DERIVATION OF PLURIPOTENT, EMBRYONIC CELL LINES FROM PORCINE AND OVINE BLASTOCYSTS

Elena Notarianni[1], Cesare Galli[2], Stuart Laurie[2], Robert M. Moor[2] and Martin J. Evans[1]
[1]Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK
[2]Institute of Animal Physiology and Genetics Research, Department of Molecular Embryology, Babraham, Cambridge CB2 4AT, UK

ABSTRACT

We have described conditions which are sufficient for the establishment of stable, pluripotent cell lines from porcine and ovine blastocysts. The general morphologies and growth characteristics of the porcine and ovine cells in culture are very similar. Furthermore, the porcine and ovine cells bear close resemblance to cells in primary outgrowths from inner cell masses, and display features which are characteristic of murine embryonic stem cells: namely, large, translucent nuclei with many prominent nucleoli, and relatively sparse cytoplasm. These observations, together with the capacity of the cells to differentiate spontaneously in culture into a range of discernible phenotypes, support the contention that the porcine and ovine cell lines represent primary ectodermal lineages. Differences in the rates and patterns of growth of ungulate compared with murine embryonic stem cells suggest that the identification and isolation of pluripotential cell lines is the same for ungulate, but different for murine species.

INTRODUCTION

Methods for the derivation of embryonic stem (ES) cells from the mouse (Evans and Kaufman, 1981; Martin, 1981) and hamster (Doetschman *et al.*, 1988), and techniques exploiting their potential (Hooper *et al.*, 1987; Kuehn *et al.*, 1987), are well established. The isolation of analogous cells from embryos of the major domestic species would be of great value (Smith and Wilmut, 1989). However, it could be predicted that the derivation of such cells from ungulate embryos would require modification of the methods formulated for the murine species due to differences in their development. Development of the inner cell mass, in particular, differs significantly between murine and ungulate species; in murine embryos proliferation of the inner cell mass occurs rapidly, whereas in ungulate embryos the inner cell mass forms a mitotically quiescent embryonic disc. This suggests that the growth characteristics and morphology of embryonic stem cells from ungulates may differ from those of mice. A problem which might beset the derivation of ovine pluripotential cells, in particular, is to obtain a population of stem cells not yet influenced by differentiated products; delamination of the endoderm occurs by the time of hatching of the blastocyst (Handyside *et al.*, 1987), and the presence of endoderm prevents the proliferation of undifferentiated stem cells (Robertson, 1987). (The inner cell masses may be isolated from hatched murine or porcine blastocysts prior to commencement of delamination of primary endoderm). We therefore attempted to isolate ovine embryonic cell lines not only by explantation of whole embryos into culture followed by rapid selection of stem-cell-like colonies, but also by immunosurgical isolation of inner cell masses.

METHODS

Porcine blastocysts were recovered by retrograde flushing from non-superovulated Large British White gilts at 7-9d post oestrus. Either intact blastocysts or mechanically-dissected inner cell masses were explanted into culture.

Welsh Mountain ewes were induced to superovulate by injection with 1200 units of PMSG on the twelfth day of the oestrus cycle, and mated with two rams at the onset of oestrus (day 0). Blastocysts, typically 3 or 4 per animal, were collected surgically by retrograde flushing of the uterine horns at 7 to 9d post-oestrus. The recovered blastocysts (totalling some several hundred) were then either explanted into culture, or subjected to immunosurgery in order to isolate the inner cell masses. Immunosurgical preparation of pure, ovine inner cell masses was performed according to Solter and Knowles (1975). Antiserum was raised in rabbits by two injections of ovine trophectodermal tissue which was dissected from 14-18d embryos. This antiserum was incubated at 56°C for 30 min to inactivate complement. Blastocysts were first mechanically dissected into inner cell masses, which then were exposed to the rabbit anti-trophectodermal antiserum (1:5 dilution in serum-free medium) for 30 min, washed in complete medium, and exposed to guinea-pig complement (1:10 dilution) for 30 min. Residual, attached endodermal and trophectodermal cells were killed by this procedure, and could be removed by mechanical disaggregation.

The culture of intact blastocysts or isolated inner cell masses, either porcine or ovine, was on mitotically-inactivated STO fibroblasts, using gelatinised tissue-culture dishes. Medium for the culture of intact porcine or ovine embryos, or their derived cell lines, was Dulbecco's modified DMEM supplemented with 10% of newborn and 5% foetal calf serum, and with 0.1 mM 2-mercaptoethanol. Both newborn and foetal calf sera were heat inactivated at 56° for 30 minutes. Neither conditioned medium nor exogenous growth factors were added. Derivative porcine or ovine cells from outgrowths of the inner cell masses were isolated by trypsinisation using 0.25% (w/v) trypsin in 0.04% (w/v) EDTA, and passaged onto fresh, inactivated STO cells using the above medium.

RESULTS

The morphology and growth characteristics of our porcine cell lines has been described (Notarianni *et al.*, in press). A typical colony and a confluent monolayer of such cells are shown in Figures 1 and 2, respectively. The cells are epithelioid with large, translucent nuclei, several prominent nucleoli and having relatively little cytoplasm - features which are characteristic also of murine ES- or EK-cells (Evans and Kaufman, 1981; Martin, 1981).

Figure 3 shows an immunosurgically-isolated, ovine inner cell mass which was explanted into culture. Outgrowths were isolated more frequently from isolated inner cell masses than from intact ovine blastocysts (at an efficiency of around 80% for 7-9d embryos), but failed to survive more than a few passages in culture. Few ovine embryos which attached gave rise to outgrowths from the inner cell masses. However, from one hatched, eight-day-old ovine embryo which had been explanted intact, a primary outgrowth was recognised as consisting of stem-like cells, and this outgrowth was collected and passaged. Eventually a stable culture was derived which was free from trophectodermal material. This culture has formed a cell line,

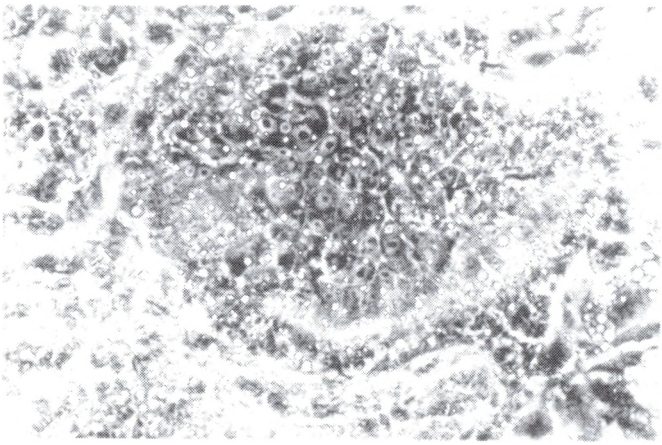

Figure 1 Colony of pluripotential, porcine cells cultured on mitotically-inactivated STO fibroblasts. Magnification x250.

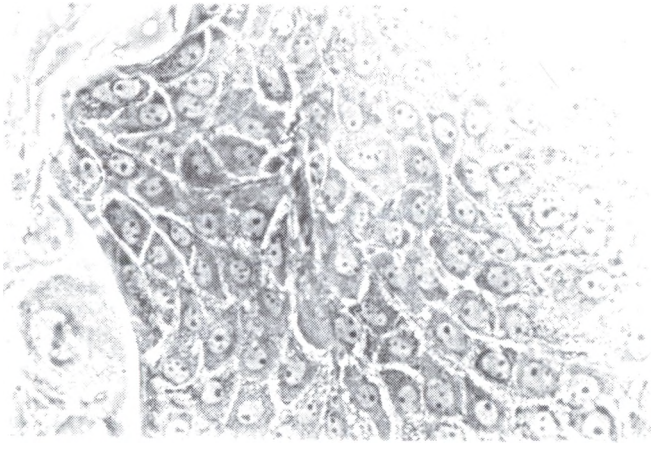

Figure 2 Confluent monolayer of porcine cells. Magnification x400.

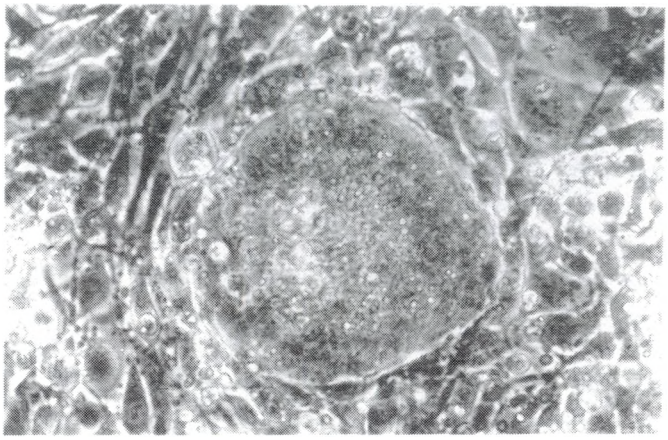

Figure 3 Ovine inner cell mass following attachment to monolayer of mitotically-inactivated STO cells. Magnification x100.

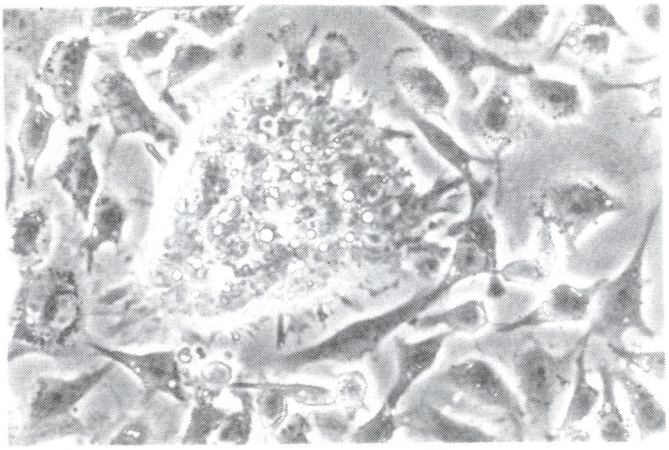

Figure 4 Colony of cells of the ovine line derived from an intact 8d blastocyst. Magnification x250.

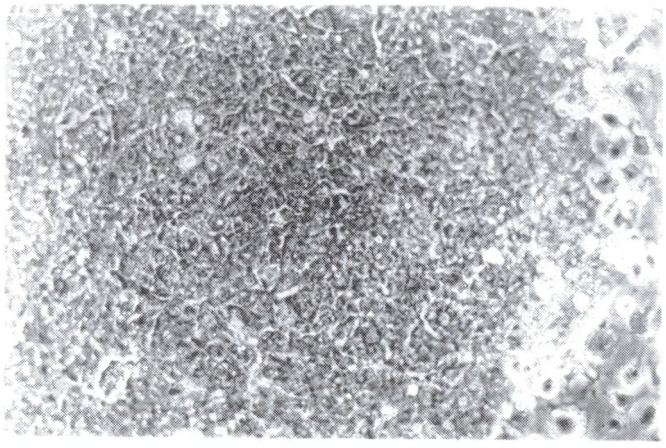

Figure 5 Culture of the ovine cell line at a high density. Magnification x100.

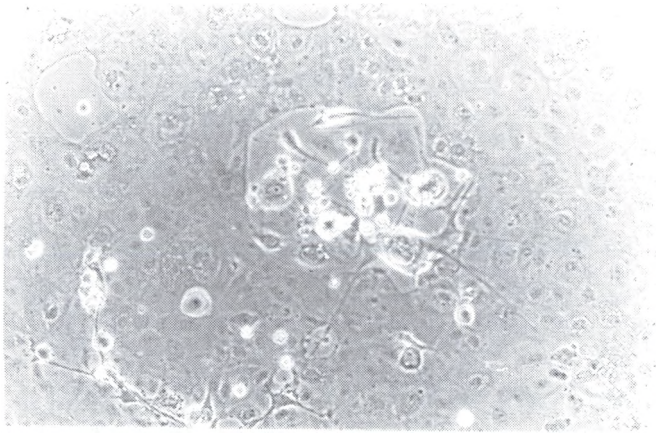

Figure 6 Porcine cell line showing spontaneous differentiation into cells of diverse morphologies. Magnification x250.

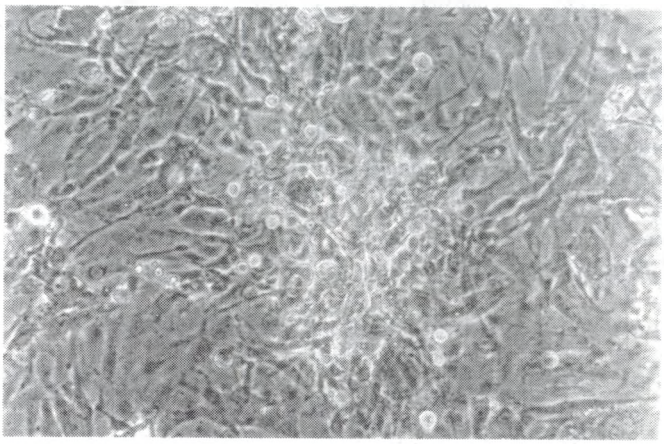

Figure 7 Ovine cell line showing spontaneous differentiation. Magnification x250.

maintaining a stable morphology (Fig. 4) closely resembling that of inner-cell-mass cells in culture (Fig. 3) as well as that of our porcine cell lines (Figs. 1 and 2): the cells are epithelial in character, and have a large nuclear to cytoplasmic ratio. The ovine cells are approximately 25um in diameter, whereas porcine and murine cells are 35-50um and 18um, respectively. Like the porcine cells, the ovine cells grow in flattened colonies, which eventually spread to form monolayers (Fig. 5). The ovine cells show a similar rate of growth to the porcine cells, and are passaged at 3 day intervals or before attaining confluency. Porcine (Fig. 6) and ovine (Fig. 7) cells spontaneously differentiate on reaching confluency into a range of morphological types, including endodermal, fibroblastic-, nerve- and muscle-like cells.

DISCUSSION

We have described the isolation of pluripotential embryonic cells from the pig and sheep, which may serve as a general method for the identification and isolation of embryonic cells from ungulate embryos.

Unlike EK cells, our porcine and ovine embryonic cells do not show a tendency to form multilayered colonies in our culture conditions, but grow in distinctive epithelioid colonies which spread to form monolayers. Cells in either case have large, clear nuclei, several prominent nucleoli and relatively little cytoplasm. The ovine cells closely resemble cells in primary cultures of inner cell masses, consistent with their having been derived from a primary ectodermal lineage.

That the porcine and ovine cells demonstrate a slower rate of division than EK cells may reflect the formation of a relatively quiescent embryonic disc by the inner cell mass in the ungulate embryo prior to implantation, compared with the rapid proliferation of cells in the inner cell mass of the murine embryo at this stage. The potencies of our cell lines are being tested by their reconstitution into embryos by blastocyst injections and nuclear transfer.

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