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Embryonic Stem Cells in Agricultural Species

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Introduction

Embryonic stem (ES) cells are undifferentiated, pluripotent cells derived in culture from early embryos. When used to describe ES cells, 'undifferentiated' means that their fate has not been restricted to a particular cell lineage. 'Pluripotent' means that the cells have the capacity to develop into many cell types; in fact, when used to refer to ES cells, pluripotent has come to mean the capacity to contribute to both somatic- and germ-cell lineages. By definition, an ES cell usually is expected to have the capacity to produce both gametes and all somatic-cell lineages.

Embryonal Carcinoma Cells

Prior to when ES cells were first isolated and cultured from preimplantation embryos, developmental biologists studied stem cells with ES-like properties found in spontaneously occurring tumours. Certain strains of laboratory mice were known to develop gonadal tumours called teratocarcinomas, which contain numerous differentiated cell types in addition to undifferentiated embryonal carcinoma (EC) cells. EC cells were known to proliferate and to maintain their malignancy in culture. If EC cells were injected under the skin of a histocompatible host, a tumour developed from the injected cells. If EC cells were injected into the blastocoel of a blastocyst, and the blastocyst was transferred to the reproductive tract of a recipient female mouse, the injected cells could combine with cells of the host embryo to differentiate and develop into tissues and organs of a normal mouse pup. These pups,

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chimeras because their cells had two distinct embryonic origins, had no higher incidence of tumour formation than other mice. Gametes produced by some of these chimeras were derived not only from the host embryo but also from the EC cells.

Discovery and Use of Embryonic Stem Cells

The similarities between EC cells and early embryonic cells did not go unnoticed, and researchers searched for methods to produce EC-like cells directly from embryos. In 1981, two laboratories independently accomplished this feat (Evans and Kaufman, 1981; Martin, 1981). The embryo-derived cell lines isolated in culture were given the name embryonic stem cells or ES cells to distinguish them from EC cells. ES cells were shown to survive indefinitely in culture, to survive freezing and thawing, and to be capable of tumorigenesis when injected under the skin of a histocompatible host. These cells also retained the capacity to develop into normal tissues, including gametes, after blastocyst injection. In fact, ES cells were shown to have a higher capacity than EC cells to contribute to both somatic and germ cell lineages (Bradley *et al.*, 1984).

ES cells are typically isolated in culture from blastocyst-stage embryos. More specifically, they develop from the inner cell mass (ICM) of the blastocyst, at least some cells of which are thought to be undifferentiated. The other cells of the blastocyst, the trophectoderm, appear to have undergone differentiation and are incapable of producing ES cells. ES cells have become an important experimental model for research involving embryogenesis, cell-lineage studies and the study of stage- and cell-specific gene expression. A great deal of interest in ES cells, and why they are a subject for presentation at this conference, has developed from their capacity to integrate foreign DNA. Unlike the random integration into the genome that occurs with microinjection of DNA into embryos, ES cells can be used to target a specific site in the genome for genetic engineering. Low-frequency events such as homologous recombination, leading to gene replacement and gene knockout, can be exploited in ES cells because of their capacity to proliferate into large numbers in culture. Even if only a few cells of the millions growing in a culture plate undergo the appropriate genetic change, various enrichment techniques allow the desired cells to survive and grow in culture, while cells without the appropriate genetic change have a reduced ability to survive. The surviving colonies are then screened for a homologous recombination event.

Despite the potential to genetically engineer ES cells in ways not possible directly with embryos, the genetic modification can be introduced into an animal only if the engineered ES cells retain their capacity for normal differentiation and development into somatic and germ cells. Two methods are possible to produce a living animal from ES cells. The most

common method is blastocyst injection whereby ES cells are injected into the blastocoel of a blastocyst. The injected cells can incorporate into the ICM of the host blastocyst and contribute to development of a chimera. If the chimeric animal produces gametes from the ES-cell line, a genetic change introduced into the ES cells can become established in a line of animals. A technique that has been developed more recently, and that has certain advantages over blastocyst injection, is nuclear transfer. Here the nucleus of a donor ES cell is introduced into an enucleated oocyte to produce a diploid embryo whose nuclear DNA is derived solely from the ES cell. An advantage of nuclear transfer over blastocyst injection for producing an animal from an ES-cell line is that nuclear transfer does not involve the uncertainty of germline transmission of the ES-cell genotype. With blastocyst injection, the chimeric animal might produce gametes only from the host embryo, or from the ES cells only at low frequency. A nuclear-transfer offspring will have the ES-cell genome in all cells of its body, ensuring that all gametes contain the ES-cell genotype. Despite rapid advances in nuclear-transfer technology, term development of nuclear-transfer embryos using ES cells as nuclear donors has not yet been reported.

Embryonic Stem Cell Technology and Species Specificity

To date, research with ES cells has been conducted almost exclusively with the laboratory mouse. The explanation for this limited application is quite simple: isolation of ES cells has not been accomplished unequivocally in other species, including in domestic livestock species. This fact could go unnoticed if one were to rely on the titles of many research articles published over the past decade in which the subject of the paper is proclaimed to be ES cells of one non-murine species or another. In fact, few of these papers provide results that adequately document that the cells under study meet the criteria to be called ES cells. Species for which the isolation of putative ES cells has been reported include the mouse (Evans and Kaufman, 1981; Martin, 1981), sheep (Handyside *et al.*, 1987), hamster (Doetschman *et al.*, 1988), pig (Piedrahita *et al.*, 1990a,b), cattle (Evans *et al.*, 1990), mink (Sukoyan *et al.*, 1992), rabbit (Giles *et al.*, 1993; Graves *et al.*, 1993), rat (Iannaccone *et al.*, 1994), monkey (Thompson *et al.*, 1995) and goat (Meinecke-Tillmann and Meinecke, 1996). This chronological listing of reports for the various species does not in all cases include papers that might be considered to contain the most convincing data, only papers that were among the first to be published for that species. If only species are listed for which chimeric offspring have been obtained after injection of putative ES cells into blastocysts, the list is considerably shorter. These species include the mouse (Evans and Kaufman, 1981; Martin, 1981), pig (Wheeler, 1994) and rabbit (Giles *et al.*, 1993; Schoonjans *et al.*, 1996). This already short list is unusual in the fact that it recently became shorter instead

of longer. In 1994 the isolation of rat ES cells that produced chimeras after blastocyst injection was reported (Iannaccone *et al.*, 1994). Recently, the paper was retracted due to concern that the rat 'ES cells' had become contaminated with mouse ES cells, and the chimeric animals were really rat–mouse interspecies chimeras (Iannaccone *et al.*, 1997). The list of species in which ES-cell isolation has been documented by chimeras with demonstrated germline chimerism (i.e. transmission of the ES-cell genome in the gametes) after blastocyst injection is even shorter; only for the laboratory mouse has this criterion been met.

What accounts for the discrepancy between the lists of species for which ES-cell isolation has been reported versus the single species for which ES-cell isolation has been fully documented with germline chimerism? The discrepancy results from the different criteria researchers have used to describe their 'ES cells' from various species. One criterion used to identify cultured embryonic cells as ES cells is a determination of their having morphology and *in vitro* developmental characteristics similar to those of murine ES cells. In other words, if the cells were cultured from an early embryo, and they looked and behaved in culture like murine ES cells, they sometimes have been described as being ES cells. Some investigators, especially among the earliest publications on the subject, used only these morphological criteria to classify their cells as ES cells. A more stringent evaluation of a cell line would include not only morphological features but developmental criteria as well. Mouse ES cells are known to undergo differentiation *in vitro*, which can either occur spontaneously or be induced. Many investigators now include *in vitro* differentiation in their descriptions of how their cell lines resemble what we know to be ES cells. The most stringent criterion for identifying ES cells is their capacity to differentiate *in vivo*. As described in the previous paragraph, for only a few species have ES-like cells been shown to be capable of *in vivo* differentiation resulting in a chimera after blastocyst injection; only in the mouse has chimerism from ES cells been shown to extend to the germline.

Conditions for Isolation of Embryonic Stem Cells in Culture

Several conditions must be met in order for ES cells to be isolated *in vitro*:

1. Undifferentiated, pluripotent cells must be present in the embryo at the time of culture. This requirement may seem obvious, but for most species the precise stage of development appropriate for isolation of ES cells has not been determined.
2. The pluripotent cells must be deprived of differentiation signals in culture.
3. The cells must be stimulated, or at least be allowed, to proliferate.

We conducted experiments to determine whether, as in the mouse, transplantable undifferentiated cells could be isolated from porcine

blastocysts. We isolated porcine ICM by immunosurgery and injected them into host blastocysts for embryo transfer and development to term. Chimeric pigs were born having characteristics similar to murine chimeras (Anderson *et al.*, 1994). Some of these chimeric pigs were shown to be germline chimeras, a requirement for ES cell technology to be useful for genetic modification. Our results confirmed that day-8 blastocysts were a source of undifferentiated embryonic cells; stimulation of proliferation and inhibition of differentiation of these cells in culture were the remaining challenges.

Culture conditions for early experiments aimed at defining conditions under which ICM-derived cells from livestock species will survive *in vitro* were based on culture systems previously defined for isolation of mouse ES cells, namely Dulbecco's modified Eagle's medium supplemented with fetal calf serum plus other additives and cultured over a monolayer of murine STO cells. Cell lines could be established from ICM culture, and these cell lines frequently maintained a morphology similar to mouse ES cells. Some cell lines were demonstrated to be pluripotent by virtue of their capacity to differentiate *in vitro* into various cell types (Piedrahita *et al.*, 1990a), but attempts to demonstrate *in vivo* differentiation were unsuccessful. These early results, though encouraging, fell short of actually documenting the isolation of ES cells. Experiments followed in which various homologous and heterologous feeder layers, or other slight modifications to the culture system, were tested (Piedrahita *et al.*, 1990b). Generally, few of these modifications to the culture system yielded results beyond those possible with culture conditions widely used for isolation of murine ES cells. Even today, in times of scarce resources, these early experiments frequently are being repeated and are yielding similar results as new laboratories become involved with livestock ES-cell research.

Some levels of success have been achieved with *in vitro* maintenance of pluripotent cells from agricultural species. Sims and First (1993) cultured bovine embryonic cells in low-density suspension culture and produced four nuclear-transfer calves after using these cells as nuclear donors. A substantially larger number of nuclear-transfer pregnancies was established, but most failed prior to term. Stice *et al.* (1996) used culture conditions more typical of those for murine ES cells to isolate ES-like cells from both *in vitro*- and *in vivo*-derived bovine embryos. In a large experiment involving more than 3000 nuclear-transfer embryos, between 12 and 40%, depending on the cell line, of the nuclear-transfer embryos developed to the blastocyst stage in culture. Up to 30% of embryo transfer recipients for these blastocysts were diagnosed pregnant at approximately 30 days of gestation, but only 20 days later at approximately 50 days gestation the percentage of pregnant recipients was only 0–15% for the various cell lines. None of the nuclear-transfer pregnancies developed to term. The authors speculated that failure of placental development was responsible for loss of pregnancy. A possible explanation for abnormal placental development in nuclear-transfer

embryos from ES cells is the stage of imprinting in the ICM and hence in ES cells, but this theory has not been proven.

Absent from this discussion of the more successful attempts at ES-cell isolation in agricultural species are the nuclear-transfer results of Campbell *et al.* (1996) and Wells *et al.* (1997) describing the birth of lambs after use of embryo-derived cell lines as nuclear donor cells. These exciting results will not be discussed here, because they are included in Chapter 5 by Dr Ian Wilmut, and also because the cell lines used by these investigators likely were not ES cells. In the first paper in which cultured embryonic cell lines were used to produce viable nuclear-transfer embryos, the authors stated:

> The (cell) line was established from early passage colonies with a morphology like that of ES cells. By the second and third passages, the cells had a more epithelial, flattened morphology … At passage 6, unlike murine ES cells, they expressed cytokeratin and nuclear lamin A/C, which are markers associated with differentiation (Campbell *et al.*, 1996).

Wells and colleagues (1997) also described their cell lines as having undergone some degree of differentiation in culture prior to nuclear transfer. Although some readers might view these distinctions as trivial given that, like ES cells, these embryo-derived cells (and differentiated somatic cells as well) could be useful for introducing genetic modifications into agricultural species, I believe that we should hesitate to refer to differentiated embryo-derived cells lines as ES cells. The biology of murine ES cells has a rich history, and researchers newly working with embryo-derived cell lines from livestock species should refrain from redefining terms based on how the cells might be used and for now should rely on definitions originally based on morphology, expression of ES-specific markers and developmental capacity.

Culture of Primordial Germ Cells and Isolation of Embryonic Germ Cells

Using information gained from research with murine embryos and ES cells, some laboratories (including ours) have worked for nearly a decade to establish conditions for isolation of ES cells in livestock species. Progress continues to be made but, while awaiting the significant breakthroughs that will facilitate isolation of ES cells in these species, alternative approaches must be considered. In 1992, two groups independently reported that pluripotent stem cells could be isolated from cultured primordial germ cells (PGC). PGC are embryonic cells that are the precursors to gametes in the adult (i.e. those cells all of whose surviving descendants form gametes). They are thought to be derived from extra-embryonic layers and to migrate into the embryo to the genital ridge. Matsui *et al.* (1992) and Resnick *et al.*

(1992) observed that when murine PGC are cultured in medium containing a cocktail of growth factors including stem cell factor, leukaemia inhibitory factor and basic fibroblast growth factor, the cells will survive and proliferate beyond the point at which they normally undergo mitotic (male germ cells) or meiotic (female germ cell) arrest in the gonad. With continued culture, the PGC lost their migratory capacity, attached to a monolayer of feeder cells and formed colonies resembling murine ES cells. The cells, designated embryonic germ (EG) cells to distinguish them from ES cells of ICM origin, were shown to share many characteristics with ES cells, including morphology, cellular markers and the capacity to differentiate *in vitro*. When injected into blastocysts, like ES cells, EG cells were shown to be capable of differentiating into normal cells of a chimeric mouse, including germ cells (Stewart *et al*., 1994). Available results from research with ES cells far exceed those with EG cells, but EG cells appear to be potentially useful for manipulations otherwise currently limited to ES cells. Debate continues over whether or not EG and ES cells, despite their different origins, are identical cell types.

In an effort to isolate porcine EG cells, we were able to collect, on average, approximately 15,000 PGC from each day-25 porcine embryo (Shim and Anderson, 1998). The cells resembled murine PGC in morphology and stained positive for alkaline phosphatase activity, a marker for murine PGC as well as for undifferentiated murine ES, EC and EG cells. When porcine PGC were cultured over a STO feeder monolayer in medium with or without growth factor supplementation, porcine PGC survived and proliferated without addition of growth factors required by murine PGC (Shim and Anderson, 1998). With prolonged culture, these cells attached to the monolayer of STO feeder cells and formed colonies similar in appearance to porcine ES-like colonies (Piedrahita *et al*., 1990a,b). Upon injection into host blastocysts, the PGC-derived cells were shown to have the capacity to differentiate into normal tissues of a chimeric piglet (Shim *et al*., 1997). Analysis of microsatellite DNA revealed that the EG cells had contributed to most somatic tissues tested. Germline transmission of porcine EG cells has yet to be demonstrated. Our results demonstrate that, as in the mouse, porcine PGC can be cultured to produce pluripotent stem cells. We are currently evaluating these cells for their ability to integrate foreign DNA as a vehicle for introducing changes into the porcine genome. One explanation for success in isolation of porcine EG cells is the substantially larger number of cells available to initiate culture from the genital ridge of day-25 porcine embryos compared with the ICM of day-8 blastocysts.

Another success at PGC culture in an agricultural species has been published in the popular press. A private company has been reported as having isolated and cultured PGC from day-30 bovine embryos. The cultured PGC ultimately were used as nuclear donors for nuclear-transfer experiments. Like most nuclear-transfer results from having used cultured bovine ICM and ES cells as nuclear donors (Sims and First, 1993; Stice *et al*.,

1996), pregnancies from nuclear transfer using EG cells were lost prior to term. When cells from nuclear-transfer embryos were retransplanted to produce second-series nuclear-transfer embryos, resulting pregnancies did survive to term. If these procedures prove to be reproducible, cultured PGC and EG cells could be useful for introducing genetic changes into the bovine genome. A description of these results is not yet available in the scientific literature, but information can be obtained over the Internet at www.absglobal.com.

Summary and Conclusions

ES cells have been demonstrated to be a powerful vehicle for targeting a specific site in the genome. To date, the technology has been developed and used almost exclusively in the laboratory mouse. Numerous embryo-derived cell lines have been established and described for agricultural species, but as yet none has yielded germline chimerism; however, some of these cell lines have yielded somatic chimerism. As an alternative to ES-cell culture, PGC can be cultured to produce EG cells with developmental capabilities similar to ES cells. The significant advances recently made in nuclear transfer using differentiated embryonic cells and somatic cells as nuclear donors have not been widely tested using ES and EG cells, and past experiments with disappointing results (e.g. low embryo and fetal survival) probably should be repeated. To end on an encouraging tone – despite lingering unanswered questions, steady progress continues toward isolation of the elusive undifferentiated stem cells in agricultural species.

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