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Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells

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Summary. To examine the effects of somatic cell support on the cleavage and viability of fertilized sheep eggs, 434 pronucleate eggs were co-cultured for 3 or 6 days on oviduct cells or fibroblasts and 77 eggs were cultured in medium alone. During the first 3 days in culture 95% of the single-celled eggs cleaved regularly to non-compacted morulae on either of the feeder-layers but only 13% underwent similar regular cleavage in medium alone. Despite the identical cleavage rates in the co-culture groups, only 33% of embryos grown on fibroblasts as compared with 80% of embryos grown on oviduct cells were fully viable as judged by their ability to develop normally after transfer to recipient animals. The viability of embryos in the oviduct group was equal to that obtained after the direct transfer of morulae from donor to recipient sheep.

After 6 days in culture 42% of embryos co-cultured with oviduct cells developed into expanded blastocysts as compared with only 4·5% cultured on fibroblasts. In both co-culture groups virtually all the remaining embryos blocked during the 4th cleavage. When transferred, 30% of blastocysts grown from the pronucleate stage on oviduct cells were viable.

We conclude that: (1) during the first 3 days after fertilization cleavage will progress at a normal rate on different feeder-layers but oviduct cells appear to be required for the acquisition of full embryonic viability; (2) in our system oviduct cells are able to support passage of embryos through the critical 4th cell cycle, whilst fibroblasts are almost entirely unable to support this critical phase; and (3) it is apparent that the factors necessary for the morphological formation of the blastocyst may be insufficient or different from those which endow it with subsequent developmental ability.

Introduction

The culture of mammalian embryos from one-cell to the expanded blastocyst stage is an uncertain process. This is particularly true of domestic animals for which only a small minority of long-term cultured embryos cleave regularly and even this minority almost invariably fail to develop further when transferred to recipient animals (reviewed by Wright & Bondioli, 1981). In the absence of reliable culture methods, study of the basic embryological processes and the manipulation of embryos by genetic engineering were severely curtailed.

At present newly fertilized eggs of farm animals cleave regularly only if transferred to the oviduct of a foster mother of the same or of an appropriate different species (Boland, 1984). On the basis of these observations we tested the hypothesis that the presence of oviduct epithelial cells in a culture system would provide an environment suitable for the regular in-vitro development of fertilized sheep eggs. The hypothesis is further strengthened by findings that specific glycoproteins, secreted by the oviduct, may play a role during fertilization and cleavage (Brown & Cheng, 1986; Kapur & Johnson, 1986). However, various other non-oviduct feeder layers have already been shown (Kuzan & Wright, 1982a, b) to facilitate the development of late morulae and blastocysts in

culture, and the cleavage of early embryos was stimulated by adding trophoblastic vesicles to the culture medium (Heyman *et al.*, 1987). It has therefore been necessary to examine the specificity of somatic cell support by comparing embryonic development on feeder layers of oviduct and non-oviduct origin.

Materials and Methods

Superovulation and collection of embryos. Welsh mountain ewes were injected with 1000 i.u. PMSG (Folligon: Intervet Laboratories Ltd, Cambridge, U.K.) on Day 10–12 of the oestrous cycle, followed after 48 h by 125 μg cloprostenol (Estrumate: ICI, Macclesfield, U.K.) and 24 h later by 0·004 μg buserelin, a gonadotrophin-releasing hormone analogue (Receptal: Hoechst U.K. Ltd, Milton Keynes, U.K.). The ewes were mated immediately after the final injection and 40 h later the embryos were flushed from the oviducts using Dulbecco's phosphate-buffered saline solution supplemented with 10% (v/v) newborn calf serum (Sera-Lab Ltd, Crawley Down, U.K.).

Source and culture of cells. Cells dislodged during the flushing of the oviducts for egg recovery provided a convenient source of epithelial cells for culture. The eggs were removed immediately after flushing and the somatic cells and medium were transferred to a conical tube and allowed to sediment for 10 min. Thereafter, the flushing medium was removed and replaced with TCM 199 with Earle's salts (Gibco Europe Ltd, Paisley, U.K.) supplemented with 10% fetal calf serum (FCS: Sera-Lab Ltd). Cells flushed from a single oviduct and resuspended in 2 ml of medium were sufficient for plating into a 4-well dish (Nunc, Gibco Europe Ltd). This technique of preparing oviduct cell cultures was simpler than that of collagenase digestion and provided superior cells with more functional cilia and without the vacuoles observed after enzymic treatment.

The fibroblast monolayers used in all the experiments originated from a cell line established in our laboratory from a primary culture of fetal sheep fibroblasts. Fibroblasts and epithelial cells were cultured at 39°C in a humidified atmosphere of 5% CO_2 in air.

The medium was changed every 3 days and the cells were used for co-culture when they had reached about 70% confluency, i.e. 3–4 days after explanation for the epithelial cells.

Culture of embryos. After removal from the flushing medium the one-celled eggs were rinsed once in TCM 199 supplemented with 10% FCS and then allocated to one of the two feeder-layer groups or cultured in medium only (control group). All cultures were maintained at 39°C in a humidified atmosphere of 5% CO_2 in air. Embryos were cultured for 3 days on their original feeder-layers. Some of the embryos were harvested at the end of this period (3-day group) and assessed as outlined below. The remaining embryos were transferred to new monolayers at 70% of confluence and cultured for a further 3 days (6-day groups). In addition, a control group of one-celled eggs was maintained under the same conditions as the co-culture groups except they were cultured without feeder-layer support.

Assessment of morphological development and viability. Embryos were assessed daily for cleavage by using an inverted microscope in a 37°C culture chamber. Eggs that did not cleave after the first 24 h in culture were fixed and stained to ascertain whether fertilization had occurred. Those that were not fertilized were excluded from the results whilst the remaining few penetrated but uncleaved eggs in this group were classified as retarded (Table 1: 1–7 blastomere group). All the eggs except the uncleaved ones were cultured for 3 or 6 days and at the end of the designated culture period some embryos were fixed, stained with lacmoid and the number of nuclei determined. The remaining embryos were transferred to recipients whose oestrous cycles were exactly synchronized with those of the original donor sheep. The recipients were allowed to go to term or slaughtered on the 13th day of pregnancy and the number of elongated conceptuses was determined.

Results

Control embryo cultures

Of the 77 embryos cultured in the absence of somatic cell support only 10 cleaved regularly reaching the 8–16-cell stage in 3 days (see Table 1). The remaining embryos cleaved irregularly and presented abnormal blastomeres or signs of fragmentation.

In view of the degenerate state of most of the embryos after culture no control embryos were maintained *in vitro* for the full 6-day culture period. Similarly, no transfers were performed on the basis of contemporary negative results obtained with embryos cultured in similar conditions by others in the laboratory (Y. Fukui & A. Glew, unpublished observations).

Embryos co-cultured with oviduct epithelial cells

Culture for 3 days. A total of 107 one-celled eggs was cultured in this group and, after 72 h *in vitro*, 104 (97%) had cleaved regularly to the early morula stage. Of these 104 embryos 44 were

Table 1. Effect of different feeder-layers on the cleavage of sheep 1-cell embryos cultured for 3 or 6 days

Co-culture with:	Length of culture (days)	No. of embryos*	Cleavage stage† after culture (%)			
			1–7 cells	8–16 cells	>16 cells	Blastocyst
Oviduct cells	3	63[a]	3 (4·8)	54 (85·7)	6 (9·5)	—
Sheep fibroblasts	3	42[a]	2 (4·8)	39 (92·8)	1 (2·4)	—
No cells	3	77[b]	67 (87)	10 (13)	0	—
Oviduct cells	6	72[c]	7 (9·7)	28 (39)	4 (5·5)	33 (45·8)
Sheep fibroblasts	6	67[d]	5 (7·4)	56 (83·6)	3 (4·5)	3 (4·5)

*The development of the groups with different superscripts is statistically different: $P < 0{\cdot}05$ (χ^2 analysis).
†The number of nuclei was detected by lacmoid staining.

Table 2. Effect of different feeder-layers and of length of culture on the viability of sheep zygotes

Co-cultured with:	Length of culture (days)	Total no. of embryos cultured*	No. of morphologically normal embryos transferred	Recovered at 13th day of pregnancy	Developed (%)
Oviduct cells	3	44[a]	44	35	28 (80)
Sheep fibroblasts	3	68[b]	65	33	11 (33·3)
Oviduct cells	6	78[b]	30	—†	9 (30)

*The viability of groups with different superscripts differ statistically: $P < 0{\cdot}05$ (χ^2 analysis).
†The pregnancies went to term.

transferred to the uteri of closely synchronized recipient ewes: 35 were recovered on Day 13 of gestation and 28 (80%) had developed normally to the elongated blastodermic vesicle stage whilst the remaining 7 had ceased development immediately after transfer or at the early blastocyst stage (Table 2).

The high rate of normal development after culture compares favourably with that obtained by transferring freshly collected embryos directly from the donor to a recipient sheep (Moore, 1982).

Culture for 6 days. After culture for 6 days, 63 embryos out of 150 (42%) had developed to the expanded blastocyst stage, 80 (53%) were arrested at the early morula stage and 7 (5%) had cleaved irregularly or degenerated (see Table 1).

Embryos which progressed through the critical 4th cell cycle invariably developed to the expanded blastocyst stage. Indeed, a few blastocysts were maintained to the 10th day in culture and appeared morphologically similar to 10-day blastocysts obtained directly from donor animals.

A total of 30 expanded blastocysts was transferred after the 6 days culture period to 17 recipient ewes allowed to go to term: 5 remained pregnant and 9 lambs were born (30%).

Embryos co-cultured with fibroblasts

Culture for 3 days. Fertilized eggs were cultured on ovine fibroblast monolayers to determine whether the positive effect of the oviduct cells on embryonic development was specific to those cells or was of a more generalized feeder-layer nature. After 3 days of culture 105 of 110 embryos (95%) in the fibroblast group had developed into early morulae. This rate of cleavage was similar to that of embryos cultured for 3 days on epithelial cell monolayers. However, the transfer of 65 of these embryos showed that their viability had been seriously impaired and only 33% developed into normal 13-day conceptuses (Table 2).

Culture for 6 days. During the second 3-day culture period differences between the support provided by the fibroblasts and oviduct cells were observed not only in viability but also in the rate of cleavage.

Only 3 out of 67 fertilized eggs (4·5%) progressed from the morula to the blastocyst stage after 6 days of culture on fibroblast feeder-layer. The low rate of progression is in marked contrast to that on epithelial cells with which nearly 10 times as many morulae underwent blastulation (63/150 or 42%).

Discussion

The presence of somatic cells increases the rate of cleavage and development of sheep zygotes in culture to levels well in advance of those achieved previously (Tervit *et al.*, 1972; Tervit & Rowson, 1974; Wright & Bondioli, 1981). However, the effectiveness of the support provided by the somatic elements depends both upon the type of feeder cells used and the developmental stage of the embryo.

For the first 3 days cleavage is supported equally by oviduct cells and fibroblasts. However, a marked difference in viability clearly emerged even in this relatively short period in culture. On one hand these differences emphasize how morphological criteria alone are totally inadequate to assess the results of an embryo culture system. On the other hand the results show that oviduct epithelium exerts a specific action even in culture, combining the stimulation of cleavage with the preservation of viability. Although fibroblasts and trophoblastic vesicles (Heyman *et al.*, 1987) can support the cleavage of fertilized eggs, in no case other than with oviduct cells is it possible to avoid a sharp drop in viability *in vitro*. The oviduct cell culture system can therefore be used with confidence in a variety of experiments including those designed to study the mechanisms underlying early embryogenesis and those involving genetic manipulation and nuclear transplantation.

Two interesting aspects of the relationship between somatic cells and embryonic development became apparent when the culture period was extended from 3 to 6 days. Firstly, the results show that somatic cells differ in their ability to support embryonic development beyond the non-compacted morula stage: oviduct cells support further development whilst fibroblasts do not. Secondly, it is clear that the total support provided by oviduct cells during the first 3 days of cleavage becomes incomplete thereafter and developmental abnormalities occur during compaction and blastulation. Indeed, we suggest that the development of the sheep embryo from the non-compacted morula to the expanded blastocyst involves two further critical phases, each with its own specific requirements. The first of these critical phases occurs in the 4th cell cycle and is probably analogous to the so-called 2-cell block in the mouse and gerbil (Goddard & Pratt, 1983; Norris *et al.*, 1985) and the 4-cell block in the pig (Polge, 1982; Davis, 1985). In each of these species this point of developmental restriction in culture corresponds closely with the stage at which the embryonic genome is activated and the first transcription occurs. Our culture results strongly suggest that specific factors from oviduct cells facilitate the passage of sheep embryos through this restriction phase. However, since fewer than 50% of embryos in our culture system overcame the 8- to 16-cell block it is clear that the requirements for transition to the 5th cell cycle are not yet

being adequately met *in vitro*. Further work on the role of the maternal environment in supporting passage through the transition phase is required, especially since oviduct explants can support the development of mouse embryos across the 2-cell block (Whittingham & Biggers, 1967).

Once the sheep embryo has overcome the restriction associated with the 4th cell cycle its development in our culture system to the expanded blastocyst stage is virtually assured. Indeed the results of Tervit & Gould (1978) demonstrate that somatic cell support is not required for the successful culture of sheep embryos removed from the reproductive tract at the 16- to 32-cell stage. The second critical step is therefore not related to the morphological development of the blastocyst but rather to the maintenance of its subsequent developmental viability. This problem is clearly highlighted in cultures of rabbit and mouse embryos. In both these species the rate of blastocyst formation is very high and exceeds that in our experiments (Kane, 1975; Tarkowski, 1977). However, the viability of blastocysts grown in culture from the most favourable inbred mouse strains seldom reaches 30% and is only 6% in the rabbit (Maurer, 1978; Papaioannou & Ebert, 1986). This developmental failure may reflect an inadequate or partial genomic activation during the transition phase. Equally it could be due to the requirements for subtle uterine signals analogous to those known to influence embryonic development in sheep between Days 9 and 12 (Wilmut & Sales, 1981; Godkin *et al.*, 1984).

It is to distinguish between these various hypotheses, and to investigate at a molecular level the interaction between the embryo and cells of the reproductive tract, that our work is now directed.

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References

Boland, M.P. (1984) Use of a rabbit oviduct as a screening tool for the viability of mammalian eggs. *Theriogenology* **21,** 126–137.

Brown, C.R. & Cheng, W.K.T. (1986) Changes in composition of the porcine zona pellucida during development of the oocyte to the 2- to 4-cell embryo. *J. Embryol. exp. Morph.* **77,** 411–417.

Davis, D.L. (1985) Culture and storage of pig embryos. *J. Reprod. Fert., Suppl.* **33,** 115–124.

Goddard, M.J. & Pratt, H.P.M. (1983) Control events during early cleavage of mouse embryo: an analysis of the "2-cell block". *J. Embryol. exp. Morph.* **73,** 111–133.

Godkin, J.D., Bazer, F.W. & Roberts, R.M. (1984) Ovine trophoblast protein 1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis. *Endocrinology* **114,** 120–131.

Heyman, Y., Menezo, Y., Chesne, P., Camous, S. & Garnier, V. (1987) In-vitro cleavage of bovine and ovine early embryos: improved development using coculture with trophoblastic vesicles. *Theriogenology* **27,** 59–68.

Kane, M.T. (1975) Bicarbonate requirements for culture of one-cell rabbit ova and blastocysts. *Biol. Reprod.* **12,** 552–555.

Kapur, R.P. & Johnson, L.V. (1986) Selective sequestration of an oviductal fluid glycoprotein in the perivitelline space of mouse oocyte and embryos. *J. exp. Zool.* **238,** 249–260.

Kuzan, F.B. & Wright, R.W. (1982a) Blastocyst expansion, hatching and attachment of porcine embryos cocultured with bovine fibroblasts *in vitro*. *Anim. Reprod. Sci.* **5,** 57–63.

Kuzan, F.B. & Wright, R.W. (1982b) Observation on the development of bovine morulae on various cellular and acellular substrata. *J. Anim. Sci.* **54,** 811–816.

Maurer, R.R. (1978) Advances in rabbit embryo culture. In *Methods in Mammalian Reproduction*, pp. 259–272. Ed. J. C. Daniels, Jr. Academic Press, New York.

Moore, N.W. (1982) Egg transfer in sheep and goat. In *Mammalian Egg Transfer*, pp. 119–133. Ed. C. E. Adams. CRC Press, Boca Raton.

Norris, M.L., Barton, S.C. & Surani, M.A.H. (1985) Changes in protein synthesis during early cleavage of the Mongolian Gerbil embryo. *J. exp. Zool.* **236,** 149–153.

Papaioannou, V.E. & Ebert, K.M. (1986) Development of fertilized embryos transferred to oviducts of immature mice. *J. Reprod. Fert.* **76,** 603–608.

Polge, C. (1982) Embryo transplantation and preservation. In *Control of Pig Reproduction*, pp. 277–291. Eds D. J. A. Cole & G. R. Foxcroft. Butterworths, London.

Tarkowski, A.K. (1977) In vitro development of haploid mouse embryos produced by bisection of one-cell fertilized eggs. *J. Embryol. exp. Morph.* **38,** 187–202.

Tervit, H.R. & Gould, P.G. (1978) Culture of sheep embryos in either a bicarbonate buffered medium or a phosphate buffered medium enriched with serum. *Theriogenology* **9,** 251–255.

Tervit, H.R. & Rowson, L.E.A. (1974) Birth of lambs after culture of sheep ova *in vitro* for up to 6 days. *J. Reprod. Fert.* **38,** 177–179.

Tervit, H.R., Whittingham, D.G. & Rowson, L.E.A. (1972) Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fert.* **30,** 493–497.

Whittingham, D.G. & Biggers, J.D. (1967) Fallopian tube and early cleavage in the mouse. *Nature, Lond.* **213,** 942–943.

Wilmut, I. & Sales, D.I. (1981) Effect of an asynchronous environment on embryonic development in sheep. *J. Reprod. Fert.* **61,** 179–184.

Wright, R.W. & Bondioli, K.R. (1981) Aspects of in vitro fertilization and embryo culture in domestic animals. *J. Anim. Sci.* **53,** 702–729.

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