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Reflections on the biology of embryonic stem (ES) cells

RICHARD L. GARDNER* and FRANCES A. BROOK

Department of Zoology, University of Oxford, Oxford, United Kingdom

ABSTRACT Remarkably little is known about mammalian embryonic stem (ES) cells despite their very widespread use in studies on gene disruption and transgenesis. As yet, it is only in the mouse that lines of ES cells which retain the ability to form gametes following reintroduction into the early conceptus have been obtained. Even in this species, most strains have so far proved refractory to the derivation of such cell lines. Apart from persisting ignorance as to how the various procedures that have been claimed to improve success actually do so, even the tissue of origin of ES remains uncertain. Furthermore, it is doubtful whether retention of pluripotency or expression of so-called "stem cell" marker molecules provide an adequate basis for classifying cells as genuine ES cells. This is because epiblast cells, their presumed precursors, lose the capacity to colonize the preimplantation conceptus well before they become restricted in the types of cell they can form or cease to express such marker molecules. In addition, it has yet to be established whether heterogeneity of cells within individual ES cell lines arises entirely during culture or is at least partly attributable to lack of homogeneity among their precursors. Finally, it has yet to be explained why ES chimeras evidently differ from those obtained by combining cells from different conceptuses in showing greater variation between tissues in the level of chimerism.

KEY WORDS: *embryonic stem cells, chimeras, blastocysts, mouse strain*

Introduction

The availability of cells that retain the ability to colonize the germline and form normal gametes following genetic manipulation and selection *in vitro* has had considerable impact on the analysis of normal gene function in the mouse. It has also enabled the generation of murine models for a variety of heritable human diseases that are attributable to mutations at single genetic loci (Clarke, 1994; Copp, 1995). However, despite the widespread and increasing use of embryonic stem (ES) cells in the mouse and repeated attempts to obtain them in a variety of other mammalian species, knowledge about their origin and biology remains surprisingly limited. Rather than provide yet another account of the various ways in which ES cells can be harnessed to probe the genetic control of development, differentiation, or physiology, our aim is to focus on how little is known about these fascinating and versatile cells. We feel that an appreciation of present ignorance is a prerequisite for identifying issues that need to be addressed if full exploitation of the potential of these cells is to be realized.

Attempts have been made to derive ES cells from peri-implantation conceptuses in a variety of mammals, principally laboratory species or those that are important agriculturally (Table 1). Cells that resemble murine ES cells in features such as morphology, marker expression, growth characteristics, and the ability to differentiate *in vitro* or *in vivo* have been obtained in such species. However, the question whether these cells also resemble their murine counterparts in ability to form viable gametes, which is the key attribute of ES cells from the point of view of genetic manipulation, remains open. Thus, although nuclei taken from 'ES-like' cells obtained in several species have been shown to be able to support normal development when transplanted into enucleated oocytes (Sims and First, 1993; Du *et al.*, 1995; Campbell *et al.*, 1996), this does not constitute proof that the cells themselves retain totipotency. That problems have been encountered in attempting to derive ES lines in mammals other than the mouse is perhaps not surprising since, in essence, what has been done is simply to apply the strategy devised for deriving ES cells in the mouse to these other species. Even in the mouse, where embryonic material is not as limiting as in other species and genetic uniformity can be assured, the present strategy has proved successful in only a very limited number of strains. Most of the germline-competent mouse ES cell lines that are in current use have been obtained in the 129 strain, and the remainder in a few other inbred strains (Table 2). One cannot expect to obtain ES lines from more than 30% of explanted blastocysts even in the 129 strain (Robertson, 1987), and success rates of around 10% appear closer to the norm (e.g. Nagy *et al.*, 1993). Various modifications to the basic procedure have been claimed to enhance the rate of success. These include attempting to denude inner cell masses (ICMs) of primitive endoderm (Handyside *et al.*, 1989), using primary embryonic fibroblasts rather than STO cells as feeders (Wobus *et al.*, 1984; Suemori and Nakatsuji, 1987), supplementing

***Address for reprints:** Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom. FAX: 1865.281310.
e-mail: richard.gardner@zoology.oxford.ac.uk

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the medium with cytokines of the IL-6 family (Williams *et al.*, 1988; Nichols *et al.*, 1990, 1994; Pease *et al.*, 1990; Conover *et al.*, 1993; Yoshida *et al.*, 1994), restricting the volume of culture medium (Hooper, 1992), and applying heat-shock (Wells *et al.*, 1991). However, while there remains a lack of basic understanding of what the production of ES cells entails, the rationale for these modifications remains elusive, as does the basis for the clearly beneficial effect of using delayed-implanting rather than non-delayed blastocysts (Evans and Kaufman, 1981; Robertson, 1987). The demonstration that germline competent ES cells can be obtained without feeder cells if the medium is supplemented with leukaemia inhibitory factor (LIF) has been interpreted as evidence that the feeder layer simply acts as a source of this or a related cytokine. However, the unusually high rates of aneuploidy found in ES lines obtained in both studies in which LIF was used in the absence of feeders has yet to be explained (Nichols *et al.*, 1990; Pease *et al.*, 1990).

Origin and features of ES cells

Whether ES cells can properly be regarded simply as superior embryonal carcinoma (EC) cells, as is often stated to be the case, is questionable. Originally, EC cells, which were shown to be the pluripotential stem cells of teratocarcinomas (Kleinsmith and Pierce, 1964), were investigated as possible vehicles for introducing genetic modifications into mice. The teratocarcinomas that were used as sources of these cells either originated spontaneously from male or female germ cells (Stevens, 1975, 1983), or were induced experimentally by grafting early postimplantation conceptuses, or just the primitive ectoderm or epiblast therefrom, to ectopic sites in syngeneic hosts (Solter *et al.*, 1975). Various lines of EC cells that were established from such tumors were found to retain the ability to participate in normal development following culture *in vitro* (Gardner, 1983; Papaioannou and Rossant, 1983). However, they colonized the germline only very exceptionally, whether maintained *in vivo* (Illmensee and Mintz, 1976) or *in vitro* (Stewart and Mintz, 1981), presumably reflecting the rarity with which they retained a normal karyotype. It was the possibility of avoiding loss of totipotency and karyotypic change through accelerating the derivation of such cells that prompted efforts to obtain them from conceptuses explanted directly into culture. This goal was realized with the production of ES cells from explanted whole blastocysts (Evans and Kaufman, 1981) or ICMs isolated by immunosurgery (Martin, 1981). The fact that ES cells consistently yield teratomas or teratocarcinomas when grafted ectopically and occasionally give rise to tumors in chimeras (Hardy *et al.*, 1990) offers some support for the view that they are essentially euploid EC cells. However, as discussed below, this is not born out by consideration of their origin.

Several groups of workers attempted to establish the embryonic origin of EC cells by using 1- or 2-dimensional gel electrophoresis to compare their pattern of polypeptide synthesis with that of tissues from both pre- and early post-implantation conceptuses (Dewey *et al.*, 1978; Martin *et al.*, 1978; Evans *et al.*, 1979; Lovell-Badge and Evans, 1980). No definite conclusions emerged from these studies since the profile for EC cells was found to be distinct from that of all tissues analyzed, presumably reflecting their adaptation to growth in an abnormal environment. Nevertheless, there was a consensus that they more closely resembled epiblastic than early ICM cells. The fact that ES cells can be obtained from isolated inner cell masses (ICMs) as well as entire blastocysts is consistent with the view that they, like EC cells, originate from cells belonging to the lineage from which the fetus is derived. However, explanted pre- rather than post-implantation conceptuses have invariably provided the starting material for deriving ES cell lines. While this does not exclude the possibility that the developmental

TABLE 1

THE ISOLATION OF ES CELLS FROM SPECIES OTHER THAN THE MOUSE

Species	Success in isolating ES lines	References*
Rat	One ES line identified by morphology and differentiation *in vitro* and *in vivo*. Gave chimeras after blastocyst injection but did not transmit through the germ-line.	Iannaccone *et al.*, 1994
Rabbit	'ES' lines identified by morphology and *in vitro* differentiation. Support normal preimplantation development after nuclear transfer but blastocysts were not transferred to pseudopregnant females.	Graves and Moreadith, 1993; Du *et al.*, 1995
Golden Hamster	4 'ES' lines identified by morphology and *in vitro* differentiation. No reported attempt to make chimeras.	Doetschman *et al.*, 1988
Mink	'ES' lines derived from morulae, ICM and blastocysts identified morphologically. Differentiate *in vitro* and *in vivo* but did not contribute to chimeras after blastocyst injection.	Sukoyan *et al.*, 1992 and 1993.
Rhesus monkey	1 'ES' line identified morphologically. Produced markers for trophoblast and endoderm after *in vitro* differentiation, and gave tumours after *in vivo* differentiation.	Thomson *et al.*, 1995
Pig	ES lines identified morphologically and by *in vitro* differentiation. High rate of chimera production after blastocyst injection. Germ -line colonisation has not yet been demonstrated.	Wheeler, 1994
Cow	Cultured inner cell mass cells were used as donors of nuclei to produce 4 calves. Cells cultured long-term have not given rise to calves.	Sims and First, 1993
Sheep	Embryonic cells with an epithelial morphology, cultured for 6-13 passages, gave rise to lambs after nuclear transfer. Cells which morphologically resemble ES cells are difficult to establish in culture and show limited differentiation *in vitro*.	Campbell *et al.*, 1996; Notarianni *et al.*, 1991

*This is not a fully comprehensive list of references but is intended to show current progress. Abstracts have not been cited as it is not possible to evaluate such studies.

TABLE 2

ES LINES OBTAINED FROM MICE OTHER THAN THE 129 STRAIN

Strain	No. of lines established/ No. of embryos*	No. lines which contributed to chimeras	No. lines which colonized the germline	Efficiency of chimera formation (% chimeras/ liveborn young)	Efficiency of germline colonization. (% male chimeras which transmitted)	References
C57BL/6	6/216 on primary embryo fibroblast feeders	2/2	1/2	42 - with BALB/c host blastocysts 11 - with 129/Sv host blastocysts	48 - with BALB/c host blastocysts 0 - with 129/Sv host blastocysts	Ledermann and Burki, 1991
C57BL/6	10/16 on human bladder carcinoma cells	-	-	-	-	Ledermann and Burki, 1991
C57BL/6 X DBA	4/92 on primary embryo fibroblast feeders	-	-	-	-	Ledermann and Burki, 1991
C57BL/6 X DBA	18/80 on human bladder carcinoma cells	-	-	-	-	Ledermann and Burki, 1991
DBA/1lacJ	3/244	3/3	1/3	14-25 - with C57BL/6 host blastocysts	89	Roach *et al.*, 1995
CD1	8/86	1/8	1/8	15 - with C57BL/6 host blastocysts	3	Suda *et al.*, 1987
C57BL/6 X CBA	-	-	-	80 - with CD1 8-cell host embryos	89	Tokunaga and Tsunoda, 1992
C57BL/6 X CBA	-	-	-	20 - with CD1 host blastocysts	0	Tokunaga and Tsunoda, 1992
C57BL/6 X CBA	-	-	-	48 - with C57BL/6 host blastocysts	0	Yagi *et al.*,1993
C57BL/6 X CBA	-	-	-	54 - with CD1 host blastocysts	0	Yagi *et al.*,1993
C57BL/6 X CBA	-	-	-	75 - with CD1 8-cell host embryos	56	Yagi *et al.*,1993
C57BL/6	12/15	4/4	3/4	12-43 - with ICR or BALB/c host blastocysts	50-100	Kawase *et al.*, 1994
BALB/c	5/204	3/4	0	14-43 - with C57BL/6 host blastocysts	0	Kawase *et al.*, 1994
MRL/Mp-1pr/1pr	14/17 delayed blastocysts	4/4	0	5-40 - with C57BL /6 host blastocysts	0	Kawase *et al.*, 1994
BXSB/MpJ-Yaa	1/4	0	-	-	-	Kawase *et al.*, 1994
C57BL/6	19/104 on Sto feeders	-	-	-	-	Suemori and Nakatsuji, 1987
C57BL/6	11/63 on primary embryo fibroblasts	-	-	-	-	Suemori and Nakatsuji, 1987
C57BL/6 X 129	20/168 on Sto feeders	-	-	-	-	Suemori and Nakatsuji, 1987
C57BL/6	7/28 from disaggregated morulae	-	-	-	-	Eistetter, 1989
C57BL/6 X 129	10/61 from disaggregated morulae	-	-	-	-	Eistetter, 1989
C3H	1/31	-	-	-	-	Brown *et al.*, 1992
C57BL/6J	1/36	-	-	-	-	Brown *et al.*, 1992
C57BL/6J X CBA	1/80	-	-	-	-	Brown *et al.*, 1992

*Lines are usually identified by morphology.

status within the epiblast lineage of progenitors of ES and EC cells is identical, such a hypothesis is difficult to reconcile with a consistent finding which seems to have been ignored in the present context. This is that colonization of the blastocyst by transplanted epiblast cells has only been achieved when the cells have been taken from implanting blastocysts, i.e. shortly after they first make their appearance (Gardner and Lyon, 1971; Gardner *et al.*, 1985). Despite repeated attempts in several laboratories, colonization of the blastocyst with epiblast cells from 6th day or later conceptuses has never been achieved (unpublished observations of R.L. Gardner, and of J. Rossant, and R.S.P. Beddington). Even during the course of the 5th day of development there is a marked decline in cloning efficiency of epiblast cells following blastocyst injection, with those from late implanting blastocysts giving about half the rate of chimerism as those from earlier ones (Gardner *et al.*, 1985). Interestingly, the success rate in obtaining ES cells from epiblast in the permissive 129 strain also seems to decline precipitously between the 5th and 6th day post coitum (Wells *et al.*, 1991). Hence, during the transition from late blastocyst to early egg-cylinder the epiblast appears to lose its ability both to colonize the blastocyst and to form ES cells. It is tempting to suggest that the two phenomena are related, although there are at present no compel-

ling data to support such a contention. While ES cells may originate from very early epiblast cells since they are derived from preimplantation blastocysts, this is clearly not the case for EC cells because, as noted earlier, those of embryonic rather than germ cell origin have usually been obtained from ectopic grafts of postimplantation conceptuses. Hence, to account for the fact that EC cells can colonize the blastocyst, it would seem necessary to postulate that epiblast cells must undergo some dedifferentiation during the process of their derivation. Re-programming of gene expression would similarly appear to be necessary to account for the fact that totipotential cells obtained through the *in vitro* culture of primordial germ cells from more advanced postimplantation conceptuses (EG cells) are also able to participate in normal development when injected into the blastocyst (Labosky *et al.*, 1994; Stewart *et al.*, 1994).

At present, the change occurring in epiblast cells during implantation that results in the loss of their ability to colonize the blastocyst is a matter for speculation. The most conspicuous early postimplantation change in the epiblast is its transformation from a solid ball of rounded cells into a pseudostratified epithelium that accompanies the formation of the proamniotic cavity. Recent work has implicated the extra-embryonic endoderm surrounding the epiblast as the key inducer of both its cavitation and epithelialization (Coucovanis and Martin, 1995). This is noteworthy in view of repeated, but as yet unsubstantiated, claims that the presence of such endoderm is detrimental to the derivation of ES cell lines (e.g. Handyside *et al.*, 1989). A very intimate relationship between the epiblast and its investing endoderm in the early postimplantation rat conceptus has been revealed by electron microscopy, with processes from the basal surface of epiblast cells making extensive contact with the endoderm through discontinuities in the intervening basal lamina (Takeuchi and Takeuchi, 1981). Therefore, it is conceivable that prolonged contact with endoderm induces changes in the adhesive properties of epiblast cells preparatory to their epithelialization and that this precludes their integrating with their forebears in the ICM. This would explain why formation of an obvious rind of endoderm on the outside of ICMs is deemed to be poor prognostically for the derivation of ES cells (e.g. Robertson, 1987).

It is important to note, particularly with regard to efforts to obtain ES lines in other mammals, that loss of the ability of epiblast cells to colonize the preimplantation conceptus in the mouse is not associated with any obvious restriction in the range of cell types that they can form. Therefore, the ability of 'ES-like' cells obtained in various mammals to form tissues representative of all three classical germ layers when grafted ectopically or made to differentiate *in vitro* cannot be taken as evidence that they will be competent to participate in the formation of chimeras.

A plausible working hypothesis is that the production of ES cells depends on the provision of conditions that secure uncoupling of the proliferation of cells of the early conceptus from their further differentiation at a stage when they retain both pluripotency and the ability to colonize the blastocyst. According to this hypothesis, while ES cells are likely to exhibit some modulation of gene expression in adapting to culture *in vitro*, they should continue to resemble their progenitors in fundamental properties such as developmental potential. The impressive facility with which ES cells participate in normal development, especially when aggregated with compromised tetraploid conceptuses (Nagy *et al.*, 1993), offers strong support for such a view. However, there is persisting uncertainty about the developmental potential of ES cells and both the identity and developmental potential of their precursors. These issues can be appreciated most readily in the context of what is known about cell lineage and commitment during the relevant period of development of the mouse conceptus. While early ICM cells do not normally form trophectoderm *in situ* (Dyce *et al.*, 1987) or following orthotopic transplantation (Gardner and Nichols, 1991), they can do so if this layer is removed (Handyside, 1978; Hogan and Tilly, 1978; Spindle, 1978; Rossant and Tamura-Lis, 1979; Nichols and Gardner, 1984). Although endoderm rather than trophectoderm is formed following later removal of the latter, there is an intermediate stage when individual ICMs can produce both types of cell (Nichols and Gardner, 1984). By the advanced blastocyst stage the superficial cells of the ICM have clearly differentiated to form the primitive endoderm, which is the progenitor tissue of the endodermal components of both the parietal and visceral yolk sacs (Gardner, 1982, 1984). Its residual deep cells remain undifferentiated morphologically as the primitive ectoderm or epiblast, the progenitor tissue of both the soma and germline of the fetus, as well as all extra-embryonic mesoderm and the ectoderm of the amnion (Gardner, 1985a). The question whether the epiblast retains the ability to regenerate primitive endoderm has yet to be resolved. Positive results obtained using immunosurgery to eliminate the primitive endoderm (Pedersen *et al.*, 1977) are to be doubted in view of evidence that this tissue is more than one cell layer thick (Gardner, 1985b). When microsurgery was used to effect complete removal of the primitive endoderm, the epiblast failed to survive beyond a short period in culture (Gardner, 1985b). A further point is the subtle change in ectoderm cells discussed earlier which results in the loss of their ability to colonize the blastocyst.

What can be learnt from a comparison of the developmental potential of ES cells with cells of the early conceptus? First, ES cells unquestionably retain the ability to form extra-embryonic endoderm and, indeed, produce these cells very readily under a variety of conditions. As noted above, whether epiblast cells share this property will remain uncertain until the tissue is grown in the absence of any contaminating primitive endoderm in conditions in which it can survive. Such conditions may be the same as those favoring the formation of ES cells, which would strengthen the case that one is dealing with the same type of cell. There is some evidence that ES cells can form trophoblast as well as extra-embryonic endoderm which, if substantiated, would suggest that they more closely resemble early ICM than epiblast cells. However, it has yet to be shown by use of an *in situ* cell marker that the occasional minor contribution of ES cells to trophoblast following blastocyst injection (Beddington and Robertson, 1989) is due to their appropriate differentiation rather than ectopic growth. Furthermore, without rigorous clonal analysis, the possibility that trophoblastic giant cells noted in some early passage ES cultures originate from a separate pool of trophectoderm cells with limited proliferative potential cannot be discounted (Evans and Kaufman, 1983). Such contaminating trophoblast would only be expected in cases where whole blastocysts rather than isolated ICMs are explanted so that there is thus the prospect of polar trophectoderm tissue remaining associated with the ICM when it is isolated for the first partial dissociation. The absence of any record of trophoblast in ES cultures derived from explanted isolated ICMs makes the possibility that it is due to the carry-over of polar trophectoderm cells seem likely. It should be noted, however, that there is at

present no reason to suppose that ES cells can only originate at a single stage in the early development of the ICM lineage. Yagi *et al.* (1993) raise the possibility that their TT2 ES line originates at an earlier stage of differentiation than most ES lines in order to explain why it gives much better colonization of the morula than the blastocyst. It would therefore be most interesting to know whether lines of ES cells established from dissociated morulae (Eistetter, 1989) can form trophoblast with the facility with which those from blastocysts or ICMs form endoderm.

As noted earlier, the most striking demonstration of the pluripotency of ES cells that has been provided so far is the production of mice that are composed entirely of these cells following their aggregation in small clumps between pairs of tetraploid morulae (Nagy *et al.*, 1993). However, a question that is left unanswered in these experiments is whether the ES cells can substitute entirely for the epiblast in such composites or whether the presence of some tetraploid epiblast cells, which may eventually be outcompeted, is required initially. Triple tissue reconstitution of blastocysts (Gardner *et al.*, 1990) using a clump of ES cells in place of the epiblast would provide an incisive way of approaching this issue.

Identifying pluripotential stem cells in ES cultures

Apart from the question of the developmental status of ES cells, there is the important practical issue of how they can be distinguished from the various types of cells with more restricted developmental potential to which they give rise. Spontaneous differentiation occurs continually in ES cultures even in the presence of high concentrations of LIF/DIA (Smith *et al.*, 1992), and might entail loss of pluripotency of the cells well before there is any discernible change in their morphology. Hence, toti- or pluripotential stem cells may constitute only a minor proportion of the total population of morphologically undifferentiated cells in ES cultures. Such a possibility would explain why it is necessary to inject many more ES than epiblast cells per blastocyst in order to obtain satisfactory rates and levels of chimerism (Gardner and Lyon, 1971; Gardner *et al.*, 1985; Bradley, 1987). That the incidence of stem cells may be very sensitive to the precise conditions of culture is suggested by the study of Beddington and Robertson (1989) who analyzed a series of ES-chimeras prenatally. These workers obtained an impressive rate of chimerism of 22% in experiments in which single ES cells were injected into blastocysts. However, their results varied strikingly when the standard 10-15 cells were transplanted, with a cell line that remained germ-line competent at passage 14 giving 100% chimeras (= 15/15) at passage 7, and a mere 6% (1/17) at passage 8. Retention of germline competence until later by this particular cell line argues against subtle genomic changes accounting for such a marked discrepancy since these would tend to be cumulative. Rather, these findings suggest that if it were possible to identify stem cells, the efficiency of chimera production could be considerably enhanced. There are now a wide variety of antigenic and molecular markers that, by virtue of being common to EC and ES cells and epiblast cells of the conceptus, are claimed to be specific to pluripotential cells. However, whether any of these would enable discrimination between stem cells and their committed, but morphologically undifferentiated, progeny is uncertain. For example, although the presence of SSEA-1 is widely held to be diagnostic of pluripotential cells, this antigen was originally characterized using F9 EC cells, which are clearly restricted in developmental potential, as immunogen. Furthermore, in the original study, primitive endoderm as well as epiblast cells were found to be positive for SSEA-1 (Solter and Knowles, 1978). If a true pluripotential cell-specific marker were available, it would obviously be most useful if its presence or absence could be detected in living dissociated cells without compromising their viability. An alternative strategy would be to select for pluripotent cells by coupling the control sequences of a gene whose expression was found to be restricted to them to the coding region of a suitable resistance gene. An obvious risk with the latter would be enrichment for cells that were no longer competent to differentiate and which therefore failed to yield either somatic or germline chimerism following blastocyst injection. The converse strategy would be to use antibodies directed against very early markers of endoderm or other differentiated derivatives of ES cells to eliminate them by complement-mediated lysis.

Heterogeneity of ES cells

A general finding is that with increasing time in culture ES lines tend to undergo karyotypic change and lose their ability to form gametes, although some lines have been shown to remain normal in both respects even at relatively high passage number (e.g. Nagy *et al.*, 1993). Whether failure of ES cells to form functional gametes is simply a consequence of karyotypic change is uncertain, particularly since it is far from clear what loss of their 'germline competence' actually entails. It could be due either to the failure of ES cells to contribute to the stock of primordial germ cells in chimeras or, additionally or alternatively, to their inability to complete normal gametogenesis thereafter.

Heterogeneity of cells within ES lines in both karyotype and competence to colonize the germline seems to be attributed entirely to stochastic processes occurring during their culture *in vitro*. Thus, in deriving ES cell lines, it is standard practice to pool all undifferentiated colonies that are obtained from an individual conceptus on the grounds that "... a cell line derived from a single embryo is effectively a clonal population in terms of genotype and sex chromosome complement" (Robertson, 1987). The possibility that the progenitors of ES cells are already heterogeneous in developmental potential, or in other respects, before explantation does not seem to have been entertained. This is surprising in view of persisting ignorance about whether all or only some early epiblast cells can contribute to the germline (Gardner *et al.*, 1985). Moreover, so long as the occurrence of conspicuous cell death in the ICM of normal blastocysts remains unexplained (El-Shershaby and Hinchliffe, 1974; Handyside and Hunter, 1986), the possibility cannot be discounted that cells which are abnormal karyotypically or in other ways, may be rescued by explantation onto feeder cells in a rich nutrient medium. Such initial heterogeneity would not be apparent until ES cell lines derived from pooled colonies were cloned, which may not be until after several passages when transfectants are first selected. Clearly, a comparison at early passage of the properties of multiple ES lines derived strictly clonally from different ICM cells of individual conceptuses is needed to address this issue.

ES-chimeras versus conventional chimeras

Aggregating genetically-dissimilar early morulae in pairs, the first and most common way of producing conventional chimeras,

was pioneered by Tarkowski (1961) and subsequently refined by Mintz (1964). It was then found that chimeras could also be produced efficiently by transplanting cells or tissues between blastocysts (Gardner, 1968), an approach that enhanced their value for analyzing cell lineage and tissue interactions. ES cell chimeras were first produced by blastocyst injection and, despite the introduction of a number of simpler ways of achieving colonization of the preimplantation conceptus with these cells, this remains the method of choice. The two simplest techniques, aggregating small clumps of ES cells between pairs of morulae (Stewart, 1993) or briefly culturing denuded 8-cell stage conceptuses on a lawn of recently dissociated ES cells (Wood *et al.*, 1993), avoid the use of microsurgery altogether. There is, however, a risk with both of the ES contribution exceeding the limit that is compatible with normal development. The third, injection of ES cells under the zona pellucida of 8-cell conceptuses (Lallemand and Brulet, 1990; Tokunaga and Tsunoda, 1992), allows control over the number of cells that are re-introduced whilst being less demanding microsurgically than blastocyst injection. However, as pointed out by Bradley (1987), all these alternatives to blastocyst injection require overnight culture in a medium that is a compromise between what is optimal for morulae and for ES cells. Even non-manipulated blastocysts grown *in vitro* from the morula stage under so-called 'optimal' culture conditions typically show a reduced ability to develop normally to term compared with those recovered directly from the uterus.

One factor complicating the comparison between conventional chimeras and ES-chimeras is that they have largely been produced using different combinations of mouse strains. Thus, the 129 strain, which features so prominently in ES-chimeras because of the relative facility with which it yields ES cell lines, has seldom been used to make conventional chimeras. Effects of strain combination on both the incidence and distribution of chimerism have been noted in morula aggregation chimeras (Mullen and Whitten, 1971). Hence, it is important to bear in mind in the following discussion that the extent to which differences between ES and conventional chimeras are attributable to the provenance rather than the genotype of the cells of which they are composed remains uncertain. Most of the relevant information comes from blastocyst injection chimeras since there is a dearth of comparative data on conventional versus ES-chimeras produced by manipulating morulae.

No cases of germline transmission were found in early experiments in which 3-5 ES cells were injected into each of a series of host blastocysts (Bradley, 1986). Transmission was obtained only when approximately 10 or more cells were injected per blastocyst (Bradley *et al.*, 1984), a practice which seems subsequently to have been adopted universally. Why it is necessary to transplant so many ES cells in order to secure functional colonization of the germline is not clear, particularly since respectable levels of somatic chimerism can be obtained with substantially fewer cells. This becomes even more puzzling when experience with transplanting the presumptive progenitors of ES cells is considered. Thus, a donor contribution to the germline was detected in 57% of chimeras produced by injecting single 5th day pc epiblast cells into blastocysts (Gardner *et al.*, 1985). This included 16 of 23 cases where the donor cell was male and 5 of 14 cases where it was female. Notwithstanding this high frequency of colonization of the germline, the donor contribution to coat pigmentation was generally modest, exceeding that of the host in only a minority of cases. In one respectable germline chimera, donor type coat color was highly restricted spatially and was estimated to be no more than 5% (Gardner *et al.*, 1985). This contrasts with the situation in ES-chimeras where a high contribution to coat pigmentation has come to be widely regarded almost as a *sine qua non* for obtaining good colonization of the germline (e.g. Bradley, 1987; Brandon *et al.*, 1995). The situation is by no means straightforward, however, since the success of germline colonization with ES cells of different genotype seems to depend in at least some cases on the way in which chimeras are produced. Thus, while injection under the zona pellucida of 8-cell stage host conceptuses seems to be far more efficient than blastocyst injection with TT2 ES cells of [C57 BL/6J x CBA] F1 origin, it was found to yield very poor results with 129-derived E14 and D3 ES cells (Yagi *et al.*, 1993). Yet a further complication is that the genotype as well as the stage of the host conceptus appears to be important (Schwartzberg *et al.*, 1989).

Several considerations bear on the issue of what determines whether ES cells colonize the germline. One, which has been touched on already, is the possibility that genomic changes occur during the culture of ES cells that are too subtle to discern karyotypically and which, whilst not obviously impairing their ability to contribute extensively to somatic chimerism, might prevent their completing gametogenesis. Another is that there is persisting ignorance about how the germ cell lineage is established during normal development in mammals. A strictly lineage-based mechanism that limited gametogenic potential to cells which inherit specific determinants from the egg, as seem to apply in both *Caenorhabditis elegans* and *Drosophila melanogaster* (Gilbert, 1994), appears untenable in mammals. However, the question whether all epiblast cells or only some of them have the potential to produce primordial germ cells among their descendants has yet to be answered (Gardner *et al.*, 1985). If only some do, then the possibility of deriving ES lines that never possessed germline potential has to be considered. Regardless of whether all or only some early epiblast cells have germline potential, it is still not known how this potential is realized during normal development. The single aspect of the problem of the early development of the germ cell lineage with which progress has been made recently is mapping the locus of origin of pgcs within the epiblast before the onset of gastrulation (Lawson and Hage, 1994). The most interesting finding to emerge from this work is that the progenitors of primordial germ cells originate from the most proximal part of the epiblast, i.e. from within the region of the tissue which otherwise forms mainly extra-embryonic mesoderm which is most remote spatially from that forming the soma of the fetus. Earlier studies on conventional aggregation and blastocyst injection chimeras hinted at the possibility that the extra-embryonic mesoderm might be derived from a pool of cells that was to some extent separate from that forming the fetus (e.g. West *et al.*, 1984). Similarly, analysis of patterns of integration of retroviruses following exposure of preimplantation conceptuses to them suggested that the fetal germline and soma did not share the same progenitors (see Gardner and Beddington, 1988; Lawson, 1994).

Hence, according to the work of Lawson and Hage (1994), in order for cells of one or other component genotype in a chimera to contribute to the germ line, it would seem to be necessary that they colonize the most proximal region of the epiblast. Whether, under normal circumstances, this is sufficient to ensure that they make such a contribution is not known. Hence, differences in the effi-

ciency with which ES cells and their progenitors colonize the extra-embryonic region of the early epiblast might contribute to the differing efficacy with which they colonize the germline. At present, relevant data on the distribution of ES cells in extra-embryonic as well as fetal tissues of chimeras are available for only a single study (Beddington and Robertson, 1989), and are therefore rather too limited to allow any firm conclusions to be drawn. Nevertheless, among the chimeras produced by injecting multiple ES cells into blastocysts in this study, both the incidence and level of chimerism appears to be lower in extra-embryonic than in fetal derivatives of the epiblast. In comparable single and multiple epiblast cell injections (Gardner and Rossant, 1979), no such bias towards colonization of the fetus as opposed to the extra-embryonic mesoderm of the visceral yolk sac is evident. In one chimera from each of the single and multiple epiblast cell injection series, the donor contribution to the extra-embryonic mesoderm actually exceeded that to the fetus. Hence, the findings from the limited comparison that can be made at least raise the possibility that ES cells may not colonize the proximal region of the epiblast as effectively as the more distal region. It is therefore conceivable that ES cells do not intermix as readily with the ectoderm cells of the host conceptus as directly transplanted ectoderm cells. Some credence is given to this notion by various indications of 'lumpiness' in ES-chimeras, also recorded earlier for EC-chimeras (e.g. Table 1 in Illmensee and Mintz, 1976), compared to their conventional counterparts. Thus, in ES-chimeras with a modest ES contribution, donor pigmentation often seems to be distributed non-randomly, being largely confined to the head and rump and absent from the remainder of the trunk (Evans and Kaufman, 1983; Robertson *et al.*, 1983). Furthermore, in a series of adult chimeras in which the distribution of ES cells was studied in detail, striking differences in the level and frequency with which they contributed to particular tissues and organs were recorded (Ioffe *et al.*, 1995). At present, there is no evidence to attribute these to consistent inhomogeneities in the distribution of ES cells rather than to selection at the tissue level. However, while striking selection for or against cells of particular genotypes at the level of the whole organism has been well documented in conventional chimeras (e.g. Mullen and Whitten, 1971), comparable differences at the level of individual parts of such chimeras have very seldom been recorded unless one component carries a mutant allele like W^v which compromises its contribution to specific cell lineages (McLaren, 1976). Finally, a functional contribution of XY cells to the germline in chimeras that develop phenotypically as females seems to occur more frequently when the genotypically male component is of ES than of conceptus origin (Bronson *et al.*, 1995; authors' unpublished observations; M.J. Evans, personal communication). This suggests that there is more often a marked disparity in the level of chimerism between germ cells and the somatic cells of the genital ridges in ES- than in conventional chimeras.

However, a systematic comparison between ES- and conventional chimeras of the same genotypic constitution, both pre- and post-natally, is needed to establish whether any or all of the differences discussed above really relate to the provenance rather than to the genotype of the cells that are combined.

Conclusion

Clearly, many questions have yet to be answered regarding both the origin and properties of ES cells. The information obtained from studies that address these questions is vital not only for extending the scope of genome manipulation in mammals, but also for gaining a better understanding of the biology of the progenitor cells of the fetus. A key to both is the ability to grow embryo-derived cells *in vitro* under conditions which enable their properties and behavior to be observed and perturbed with a precision that is unattainable in the intact conceptus. This represents a further refinement in the process of making postimplantation mammalian development more accessible to study that began with Denis New's pioneering work on whole embryo culture.

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