J. Mamm. Ova Res. Vol. 15, 17–21, 1998

Co-Culture of Rat Ova with Oviductal Cells in m199 FCS

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Abstract: *This experiment was designed to evaluate the ability of 4 different types of somatic cells to promote development of early cleavage in rat embryos. Embryos were collected from Wistar-Imamichi rats. Four and 8-cell rat embryos were co-cultured with rat granulosa, oviductal, uterine and kidney monolayer cells. The culture was performed in TCM 199 supplemented with 10% fetal calf serum, sodium lactate and pyruvate alone (m199FCS). Among the co-culture systems, morula and blastocyst development was better with granulosa, oviductal and uterine cells (98.6, 95.6 and 94.1%, respectively) than with m199FCS alone and kidney cells (77.6 and 73.7%, respectively). Similarly, 4-cell rat embryo development also followed the same trend and the development percentages with granulosa, oviductal, uterine, kidney cells and m199FCS alone were 32.0, 30.7, 29.3, 0 and 2.7%, respectively. Nevertheless, 8-cell embryos were developed to hatched blastocysts in co-culture with oviductal, uterine cells and in the conditioned medium of oviductal cells, 21.0, 19.0 and 2.1%, respectively. But co-culture with granulosa, oviductal and uterine cells represented the best physiologic approach and showed superiority to the other cell types. Forty-four of 184 1-cell embryos co-cultured with oviduct cells developed to the morula/blastocyst stage. The co-culture of early rat embryos in a medium with oviductal explants can support further development.*

Key words: *Rat embryo, Co-culture, Oviduct cell, Uterine cell, Hatching.*

Rat embryos at early cleavage stages do not readily develop beyond the 2-cell and 4-cell stages due to a developmental block under *in vitro* conditions [1], but 1-cell rat embryos pass through that block [2, 3] to blastocysts in HECM-1 when glucose and phosphate are removed from the culture medium. The ability to culture pre-implantation mammalian embryos *in vitro* by using simple chemically defined media [4, 5] has been achieved for many species. Attempts to improve existing culture systems by modifying compositional or physical parameters have failed to yield consistent results but, most of the attempts to optimize culture media in order to obtain both embryo cleavage rates and embryo viability that are comparable to those *in vitro* have been largely unsuccessful. The failure of *in vitro* culture systems to support early development may be alleviated in some species by co-culturing embryos with oviductal tissue. Co-culture of embryos with cells of the reproductive tract has been shown to improve embryo development [6–8].

The object of this study is to evaluate the effect of co-culture with granulosa, oviduct, uterine and kidney cells on the development of 1- and 8-cell rat embryos to morulae and blastocysts. In most of the cases hatching was also considered.

Received: August 28, 1997
Accepted: December 8, 1997

Materials and Methods

Animals and Embryo collection: Adult virgin female rats of Wistar-Imamichi strain weighing from 200 to 240 g were used. They were maintained under controlled light conditions [12 h light: 12 h darkness: light on at 6:00 h) and allowed free access to a pelted diet and water. Vaginal smears were taken in the morning for assessment of proestrus and graded. They were mated overnight with fertile males of the same strain. The day when spermatozoa were detected in the vaginal smear was considered day 1 of pregnancy. Tissue culture medium m199 FCS was supplemented with sodium lactate 2.253 g/l, sodium pyruvate 0.056 g/l and 10% fetal calf serum. Embryos were recovered by flushing the oviduct with m199 FCS culture medium from day 1 to day 4.

Collection and preparation of somatic cells: Granulosa cells were obtained from immature (28–29 day old) rats 48 h after a subcutaneous injection of 20 IU PMSG

(Peamex, Sankyo Co., Japan). The oviduct and uterus were trimmed free of ligaments and slit lengthwise on the side of the mesosalpinx. Cells from the oviduct and uterus were collected by lightly scraping the luminal surface with a scalpel. The cells were suspended in m199 FCS in a 4-well Multi-dish (Nunc, Denmark). The oviduct and uterine cells were cultured at 37.5°C in an atmosphere of 95% air and 5% CO_2. Kidney cells were collected by placing minced kidney medulla of immature rats (0.5 g) in 2.5 ml m199 FCS with 0.5 g/ml collagenase (Sigma, USA) and were then incubated for 18 h at 37°C with shaking. The cells in the suspension were washed and used to seed a 4-well Multi-dish. Kidney and granulosa cell monolayers were cultured under the same conditions as oviductal cells. In all cases, confluent monolayers were developed for 4 days to 70–80% confluence and the supernant was collected and used as conditioned medium.

Embryo culture: For each replicate experiment, embryos were randomly assigned to each treatment group from the pool of embryos collected. All incubations were carried out 48 h after the culture start. For co-culture the medium was the same, m199 FCS. All the culture media were supplemented in 20 IU/ml penicillin and 20 μg/ml streptomycin. Incubation was carried out as described before.

Experiment 1. Four- and 8 -cell embryos were collected and were cultured in m199FCS alone or co-cultured with a monolayer of granulosa, oviductal and uterine cells and in m199 FCS alone. Similarly, the 8-cell embryos were cultured in conditioned medium as above. Embryos were evaluated for development to the compact morula and blastocyst stage or hatched blastocyst.

Experiment 2. One-, 2-, 4- and 8-cell embryos were collected for this experiment and cultured in m199FCS alone and co-cultured with a monolayer of oviductal cells to test their further development.

Statistical analysis: Each experiment was repeated for 6 times and data were evaluated for statistically significant differences by χ^2-test.

Results

In vitro development of 8-cell rat embryos in the co-culture system and in condition medium are shown in Table 1. In co-culture with granulosa, oviductal, uterine and kidney cells, 8-cell rat embryos which developed to morula and blastocyst were 98.6, 95.6, 94.1 and 73.7%, respectively but in conditioned medium with the same type of somatic cells morula and blastocyst development percentages were 94.0, 92.7, 92.0 and 74.0%, respectively. Morula and blastocyst development with granulosa, oviduct and uterine cells was similar in both groups and higher ($p<0.001$) than that of m199 FCS alone and kidney cells.

Similarly, further development (blastocysts and hatched blastocysts) of 8-cell rat embryos, which were co-cultured with various somatic cells is shown in Table 2. There was a difference ($p<0.001$) in the subsequent embryo development between the 8-cell to blastocyst and hatched blastocyst stages with granulosa, oviduct and uterine cells and that of others (m199 FCS alone and kidney cells). In the conditioned medium, hatched blastocyst was lower than co-culture system.

In Table 3, *in vitro* development of 4-cell rat embryos in various culture systems is shown. Morula and blastocyst development percentages with granulosa, oviduct and uterine cells were 32.0, 30.7 and 29.3%, respectively, and these values were higher ($p<0.001$) than that of m199 FCS alone (2.7%) and kidney cells (0.7%).

Development of rat embryos when culture initiated in an early stage and there was co-culture with oviduct epithelium cells is shown in Table 4. The morula and

Table 1. *In vitro* development of 8-cell rat embryos in various culture systems

Treatments	Co-culture				Condition medium			
	No. of embryos	Development			No. of embryos	Development		
		Morula	Blastocysts	(%)		Morula	Blastocysts	(%)
m199FCS alone	210	16	147	77.6	150	11	105	77.3
Granulosa cells	218	1	214	98.6*	150	14	127	94.0*
Oviductal cells	205	2	194	95.6*	150	15	124	92.7*
Uterine cells	204	2	190	94.1*	150	18	120	92.0*
Kidney cells	209	24	130	73.7	150	13	98	74.0

* Differ significantly from the control (m199 alone) within the same column ($p<0.001$)
(%): Morula+Blastocyst.

blastocyst development from 1-, 2-, 4- and 8-cells were 23.9, 27.8, 29.7 and 94.2, respectively, and these values were higher ($p<0.05$) than those of their respective controls.

Discussion

Results of experiments reported here indicate that early *in vitro* development of rat embryos is facilitated by co-culture with rat oviductal epithelial cells.

The success of Gandolfi and Moor [6] with respect to the culture of sheep embryos with oviduct cells indicates the ability of these cells to support "normal" development *in vitro*. Transfer of these embryos following *in vitro* culture resulted in a high percentage of embryos continuing to develop *in vivo*. These results established this technique as superior to other successful culture techniques involving medium alone or co-culture with other cell types such as trophoblastic vesicles [9] or fibroblast monolayers [10].

Table 2. Further development from 8-cell to the blastocyst and hatched blastocyst after co-culture *in vitro* in each of the treatment groups

Treatments	No. of embryos	Development					
		Early blastocyst	(%)	Expanded blastocyst	(%)	Hatched blastocyst	(%)
m199FCS alone	210	133	(63.3)	14	(6.7)	0/14	(0)
Granulosa cells	218	1	(0.5)*	213	(97.7)*	50/213	(23.5)*
Oviductal cells	205	1	(0.5)*	193	(94.1)*	41/193	(21.2)*
Uterine cells	204	11	(5.4)*	179	(87.7)*	34/179	(19.0)*
Kidney cells	209	130	(62.2)	0	(0)	0/0	(0.0)
Condition medium of oviductal cells	215	2	(0.9)*	194	(90.2)*	4/194	(2.1)

* Differ significantly from the control (m199 FCS alone) within the same column ($p<0.001$).

Table 3. *In vitro* development of 4-cell rat embryos in various culture media

Treatments	No. of embryos	Development		
		Morula	Blastocyst	(%)
m199FCS alone	150	4	0	2.7
Granulosa cells	150	10	38	32.0*
Oviductal cells	150	9	37	30.7*
Uterine cells	150	8	36	29.3*
Kidney cells	150	1	0	0.7

* Differ significantly from the control (m199 FCS alone) ;$p<0.001$.

Table 4. Development of pre-implantation rat embryos in co-culture with oviductal tissue

Observation	Stage of development							
	1-cell		2-cell		4-cell		8-cell	
	control	co-culture	control	co-culture	control	co-culture	control	co-culture
No. of embryos	117	184	121	187	139	158	123	120
Compaction Morula [a]	0	25	1	21	4	9	7	10
Blastocyst [b]	0	19	0	32	0	38	87	103
Development a+b (%)	0	23.9*	0.8	28.3*	2.9	29.7*	76.4	94.2 *

*Differ significantly from their respective controls (m199 FCS alone) ; $p<0.05$.

The embryo development was slower if the culture was initiate in 1-, 2- or 4-cell stages than 8-cell stage. This may be due to blocked development. i.e., some embryos may have their development arrested. The *in vitro* block appears to be related to embryonic genome activation, increased DNA synthesis, and both qualitative and quantitative changes in amino acid uptake and morphological maturation of organelles [11]. The results of my present experiments indicated that oviduct cells provide factors necessary for cleavage of rat embryos through the 2- and 4-cell blocks. At best, the embryos developed in co-culture are equivalent in terms of morphologic appearance and ability to establish viable pregnancies similar to embryos developed *in vivo* [12–14]. Phosphate is responsible for cell block in rat [3]. Less embryo development was observed with kidney cells in co-culture. It is possible to speculate that all other somatic cells used in these experiments expect kidney cells may have the ability to change the negative function of phosphate in a co-culture system. There are some reports of the "toxic" effect of certain co-culture systems [15–17], but variability in the co-culture systems used, such as helper cell type, embryo species and culture conditions, make comparison of data between studies difficult, but some consistencies do emerge.

It is attributed to specialized functions of growth factor during the second part of preimplantation embryo development, such as supporting special nutritive functions; for example, transport of nutrients e.g. insulin [18–20], blastocyst expansion epidermal growth factor (EGF), transforming growth factor α (TGF α) [21], or cellular changes directed towards implantation. Of greatest potential importance to embryo culture are growth factor ligands that are expressed in reproductive tract tissues during the first few days of pregnancy and whose cognate receptors are present in preimplantation embryos. This situation suggests a possible paracrine relationship between the maternal tract and the embryo in which the maternal tissues act on the embryo.

One growth factor that is present in the female reproductive tract and whose target receptors are present on the embryo is EGF. EGF ligand has been localized to the apical membrane domain of uterine epithelial cells on day 4 of mouse pregnancy [22], but the presence of EGF receptor mRNA and protein has been demonstrated in mouse embryos in increasing quantities from the 4-cell to the blastocyst stage [23, 24]. Mouse embryos bind [^{125}I] EGF from the 8-cell stage onwards, and this binding can be inhibited by excess unlabelled EGF, as well as TGFα [25]. Biochemically functional receptors are indicated by the stimulation of tyrosine phosphorylation seen in response to incubation of embryo homogenates with EGF [26].

Most of the available evidence indicates that EGF receptors in the blastocyst are confined mainly to the trophectoderm [23, 25], although they have also recently been found in the inner cell mass [24]. The juxtaposed relationship of the EGF receptor in the trophoectoderm and EGF ligand on the apical domain of the uterine epithelial cells is suggestive of a paracrine role for EGF in utero. Functional assays implicate trophoectoderm as the major target of EGF (or TGF α) action. The overall hatching rate was low in conditioned medium compared to the co-culture system observed in the present experiment. This suggests that the co-culture system may have the ability to produce EGF. EGF stimulates mouse blastocyst outgrowth with increased numbers of trophoblast giant cells [27]. Other responses to EGF *in vitro* include more rapid accumulation of blastocoel fluid [28], higher percentages of embryos hatching [25] and an increase in the change of [^{3}H] leucine into protein of the trophoectoderm but not ICM [29].

This study indicted that a range of embryo-cell co-culture systems could be used to overcome cleavage blocks *in vitro*. Within a relatively short time embryo co-culture has become a routine method of embryo support *in vitro*.

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