Manipulation of Blastodermal Cells

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ABSTRACT Blastodermal cells isolated from newly
laid, unincubated eggs are virtually uncommitted cells
that exhibit many of the properties of pluripotential
stem cells. They can be transferred from donor to
recipient embryos and contribute to both somatic tissues
and the germline. Blastodermal cells that have been
maintained in culture for 7 d express the epitopes
ECMA-7 and SSEA-1, which are also expressed by
mouse embryonic stem cells. After culture for up to at
least 7 d, blastodermal cells retain the ability to
differentiate into somatic tissues and the germline both
in vivo and in vitro. Proliferation in the absence of
differentiation of blastodermal cells is stimulated by the
presence of Leukemia Inhibitory Factor (LIF) and other
ligands that interact with the gp130 receptor, and
differentiation is stimulated by exposure to retinoic acid.
Blastodermal cells also possess high levels of telomerase
activity, which is shared by immortalized cells and cells
within the germline. Blastodermal cells can be trans-
fected and will express foreign genes both in vivo and in
vitro. Transfected cells can be isolated by fluorescence
activated cell sorting and can be cryopreserved without
losing their ability to contribute to either somatic tissues
or the germline. These properties of blastodermal cells
make them ideal vectors for introducing genetic modifi-
cations to the germline.

(Key words: pluripotential precursors, stem cells, blastodermal cells, chimeras)

1997 Poultry Science 76:1075–1083

INTRODUCTION

Between fertilization and development of a fully
formed embryo, cell division is usually accompanied by
differentiation. Within the first few divisions, cell
lineages are formed and cells within these lineages are
committed to specific fates such as development of skin,
eyes, bone, and nerves. In mice, a few cells can give rise
to an uncommitted (i.e., pluripotent) embryonic stem
cell lineage and it is postulated that an equivalent
lineage exists in, or can be derived from, other vertebrate embryos.

Fertilization in the hen occurs in the infundibulum of
the reproductive tract within 15 min following ovulation
and the first nuclear division is usually in progress
when the egg enters the shell gland approximately 5 h
after fertilization. During the next 18 to 20 h, cell
divisions occur at approximately 45-min intervals to
produce an embryo containing 40,000 to 60,000 cells at
oviposition. The extremely short cell cycle throughout
this initial “oviductal” phase of development probably
precludes irreversible commitment to differentiation of
all cells within the embryo during this stage of
development. It seems likely, therefore, that populations
of cells derived from Stage X (Eyal-Giladi and Kochav,
1976) embryos contain pluripotential precursors that can
contribute to a diverse array of tissues.

The prediction that there are populations of “em-
broynic stem” cells within Stage X embryos is supported
by their undifferentiated morphology and their ability to
contribute to the germline, ectodermal, mesodermal, and
endodermal lineages (Petitte et al., 1990; Watanabe et al.,
1992; Carsience et al., 1993; Fraser et al., 1993; Thoraval
et al., 1994; Kagami et al., 1995; Etches et al., 1996; Pain et
al., 1996). Although the cells within Stage X (Eyal-Giladi
and Kochav, 1976) embryos have a relatively uniform
morphology, they may not be uniformly undifferen-
tiated and are unlikely to respond as a single population
when transferred to different environments in vivo or in
vitro. For example, some cells within Stage X (Eyal-
Giladi and Kochav, 1976) embryos express the EMA-1
epitope that is produced by primordial germ cells
(Urven et al., 1988). Cells taken from the posterior, but
not the lateral, edges of Stage X embryos (Eyal-Giladi
and Kochav, 1976) can induce formation of the primitive
streak (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-
Giladi, 1989) and cells from the central disk of Stage X
embryos (Eyal-Giladi and Kochav, 1976) form somatic
and germline chimeras approximately 10 times more
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TABLE 1. Contributions of cells grown on feeder cells, of cells grown in the absence of feeder cells and of explanted embryos to somatic tissues and the germline following injection of approximately 500 cells into the subgerminal cavity of irradiated recipient embryos. In some cases, the cultures were enriched with 20 ng/mL of Leukemia Inhibiting Factor (LIF), 20 ng/mL basic Fibroblast Growth Factor (FGF), and 100 ng/mL chicken Stem Cell Factor (SCF). From Etches et al. (1996).

<table>
<thead>
<tr>
<th>Method of culture</th>
<th>Number of chimeras hatched</th>
<th>Number of somatic chimeras</th>
<th>Number of putative chimeras</th>
<th>Number of germline chimeras among:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolayer</td>
<td>52</td>
<td>4 (8%)</td>
<td>48 (92%)</td>
<td>Somatic chimeras 0/2 Putative chimeras 0/2</td>
</tr>
<tr>
<td>Feeders with LIF, FGF, SCF and mercaptoethanol</td>
<td>14</td>
<td>6 (43%)</td>
<td>8 (57%)</td>
<td>Somatic chimeras 1/1 Putative chimeras 1/1</td>
</tr>
<tr>
<td>Feeders without LIF, FGF, SCF and mercaptoethanol</td>
<td>24</td>
<td>10 (42%)</td>
<td>14 (58%)</td>
<td>Somatic chimeras 0/2 Putative chimeras 2/2</td>
</tr>
<tr>
<td>Explants</td>
<td>37</td>
<td>8 (22%)</td>
<td>29 (79%)</td>
<td>Somatic chimeras 2/2 Putative chimeras 0/2</td>
</tr>
<tr>
<td>No culture, fresh cells</td>
<td>22</td>
<td>17 (77%)</td>
<td>5 (23%)</td>
<td>Somatic chimeras 7/1 Putative chimeras 1/1</td>
</tr>
</tbody>
</table>

1Esgro, Australia.  
2Sigma Chemical Co., St. Louis, MO 63178-9916.  
3Kindly provided by Amgen, Thousand Oaks, CA 91362.  
4Somatic chimeras are distinguished at hatch by the presence of black down; putative chimeras develop from injected embryos but do not exhibit donor-derived plumage.

The contributions of cells grown on feeder cells, of cells grown in the absence of feeder cells and of explanted embryos to somatic tissues and the germline following injection of approximately 500 cells into the subgerminal cavity of irradiated recipient embryos. In some cases, the cultures were enriched with 20 ng/mL of Leukemia Inhibiting Factor (LIF), 20 ng/mL basic Fibroblast Growth Factor (FGF), and 100 ng/mL chicken Stem Cell Factor (SCF). From Etches et al. (1996).

**Culture for 48 h**

In an initial series of experiments, Stage X embryos were maintained in medium 199 containing fetal bovine serum (FBS), fibroblast growth factor (bFGF), stem cell factor (SCF), and insulin-like growth factor-1 (IGF-1) as explanted whole embryos, as dispersed cells isolated from the central region of the embryo, or as dispersed cells grown on a confluent layer of feeder cells (Etches et al., 1996). Cells cultured in each of these situations develop a unique morphology shown in Figure 1, but in each case, at least some cells contributed to both somatic tissues and the germline (Table 1). Although these data provided evidence that uncommitted cells could be maintained in vitro, they also demonstrated that the contributions to the somatic tissues and the germline can be unequal and unrelated. For example, the use of mouse embryonic fibroblast feeder cells greatly increased the frequency of somatic chimerism, but the frequency of germline chimerism was unchanged (Table 1).

**Culture for 7 d**

Mouse embryonic stem cells are distinguished by their endogenous alkaline phosphatase activity (Strickland et al., 1980), by their expression of the Stage Specific Embryonic Antigen-1 (SSEA-1) (Solter and Knowles, 1978) and by expression of the antigen recognized by Embryonal Carcinoma Monoclonal Antibody-7 (ECMA-7) (Kemler et al., 1981). Cultures of chicken blastodermal cells also contain islands of alkaline phosphatase positive cells (Figure 2) and in many cases, alkaline phosphatase positive cells also express SSEA-1 and ECMA-7 (Figure 3).

**TABLE 2. Production of somatic and germline chimeras from cells carrying pm{iwZ} that were isolated by fluorescence activated cell sorting (Speksnijder, 1996)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Embryos injected with cells:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expressing lacZ</td>
</tr>
<tr>
<td>Number of embryos surviving to &gt; 14 d</td>
<td>69</td>
</tr>
<tr>
<td>Number and percent of somatic1 chimeras</td>
<td>37 (53%)</td>
</tr>
<tr>
<td>Percentage donor-derived plumage</td>
<td>19</td>
</tr>
<tr>
<td>Number of germline chimeras/Number of somatic chimeras</td>
<td>6/9</td>
</tr>
<tr>
<td>Number of germline chimeras/Number of putative2 chimeras</td>
<td>0/6</td>
</tr>
<tr>
<td>Percentage germline transmission</td>
<td>19%</td>
</tr>
</tbody>
</table>

1Somatic chimeras are identified at hatch by donor-derived plumage.  
2Putative chimeras have been injected with donor cells but do not have donor-derived plumage.  
3The individual rates of germline transmission were 1,2,3,4 and 99%.
FIGURE 1. The morphology (100×) of Stage X embryos maintained as explants (A), maintained as dispersed cells (B), and maintained as dispersed cells on mouse embryonic feeder cells (C). Within an explant, some cells at the periphery become epithelial like whereas some cells within the interior of the explant retain an undifferentiated morphology (D). From Etches et al. (1996).
In addition, both mouse embryonic stem cells and chicken blastodermal cells express an epitope that is detected on mouse and chicken primordial germ cells by the antibody EMA-1 (Figure 3). Both the expression of alkaline phosphatase activity and the epitope profile of blastodermal cells have been used to evaluate the addition of various cytokines and growth factors that support the growth of blastodermal cells with an undifferentiated morphology.

Proliferation of undifferentiated mouse embryonic stem cells is promoted by members of a family of cytokines that act through the gp130 receptor including leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostation (OSM), interleukin-6 (IL-6), and interleukin-11 (IL-11) (Yoshida et al., 1994). Addition of each of these cytokines to cultures of chicken blastodermal cells increases the number of alkaline phosphatase positive colonies. Therefore, blastodermal cells were routinely cultured in the presence of a mixture of LIF, IL-6, and IL-11. The removal of LIF from the culture decreased the proportion of cells that expressed the EMA-1, SSEA-1, and ECMA-7 epitopes within 12 d (Figure 4).

To further induce the proliferation of blastodermal cells in the absence of differentiation, endogenous retinoic acid in the media was rendered inactive as a complex with a monoclonal anti-retinoic acid antibody. Retinoic acid is known to be a strong inducer of differentiation in many animals including birds and will induce differentiation of chicken blastodermal cells (see below).

Cell lines that have been isolated from spontaneous tumors, transformed cell lines, cells committed to the germline, and mouse embryonic stem cells contain high levels of telomerase (Kim et al., 1994; Pain et al., 1996). Telomerase is a ribonucleoprotein that synthesizes telomeric DNA onto chromosome ends using a segment of its RNA as a template. Cells that have a limited life span lack telomerase activity and, therefore, their telomeres shorten by 50 to 200 base pairs at each cell division. Senescence is believed to occur in mortal cell lines when replication is no longer possible because the telomere is too short. Blastodermal cells maintained in culture have high levels of telomerase activity (Figure 5), which is immediately lost after exposure of the culture to retinoic acid. It appears likely, therefore, that blastodermal cells share this characteristic of cells that are immortalized or committed to the germline.

Blastodermal cells maintained in culture for 7 to 8 d are capable of contributing to both somatic tissues and to the germline after injection into Stage X (Eyal-Giladi and Kochav, 1976) recipient embryos. More than 50% of the chicks that hatched contained donor-derived pigmentation in their plumage and on average, 33% of the plumage pigmentation was donor-derived. To date, two germline chimeras have been identified among chimeras made with cells cultured for 7 to 8 d (Pain et al., 1996 and unpublished observations).

When differentiation of cultured cells was induced by transfer to non-tissue culture dishes in the absence of LIF, embryoid-like bodies were formed and differentiation was initiated. Embryoid-like bodies are composed of an outer layer of “endodermal-like” cells enclosing a fluid filled cavity and an internal layer of “ectodermal-like” cells (Robertson, 1987). After 10 d, some cells exhibited the morphological features of nerve cells (Figure 6). Differentiation of ectodermally derived tissues in the embryoid body was confirmed by staining the putative nerve cells with an antibody raised against neural cell adhesion molecule (N-CAM). Morphologically functional muscle cells were also observed in the culture and stained with an anti-myosin antibody to confirm the derivation of a mesodermal tissue within the culture. Differentiation of mesoderm was also confirmed by the presence of hematopoietic precursors in the culture (Figure 6).

It is clear, therefore, that the population of cells extracted from Stage X embryos contains precursors that contribute to the somatic tissues and the germline. These cells can be induced to differentiate by appropriate stimuli but will proliferate without differentiating in the presence of LIF, CNTF, OSM, IL-6, and IL-11. Future effort will be directed to identification of the cell type or types that give rise to each of the somatic tissues and the germline.

**FIGURE 2.** Cultures of mouse embryonic stem cells (A) and chicken blastodermal cells (B) contain colonies of undifferentiated cells that are alkaline phosphatase positive. From Pain et al. (1996).
FIGURE 3. After 5 d in culture, alkaline phosphatase positive chicken blastodermal cells (A) usually express ECMA-7 (B). Similar cells shown in phase contrast in (C) are SSEA-1 positive (D). Both mouse embryonic stem cells and chicken blastodermal cells (shown in phase contrast in (E) and (G), respectively are EMA-1 positive (F and H, respectively). From Pain et al. (1996).
Transfection

Foreign DNA has been introduced into chicken blastodermal cells by lipofection and cells expressing the introduced gene have been incorporated into somatic tissues (Brazolot et al., 1991; Fraser et al., 1993). The process of lipofection, however, requires extensive exposure to the lipofection reagents, which may have the potential to alter differentiation of the cells. Whereas other methods of introducing DNA into cells have received considerable attention in mammalian species, they have not been widely investigated in birds.

Transfection of foreign DNA into chicken blastodermal cells can be accomplished effectively using electroporation to produce populations in which more than 50% of the cells express the transgene (Zajchowski and Etches, unpublished data) (Figure 7). Injection of these cells yielded somatic chimeras indicating that the cells were not debilitated from either the exposure to electroporation conditions or the presence of the plasmid pmiwZ, which encodes the bacterial enzyme β-galactosidase. When exposed to 250 V, however, both the frequency and extent of somatic chimeras was lower than expected but more than 90% of the chicks had incorporated electroporated donor cells when exposure was reduced to 200 V. Although electroporation is attractive because blastodermal cells are exposed to in vitro conditions for only a brief period before being introduced to a recipient embryo, the bacterial lacZ gene was not detected in any of the chimeras following extraction of DNA from erythrocytes and amplification of the gene by PCR. The absence of the lacZ construct in tissues from chimeras made with electroporated cells indicates that it is stably integrated into the genome of chicken blastodermal cells only rarely.

Isolation of Transfected Cells by FACS

The probability of obtaining offspring from a chimeric chicken carrying a genetic modification that was introduced into donor blastodermal cells is the product of the individual probabilities of 1) the rate of germline transmission, 2) the formation of germline chimeras, and 3) the frequency of stably transfected cells within the injected population that have retained the ability to enter

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fresh cells</th>
<th>Nycoprep gradient</th>
<th>Percoll gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of chimeras tested</td>
<td>16</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Number of germeline chimeras</td>
<td>9</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Germline transmission, %</td>
<td>30</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
FIGURE 6. Differentiation of blastodermal cells in culture. In the presence of LIF the culture remains undifferentiated (A). Removal of LIF and transfer to a non-tissue culture dish induces differentiation into an embryoid-like body (B). Nerve cells differentiate from the culture and are evident under phase contrast (C) and after staining with N-CAM. Hematopoietic precursors can also be observed under phase contrast (E) and after staining with MEP21 (F). Muscle cells differentiated in the culture and were viewed under phase contrast (G) and after staining with anti-myosin (H). From Pain et al. (1996).
Hoefer Scientific Instruments) was set at 200 V and 980 μF with a discharge interval of 1 s. The cells were then cultured for 45 h and stained containing 10 mM MβCD. Cells were exposed to 4 mM 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG) and FACS (Speksnijder, 1996).

Isolation of cells by FACS requires that cells be held for up to 27 h before injection without compromising the capacity of blastodermal cells to contribute to the germline. After exposure to pmiiwZ and Lipofectin™ as described by Brazolot et al. (1991) and Fraser et al. (1993) the cells were incubated in conditioned medium from mouse SNL 76/7 fibroblast cells (kindly provided by Alan Bradley, Baylor College of Medicine, Houston, TX 77030). The cells were then incubated with C12FDG and transfected cells were isolated using a Coulter EPICS Elite ESP FACS. The cells were then either cultured for 3 d or immediately injected into recipient embryos. Cells in which the lacZ gene was expressed contributed to both somatic tissues and the germline of chimeras at approximately the same frequency as cells from the negative pool from the FACS sorter (Table 2) indicating that cells expressing the foreign gene were neither debilitated nor prevented from differentiating into somatic tissues or the germline. After 3 d in culture, exposure to the chromogenic substrate, X-gal revealed that essentially all of the cells in which β-galactosidase activity was detected by FACS continued to express β-galactosidase. The lacZ gene was detected in three chimeras between hatching and sexual maturity by PCR or Southern analysis indicating that pmiiwZ had integrated into some cells that contributed to somatic tissues. The lacZ gene, however, was not detected in sperm or offspring from any of these birds (Speksnijder, 1996).

Frozen-Thawed Cells

Analysis of genetic modifications to blastodermal cells could be facilitated if donor cells were cryopreserved. For example, genetically modified cells could be maintained as a frozen stock while the nature of the genetic modification was determined. If the desired changes were present, the cells could be thawed and injected to make germline chimeras that would transmit the genetic modification to their donor-derived offspring. In an initial series of experiments, Reedy et al. (1995) demonstrated that somatic chimeras could be made using cryopreserved blastodermal cells, although the viability of cells was compromised and donor-derived contributions to the resulting chimera were reduced. Subsequently, the cooling and warming rates for blastodermal cells were optimized and density gradient centrifugation was used to separate damaged cells from the population (Kino et al., 1995, 1997). These modifications to the protocol increased the frequency and extent of donor-derived somatic contributions to the germline to a level that was not significantly different from that observed using fresh cells. In addition, cryopreserved cells entered the germline (Table 3) although the frequency and extent of donor-derived contributions were lower than that observed when fresh cells were injected. Nevertheless, it is clearly possible to maintain the genome of blastodermal cells in a frozen state and reinstate genetic information within the germline after injection into recipient embryos. The ability to freeze and thaw blastodermal cells provides the opportunity to evaluate modifications to the genome of blastodermal cells and in addition provides a technology that can be used to cryopreserve and reconstitute stocks that are potentially useful but not in current use (Reedy et al., 1995; Kino et al., 1997).

ACKNOWLEDGMENTS

This work was supported in part by the Ontario Ministry of Agriculture, Food and Rural Affairs and the Natural Sciences and Engineering Research Council of Canada.

REFERENCES


Eyal-Giladi, H., and S. Kochav, 1976. From cleavage to sexual maturity by PCR or Southern analysis indicating that pmiiwZ had integrated into some cells that contributed to somatic tissues. The lacZ gene, however, was not detected in sperm or offspring from any of these birds (Speksnijder, 1996).

FIGURE 7. Cells expressing lacZ following transfection by electroporation. Cells were exposed to 4 μg of linearized pmiiwZ in Optimem™ containing 10 mM HEPES. The electroporator (PG 200 Progenetor II, Hoefer Scientific Instruments) was set at 200 V and 980 μF with a discharge interval of 1 s. The cells were then cultured for 45 h and stained with xgal (Zajchowski and Etches, unpublished data).

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