Non-flattened	Flattened	Prematurely compacted	
Co-culture	8 (8.4)	73 (76.8)	14 (14.7)	53.1 (95)
P(−) culture	58 (95.1)	3 (4.9)	0 (0.0)	2.5 (61)

The flattening index was obtained by dividing the total score by twice the number of embryos, and multiplying by 100 [13].

Immunocytological staining

Embryos used for immunocytological staining were recovered 34 h after release from nocodazole and the zona pellucidae were removed by brief incubation in acid Tyrode's solution [15]. Zona free embryos were rinsed with Tris-buffered saline (TBS, pH 7.4) containing 1 mM $CaCl_2$ and 10 mg/ml BSA (TBS/BSA), and incubated in 10% normal goat serum in TBS for 1 h. Immunological staining was done with anti-E-cadherin monoclonal antibody (ECCD-2, Takara Shuzo Co., Ltd, Japan) diluted 1/200 in TBS/BSA for 1 h followed by fluorescein-labeled anti-rat IgG antibody (EY Laboratories, Inc., CA, USA) for 1 h. After rinsing 3 times with TBS/BSA for 20 min each, the embryos were mounted on a glass slide and observed under a fluorescein microscope (Olympus Co., Ltd., Japan). The normal fixation step was eliminated because it was reported that the pattern of immunofluorescence was the same in fixed and unfixed cells [14].

Statistical analysis

The data shown in Table 1 are expressed as 0-1 variables and were analyzed by Duncan's multiple range test for variables by using the GLM procedure of the statistical analysis system (SAS Institute, Cary, NC). $P<0.05$ was considered statistically significant.

Results

Developmental competence of mouse embryos treated with nocodazole and cell flattening at the 4-cell stage

As shown in Table 1, the percentages of blastocysts that developed in phosphate-free medium and in co-culture were 62.0% and 72.5%, respectively, after synchronization by nocodazole treatment at the 1-cell stage. The percentage of the blastocysts that developed in phosphate-free medium without nocodazole treatment was 71.2% and there was no significant difference among treatments (Table 1). In addition, phosphate-free culture and co-culture with oviductal tissue were both effective for the further development of 2-cell embryos to the blastocyst stage (62.0%, 72.5% vs 14.9%). The percentages of the nocodazole-treated embryos cultured in phosphate-free medium and in an oviductal environment that reached the 4-cell stage were 84.7% and 79.2%, respectively, suggesting that the nocodazole treatment is effective in synchronizing the developmental stage of 1-cell embryos at the 4-cell stage.

The flattening index of embryos was determined according to the morphology of 4-cell embryo (Fig. 1). The index score of 4-cell embryos cultured in an oviductal environment was 53.1 and that of embryos cultured in phosphate-free medium (without an oviductal influence) was 2.5 (Table 2).

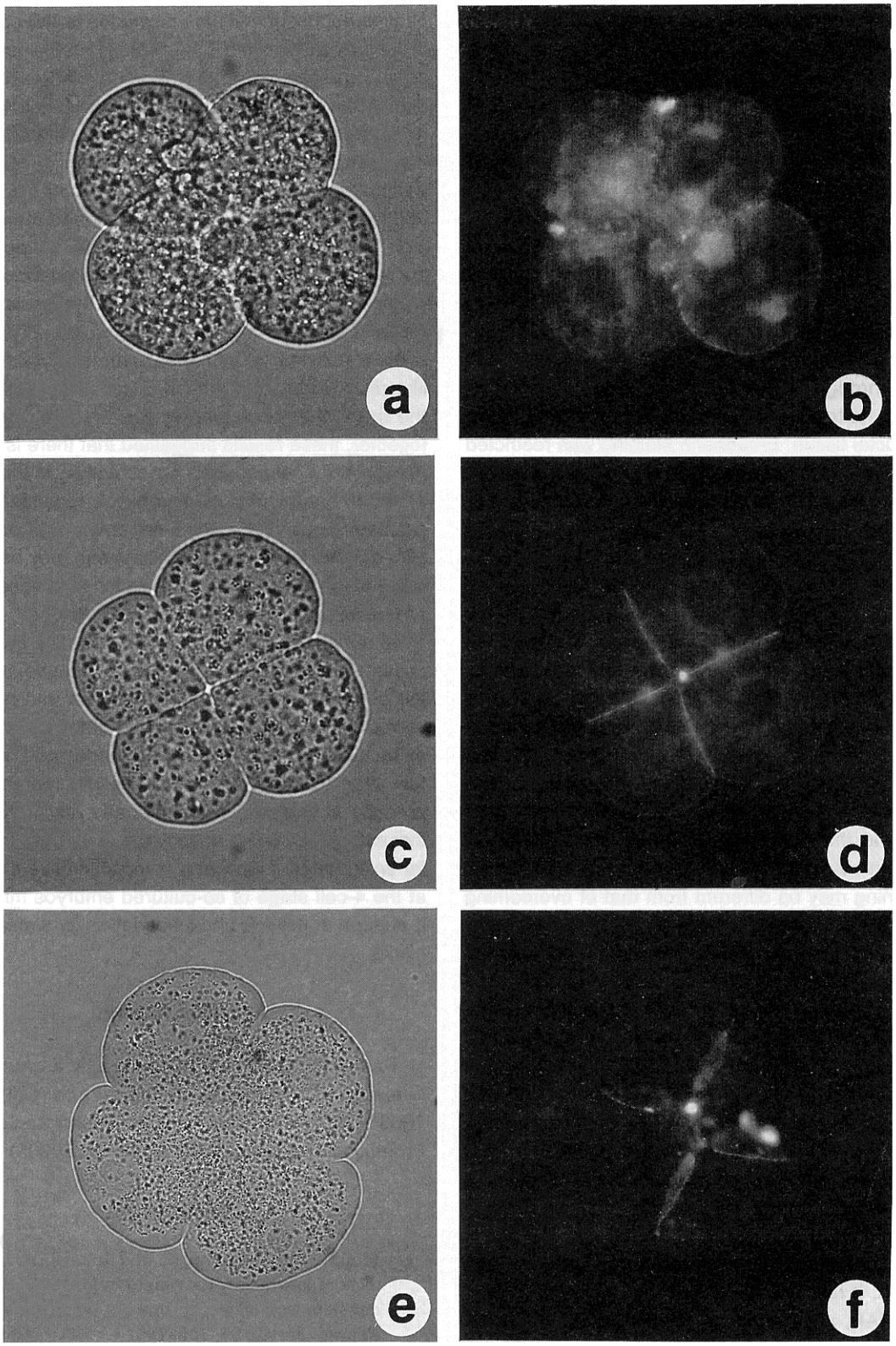

Fig. 2. E-cadherin localization in 4-cell embryos. Bright field (a, c, e) and fluorescence (b, d, f) photomicrographs of the same 4-cell embryos. Embryo developed in P(−)m-WM (a, b), embryo developed *in vivo* (c, d) and embryo developed in co-culture (e, f).

Cell flattening of embryos is associated with E-cadherin localization

To examine whether the oviduct-induced cell flattening was associated with the presence of E-cadherin (cell adhesion molecule) localization, 4-cell embryos developed under the influence of an oviduct were stained with anti-cadherin monoclonal antibody ECCD-2.

In control embryos developed in P(−)m-WM, E-cadherin was distributed evenly on the surface of blastomeres (Fig. 2a, b). In some 4-cell embryos that developed in vivo and that exhibited cell flattening to some extent, E-cadherin appeared to localize in the regions of cell-to-cell contact, but staining was still observed through the surface of each blastomere (Fig. 2c, d). In flattened embryos developed in co-culture with oviductal tissue, E-cadherin staining was restricted to the cell contact regions (Fig. 2e, f). This staining pattern was the same as that observed in compacted 8-cell embryos [8].

Discussion

Mouse 1-cell embryos cultured in an oviductal environment can develop normally into blastocysts without exhibiting a developmental block in vitro [1]. It has also been reported that mouse 1-cell embryos cultured in medium containing EDTA [16] or in medium without phosphate [17] develop to the blastocyst stage, but embryos developed in these media do not exhibit cell flattening at the 4-cell stage. From these data, the effect of an oviductal environment on the induction of cell flattening may be different from that of overcoming the developmental arrest *in vitro.*

It has been reported that the developmental arrest at early stages of in vitro development is overcome by culturing embryos in an oviductal environment [1, 18–22], but details of the mechanisms of release from this developmental arrest remain unclear.

It was recently reported that 4-cell embryos treated with PKC activators, PMA and DAG [14, 23] had premature cell flattening. These authors suggest that PKC plays a role in the start of compaction. PKC is activated by diacylglycerol, one of the products of receptor-mediated hydrolysis of membrane phospholipid, and sends the extracellular signal across the cell membrane [24]. In addition, it has been reported that embryos treated with 6-DMAP, an inhibitor of serine/threonine kinase, also have cell flattening and premature formation of gap junctions between blastomeres at the 4-cell stage [13]. The paradoxical effects of 6-DMAP and PMA are thought to be caused by the existence of complicated phosphorylation/dephosphorylation cascades in the compaction process. Bloom and McConnell (1990) indicate that some changes in protein phosphorylation associated specifically with passage through the 8-cell stage may be related to the cell flattening and polarization occurring at normal compaction [25]. During compaction, each blastomere becomes flattened and cell adhesion between adjacent cells increases due to the cell adhesion molecule, E-cadherin [10, 11]. The mechanisms involved in compaction are also post-translational, since inhibition of both RNA and protein synthesis does not prevent cell flattening or polarization [26, 27]. Our data show that oviductal influences can induce compaction-like morphology and induce E-cadherin localization to the cell adhesion region at the 4-cell stage. Taken together, these results suggested that there is phosphorylation and/or dephosphorylation activity in the oviductal environment *in vitro.* In general, localization of E-cadherin is observed in the 8-cell embryos at compaction, although the localization of E-cadherin may begin at the 4-cell stage because the localization was observed in *in vivo* developed 4-cell embryos (Fig. 2b).

In conclusion, it is suggested that the oviduct may activate or inactivate some protein kinases and control the cell adhesion of each blastomere that may be involved in the normal development of the embryo. Many extracellular signals such as hormones and growth factors bind to receptors in the plasma membrane and produce changes in protein kinase activity directly or indirectly [28]. Some unknown factors from the oviductal environment inducing a compaction-like morphology at the 4-cell stage of co-cultured embryos might cause a change in protein phosphorylation by similar mechanisms.

Acknowledgments

Part of this work was supported by a grant from the Ministry of Education, Science and Culture (No. 10660270) and a grant from the Japan Society for the Promotion of Science (JPS-RFTF 97L00905).

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