

## An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo

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### Summary

Embryonic stem cells (ES) cells were injected into host blastocysts either in groups of 10–15 cells or as single cells in order to test their developmental potential in the developing embryo. The analysis of midgestation chimaeras, by electrophoretic separation of glucose phosphate isomerase (GPI) isozymes, showed that ES cells

were capable of colonizing trophectoderm and primitive endoderm derivatives at a low frequency, as well as producing a high rate of chimaerism in tissues of the fetus and extraembryonic mesoderm.

Key words: embryonic stem cells, mouse embryo, chimaera.

### Introduction

Embryonic stem cells (ES cells) have proved to be extremely efficient at forming chimaeras and colonizing the germ line following injection into the blastocyst (Bradley *et al.* 1984). The consistent widespread chimaerism in liveborn mice and the absence of tumours in adult ES cell chimaeras, unlike those obtained using a variety of embryonal carcinoma (EC) cell lines (Papaioannou & Rossant, 1983), is directly comparable to the behaviour of normal embryonic cells introduced into the blastocyst or aggregated with cleavage-stage embryos (Robertson & Bradley, 1986). However, characterization of chimaeras after birth cannot reveal the true potential of ES cells as it precludes analysis of extraembryonic tissues. Consequently, it is not known which cell type from the preimplantation mouse embryo ES cells most resemble. This is of some interest in considering both the origin of ES cells, the tissues in which alterations of gene expression may be studied using ES cell chimaeras, and the use of ES cells as an *in vitro* model system for certain aspects of early mouse development.

In the absence of suitable biochemical markers to distinguish between cells at successive preimplantation stages with the potential to contribute to the fetus, the most direct means of establishing the identity of ES cells is to examine their fate after injection into the blastocyst. For this reason, single and multiple ES cells were introduced into host blastocysts and their contribution to derivatives of the three primary tissue lineages (trophectoderm, primitive endoderm and epiblast) was analysed in midgestation conceptuses. From the results obtained it would appear that ES cells resemble early

inner cell mass (ICM) cells in their developmental potential.

### Materials and methods

#### *Derivation and maintenance of ES cell lines*

The CP1 and CP3 ES cell lines used in this study were derived from embryos recovered from matings between mice of the 129/Sv/Ev inbred strain. The lines were isolated from single implantationally delayed blastocysts, using procedures previously described (Evans & Kaufman, 1983; Robertson, 1987). Both these lines, which are karyotypically XY, have been shown to contribute to the germ line of chimaeric males up to at least the 14th passage *in vitro* (Bradley *et al.* 1984; Bradley & Robertson, unpublished observations). Karyotypic analysis of both lines was undertaken at the 6th subculture generation. A total of 40 chromosome spreads from CP1 were counted and 32 (80%) contained 40 chromosomes. 23 of these spreads were G-banded and 18 (78.3%) were euploid. Of the 4 (17.4%) that had lost chromosomes a different chromosome in each case was missing. One spread (4.3%) contained an extra chromosome. CP3 cells showed a similar profile with 75% of spreads appearing euploid and no evidence for any consistent abnormality after G-banding. Considering that some observed aneuploidy is probably the result of technical artifact the vast majority of cells in both lines were euploid. However, it is impossible, given a certain inevitable frequency of aneuploidy in tissue culture cell lines, to be sure that only chromosomally normal cells were used for injection.

For the experiments described here, the lines had been maintained for between 7 and 9 subculture generations (approximately 40–50 cell doublings) on feeder layers of fibroblasts treated with mitomycin C (Sigma), as described elsewhere (Robertson, 1987). For blastocyst injection experiments, a single cell suspension was prepared by trypsin

**Table 1.** Frequency of normal development and chimaerism among injected blastocysts

| ES cell line   | Number of ES cells injected | Number blastocysts injected* | Number empty decidua (%) | Number retarded embryos (%) | Number normal embryos (% of blastocysts injected) | Number normal chimaeras (% of normal embryos) |
|----------------|-----------------------------|------------------------------|--------------------------|-----------------------------|---|---|
| CP3p.6         | 10–15                       | 18                           | 0                        | 0                           | 15 (83.3 %)                                       | 15 (100 %)                                    |
| CP3p.7         | 10–15                       | 41                           | 10 (24.4 %)              | 3 (7.3 %)                   | 17 (41.5 %)                                       | 1 (5.9 %)                                     |
| CP3p.7         | 1                           | 18                           | 0                        | 0                           | 12 (66.7 %)                                       | 3 (27.3 %) <sup>†</sup>                       |
| CP1p.7         | 1                           | 15                           | 1 (6.7 %)                | 1 (6.7 %)                   | 8 (53.3 %)  | 1 (12.5 %)                                    |
| CP1p.8         | 1                           | 18                           | 0                        | 1 (5.6 %)                   | 14 (77.8 %)                                       | 5 (35.8 %)                                    |
| CP1p.8 'small' | 1                           | 18                           | 0                        | 0                           | 11 (61.1 %)                                       | 1 (9.1 %)                                     |
| CP1p.8 'large' | 1                           | 18                           | 0                        | 0                           | 9 (50 %)  | 2 (22.2 %)                                    |

\* Blastocysts transferred to recipients which did not become pregnant are not included.

<sup>†</sup> Only 11 of the normal conceptuses were analysed.

digestion (0.25 % trypsin, 0.04 M-ethylenediaminetetra-acetic acid (EDTA) for 4 min at 37°C). The cells were collected in Dulbecco's Modified Eagles Medium (DMEM – Flow Laboratories, UK) containing 10 % v/v fetal bovine serum and kept at room temperature until required.

#### Blastocyst injection

Embryos for microinjection were obtained from matings between outbred MF1 strain animals (homozygous for the *Gpi-1<sup>b</sup>* allele). Expanded blastocysts were recovered 3–5 days *post coitum* (d.p.c.) and cultured in drops of DMEM plus 10 % v/v fetal bovine serum in a 37°C, 6 % CO<sub>2</sub> humidified incubator. Groups of 5–10 blastocysts were transferred to a microinjection chamber containing DMEM, 5 % fetal bovine serum and 20 mM-Hepes (Sigma). A small number of ES cells were added. Transfer of ES cells into blastocysts was performed using the method described by Bradley (1987). Two series of injections were carried out: one in which multiple cells (10–15) were injected into blastocysts, and the other in which a single ES cell was placed in the blastocoel cavity. For the latter series, in some experiments ES cells were selected according to size i.e. 'large' cells (diameter approximately 15 µm) and 'small' cells (diameter approximately 10 µm). This was done in order to determine whether there was any difference in the differentiation abilities of different sized cells.

Following injection, the embryos were returned to the incubator for 4–5 h, after which time they were inspected in a phase contrast microscope (×200). Any embryos in which the ES cells had been excluded from the blastocoel cavity (i.e. clearly visible between the trophectoderm and zona pellucida) were discarded. The remainder were transferred, in groups of 5 or 6, into the uterine horns of pseudopregnant (C57Bl × CBA)F<sub>1</sub> recipient females.

#### Dissection of midgestation tissues

Conceptuses were recovered at midgestation when the majority were at a developmental stage equivalent to 10th day embryos which had just completed turning. This is the stage at which it is easiest to isolate from the same conceptus tropho-ectoderm, parietal endoderm, visceral yolk sac (VYS) endo-derm and VYS mesoderm. Recipient females were killed 7 days after blastocyst transfer and the decidual swellings transferred to PB1 (Whittingham & Wales, 1969) containing 10 % (w/v) fetal calf serum (FCS) instead of bovine serum albumen. The decidual tissue was removed using fine watch-makers' forceps. The constituent tissues of the conceptus were isolated as described below, rinsed in phosphate-buffered saline (PBS) and placed in microdrops of PBS in

microwell plates (MicroWell, Nunc.) at 4°C. Abnormal embryos were isolated into fractions wherever possible. Otherwise, they were treated as single samples. Samples were stored at –20°C.

Trophoblast giant cells were detached from Reichert's membrane with forceps. The placenta was removed by making a single cut with iridectomy scissors in the region of parietal and VYS insertion. The majority of maternal tissue was peeled away from the surface of the placenta. The parietal endoderm, together with Reichert's membrane, was removed and any residual adherent trophoblast giant cells picked off and discarded. The remaining part of the conceptus was separated into VYS, amnion and fetal fractions. The VYS was further separated into its mesoderm and endoderm components by first incubating it in 0.5 % (w/v) trypsin (Sigma) plus 2.5 % (w/v) pancreatin (Difco, USA) in Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free Tyrode Ringer's solution (pH 7.6) at 4°C for 3 h and then separating the endoderm and mesoderm with fine forceps. The dissections resulted in each normal conceptus being divided into 7 fractions: trophoblast giant cells, placenta, parietal endoderm, VYS endoderm, VYS mesoderm, amnion and foetus. The placental fraction was not analysed because, being composed of derivatives of all three of the founder tissue lineages, it is not informative. The amnion was only analysed as separate fraction in the clonal injection series.

#### Electrophoretic analysis

Tissue fractions were frozen and thawed at least once. Samples were run on Titan III plates (Helena Laboratories) using the method described by McLaren & Buehr (1981) except that the concentration of Tris–glycine bridge buffer was reduced to 0.025 %. The plates were stained using a standard procedure (Eicher & Washburn, 1978) and the relative contributions of ES and embryo allozyme activity estimated by eye.








## Results

#### Frequency of normal development

A summary of the development in pseudopregnant recipients of injected blastocysts is shown in Table 1. A total of 59 blastocysts were injected with 10–15 CP3 cells and transferred to 9 recipients. 32 normal and 13 abnormal or resorbing conceptuses were recovered. 51 blastocysts were injected with single unsorted CP1 and CP3 cells and these generated 34 normal conceptuses, 2 retarded embryos and 1 resorption. 'Large' single CP1



**Table 2.** Distribution of ES cells in multiple cell injections

| Chimaera number         | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|-------------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| Fetus                   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |
| Unseparated VYS*        |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |
| VYS mesoderm            |   | M |   |   |   |   |   |   |   |    |    |    |    |    | M  |    |
| VYS endoderm            |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |
| Parietal endoderm       |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |
| Trophoblast giant cells |   | 0 |   |   |   | ? | ? |   | 0 |    |    |    |    | ?  | 0  |    |

|   |  |   |                                   |
|---|--|---|-----------------------------------|
|  | = greater than 50 % ES cell contribution |  | = sample missing                  |
|  | = 10–50 % ES cell contribution           |  | = no activity in sample           |
|  | = less than 10 % ES cell contribution    |  | = trace contribution?             |
|  | = no detectable ES cell contribution     | *   | = VYS not separated in chimaera 1 |

**Table 3.** Distribution of ES cells in single cell injections

| Chimaera number*        | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-------------------------|---|---|---|---|---|---|---|---|---|----|----|----|
| Fetus                   |   |   |   |   |   |   |   |   |   |    |    |    |
| Amnion                  |   |   |   |   |   |   |   |   |   |    |    |    |
| VYS mesoderm            |   |   |   |   |   |   |   |   |   |    |    |    |
| VYS endoderm            |   |   |   |   |   |   |   |   |   |    |    |    |
| Parietal endoderm       |   |   |   |   |   |   |   |   |   |    |    |    |
| Trophoblast giant cells |   |   |   |   |   |   |   |   |   |    |    |    |

|   |                                       |
|---|---------------------------------------|
|  | = 10–50 % ES cell contribution        |
|  | = less than 10 % ES cell contribution |

- \* chimaera nos 3 and 7 resulted from injection of "large" cells  
chimaera no. 4 resulted from injection of a "small" cell

cells were transplanted into 18 blastocysts and produced 9 normal 10th day embryos. 'Small' single CP1 cells were injected into 18 blastocysts and 11 developed normally.

#### Colonization pattern of ES cells

Injection of multiple CP3 cells resulted in 16 chimaeric conceptuses (16/32 = 50%). In addition, 2/5 (40%) of abnormal embryos were chimaeric. The remaining 8 resorptions did not contain sufficient GPI activity for analysis. The distribution of ES cell progeny in normal chimaeras and the relative contribution to the various tissues analysed is shown in Table 2. The most frequently colonized tissues and those with consistently the highest ES cell contribution were the fetus and extraembryonic mesoderm. However, donor enzyme activity was also present in visceral and parietal endoderm fractions in 25% of chimaeras and a contribution to trophoblast giant cells was detected unequivocally in 4 conceptuses.

Single-cell injections produced a lower percentage of chimaeras. Pooling all the clonal experiments gives a rate of blastocyst colonization of 22.2% (12/54). Neither of the 2 retarded embryos proved to be chimaeric. The distribution of clones is shown in Table 3. Again the predominant pattern is chimaerism restricted to the fetus, amnion and VYS mesoderm. One clone had descendants in both the fetus and parietal endoderm. Both large and small CP1 cells adhered to this pattern and showed no obvious difference in their colonization potential. No clone contributed to the trophoblast fraction and, as one might expect, the extent of donor cell contribution in chimaeric tissues was less than that seen with multiple-cell injections.

#### Discussion

Microsurgical methods for reconstituting blastocysts (Gardner *et al.* 1973; Papaioannou, 1982) and injection

of multiple or single ICM cells into genetically dissimilar blastocysts (Gardner & Rossant, 1979; Gardner, 1982, 1985a; Gardner *et al.* 1985) have shown that the late blastocyst is composed of three distinct tissue lineages (trophoblast, primitive endoderm and epiblast) each of which gives rise to its own subset of midgestation embryonic or extraembryonic tissues. The segregation of cells into these respective lineages appears to occur sequentially. Early ICM cells injected into the blastocyst contribute mainly to the fetus and extraembryonic mesoderm but can also give rise to trophoblast and primitive endoderm (Gardner, 1985a). By the expanded blastocyst stage injected ICM cells can no longer give rise to trophoblast (Gardner, 1985a). In the 5th day blastocyst, the ICM is composed of two distinct populations, one which generates primitive endoderm derivatives and one, epiblast, which colonizes only the fetus, extraembryonic mesoderm and amniotic ectoderm (Gardner & Rossant, 1979; Gardner, 1982).

ES cells are known to give rise to widespread chimaerism in liveborn mice following their introduction into the blastocyst (Bradley *et al.* 1984; Evans *et al.* 1985) and to a variety of mature somatic tissues in solid tumours (Martin, 1981; Evans & Kaufman, 1983). However, it is not known whether this pluripotency reflects their similarity to epiblast cells or stems from an identity with an earlier embryonic cell type capable of generating trophoblast and primitive endoderm derivatives. It has been argued from biochemical comparisons that the closest counterpart to ES cells in the embryo is 6th day epiblast (Evans *et al.* 1979; Evans & Kaufman, 1981). However, the results presented in this paper demonstrate that injected ES cells can contribute to all three tissue lineages, unlike embryonic epiblast tissue (Hogan *et al.* 1983; Gardner & Rossant, 1979; Gardner, 1985b). In both multiple and single cell injections, there is a clear bias towards colonization of the fetus and extraembryonic mesoderm, characteristic of epiblast tissue. However, a quarter of chimaeras produced by multiple cell injection have ES cell progeny in parietal or visceral endoderm (Table 2). Three further chimaeras (18.8%) have an unequivocal contribution to trophoblast giant cells which cannot be explained by residual endoderm contamination because the endoderm was not chimaeric in these conceptuses (Table 2). In addition, there is one example of an ES cell clone (Table 3) which embraces both epiblast and primitive endoderm derivatives. Although no giant cell contribution was seen in single cell injections it is, perhaps, worth noting that supposed extraembryonic endoderm and large cells, reminiscent of trophoblast giant cell outgrowths, are often seen in clonally derived ES cell cultures (Evans & Kaufman, 1983; Robertson & Bradley, 1986; Robertson, 1987; Doetschman *et al.* 1985).

One could conclude that ES cell cultures are a heterogeneous mixture of epiblast and more primitive cells, with those of epiblast character predominating. However, such a conclusion is not easily reconciled with the repeated passaging of clonally derived cultures

without loss of totipotent cells. The simplest explanation of these results, by comparison with experiments using normal preimplantation embryonic cells (Gardner, 1985a), is that ES cells most closely resemble early ICM cells. The bias towards colonization of the fetus and extraembryonic mesoderm may reflect either the position that injected cells assume in the host ICM, which favours epiblast formation, or a failure to be assimilated into the ICM until after opportunities for trophoblast or endoderm differentiation have passed.

The conclusion that ES cells are analogous to early ICM cells is somewhat surprising considering that CP1 and CP3 were originally isolated from delayed blastocysts. Electron microscopic studies of blastocysts during lactational- or ovariectomy-induced delayed implantation have clearly shown that primitive endoderm, distinguished as cells rich in rough endoplasmic reticulum, is present from the earliest stages following induction of delay (Gardner *et al.* 1988). In normal development, this differentiation appears to coincide with subdivision of the ICM into epiblast and primitive endoderm tissue lineages (Gardner, 1982). It is possible that epiblast does not develop in concert with primitive endoderm during delay. However, ICM cells from delayed blastocysts do not behave like earlier ICM cells in that they do not produce overt chimaerism in liveborn mice following injection into host blastocysts (Gardner *et al.* 1985). Alternatively, ES cells may be derived from epiblast present in implantationally delayed embryos but on explantation *in vitro* they revert to a more primitive cell type. If the restriction in potential characteristic of epiblast cells is dependent on cellular interactions it may not be possible to retain such restriction in clonal cell cultures (Gardner & Beddington, 1989).

These colonization patterns described for two different ES cell lines at midgestation are also of some practical relevance. ES cells provide a unique means of studying the effects of certain genes, manipulated *in vitro*, in chimaeras. There are many examples of genetic effects that may be lethal if expressed in all cells but which can be rescued in chimaeras (e.g. parthenogenetic cells (Stevens *et al.* 1977); trisomy 17 (Epstein *et al.* 1982); trisomy 15 (Epstein *et al.* 1984)). The fact that ES cells can contribute to all tissues of the conceptus means that they can be used to study specific genetic effects, not only in the fetus, but also in important extraembryonic organs such as the placenta and visceral yolk sac.

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