Production of Transgenic Animals Using Embryonic Stem Cells and Cloning

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Improved agriculture through transgenic technology has been intensely researched by both the private and public sectors. On the plant side a transgenic tomato have reached the market place. Also, there are several plant transgenic products that are being commercialized. However, transgenic farm animal have not reached the market place and none of the transgenic animals produced to date are expected to have a commercial impact. Why is this? One might suggest that farm animals have reached their limit of productivity. However, in cattle, genetic gain through traditional selective breeding is continually contributing to an annual increase in milk production of 200 pounds of milk per cow in the US with no signs of this progress tailing off. In addition, there are certain individual cows that produce two to three time more milk per year than the national average. Protein content in milk and percentage also varies considerably in the cow population. Again, some dairy cows produce three times the amount or percentage milk protein than the national average. Clearly, the average milk production and protein composition can be increased dramatically without seriously affecting the general physiology of the animal. Why is it then that most attempts to increase farm animal productivity through transgenics has failed to accomplish this goal? By far, the biggest obstacles in commercializing large animal transgenic technology are the low efficiency of the procedure, resulting in expensive and time consuming experiments and the inability to place important transgenes in specific and beneficial location in the genome.

Commercial benefits of domestic animal embryonic stem cells

Embryonic stem (ES) cell technology will have two major benefits in future attempts to commercialize transgenic farm animals. The first benefit will be the reduced cost of producing a transgenic animal. These costs can run into the millions of dollars for each calf when traditional microinjection techniques are used. An ES cell approach will reduce costs considerably by using fewer genetically valuable embryos. Secondly, ES cells enable the researcher to place new genes in advantageous places in the genome or to remove deleterious genes. Previous experiments using milk gene constructs have demonstrated that the position of a transgene in the genome greatly affects expression levels of a transgene in the mammary gland. These two principle advantages of ES cells will greatly facilitate progress towards the production of valuable transgenic cattle.

Researchers have found, by using microinjection techniques, approximately 1000 to 3000 one-cell embryos are required to produce one transgenic calf. The cost of producing this number of embryos is not insignificant, especially if the transgene must be injected into genetically valuable
embryos. Embryos derived from high value genetic parentage often sell for $1,000 to $5,000 each. If it was technically feasible to retrieve one-cell embryos from these valuable animals, which it is not, the cost alone would be prohibitive, and run in excess of $1,000,000. Even if an inexpensive source of embryos is obtained, the costs associated with maintaining a large recipient pool of surrogate females can be prohibitive. A pool of over two hundred recipients would likely be needed to produce one transgenic calf. Our experiences in the past suggest that total cost associated with such a pool are over $500,000 per year. Because pigs are litter bearing species the cost of producing a transgenic animal does go down. This might be as low as $25,000 for animals having a nonspecific genetic background but this cost could increase dramatically if the transgene needs to be inserted into valuable pedigree or grandparent stock. For cattle, one transgenic calf derived from top genetic embryos could easily cost between 2 to 20 million dollars.

Transgenic ES cells could be less labor intensive and less expensive than traditional methods of producing transgenic cattle. Currently, the efficiency of producing ES-like cell lines derived from pre-implantation embryos is approximately 50% (1). In other words, only two embryos are needed to produce a cell line. The cost of using a superior genetic background in transgenic studies is minimal if ES cells are used. Traditional transgenic technology has other economic drawbacks. Because of the limitations in the technology, a large number of non-transgenic embryos are transferred into recipient females to obtain a limited number of transgenic pregnancies. Transgenic ES cells would eliminate the transfer of nontransgenic embryos, greatly reducing recipient herd costs. ES cells can be screened for the transgenes as they are being cultured in vitro and only transgenic cells used in the production of offspring. Therefore, costs of producing a transgenic cow are greatly reduced if transgenic ES cell technology is developed.

Technically, the traditional microinjection technique has other drawbacks which ES cells might alleviate. ES cell and homologous recombination technologies can be used to insert a transgene into a specific location in the genome. Additional milk protein could be produced if the milk gene is inserted into a high expression region of the genome. Another possibility is to use ES cells to "knockout" a milk protein that is detrimental to cheese or yogurt production. These are just a few of the many opportunities cattle transgenic ES cell technology present, and would be impossible using current cattle transgenic technology.

Techniques used in developing transgenic pig and cattle ES cells are likely to be of value in the biomedical industry. Transgenic pig ES cells would be a breakthrough in the advancing field of xenotransplantation. Pigs generated from modified ES cells, may decrease rejections problems that result when a pig's organ transplant into a human. In particular, "knocking out" galactosyl epitopes in pig ES cells may prevent a large portion of the hyperacute immunological rejection in xenograft transplants.

**Production of mammalian embryonic stem cells**

In 1981, the term embryonic stem cells or ES cells was used to denote a cell line isolated directly from mouse embryos whereas, embryonal carcinoma cells (EC) were derived from teratocarcinomas (2,3). ES cells and EC cells share many characteristics. Morphologically, these cells were indistinguishable from each other (2). Initial descriptions of mouse ES cells relied on
how they were derived, cytogenetics, in vitro pluripotent properties and tumorigenicity. Since then, the in vitro characteristics of mouse ES cells analyzed and useful ES cell specific markers developed (4).

For many researchers, the definition and utility of ES cells changed when Bradley and coworkers (5) demonstrated that mouse ES cells formed stable germ-line chimeras (Fig. 1). This was important because it showed that ES cells' genetics were passed on to offspring. Thus, genetic modifications of ES cells in vitro resulted in transgenic offspring. Since then, germ-line transmission of the ES cell genetics has not been reported in any species other than the mouse. Therefore, domestic animal ES-like cell lines have not met this most stringent and useful ES cell characteristic.

Figure. 1. (a) ES cells (shaded areas) are cultured in a petri dish while new genes are added or endogenous genes disrupted (gene targeting). (b) Then ES cells are injected into a host embryo (black) to produce a chimeric embryo. (c) Chimeric offspring have some cells derived from the host embryo (black) and some cells derived from the ES cells (shaded). (d) Offspring derived from the germ-line chimera are either derived from the genetics of the ES cells or that of the host embryo.

**Screening candidate lines derived from pig embryos for possible ES cell characteristics**

Domestic animal ES-like cells have some in vitro characteristics in common with mouse ES cells. The presence or absence of intermediate filaments (cytokeratin) has been used to characterize domestic animal ES-like cells (6,7,8). When probed for cytokeratin, some cell lines were negative for this ES cell marker. In the mouse, cytokeratin is restricted to the trophoderm and not found in the inner cell mass (ICM) (9). However, unlike the mouse ES cells, the cytokeratin negative ES-like cells have not produced chimeric offspring (6,7). Thus, by itself, cytokeratin negative ES-like cells may not produce germ-line chimeras.

One marker often associated with undifferentiated mouse ES cells is alkaline phosphatase (AP) (10). Mouse ES cells lose AP activity upon differentiation in vitro. The ICM of sheep and pig
embryos is positive for AP, but cell lines derived from the ICM often lose AP activity after in vitro propagation (11). Since ES cells should be similar to ICM cells, there is reason to expect that an ES-like cell line should also be positive for AP.

Additional information is obtained if more than one marker is used to isolate potential ES cell lines. We have done this with porcine ES-like cell lines by testing them for both cytokeratin and AP. The use of these two very different markers has produced some very interesting results (Table 1). Two different types of cell lines have been isolated. Cell colonies from the first cell type have a flat epithelial appearance and are cuboidal. These cells are AP positive and cytokeratin positive. The second type of cell grows as multiple layers and is AP negative and cytokeratin negative. Therefore, neither of these two lines have the same marker characteristics that mouse ES cells possess.

Table 1. Morphological and cell marker characteristics of potential ES-like cell lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>morphology</th>
<th>Alkaline phosphatase activity</th>
<th>cytokeratin 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICM (type I)</td>
<td>flat, cuboidal</td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td>ICM (type II)</td>
<td>multilayer, small cells</td>
<td>absent</td>
<td>absent</td>
</tr>
</tbody>
</table>

The production of chimeric offspring is the most important test used to define a cell line as being an ES cell line. Under ideal circumstances, both ES-like cell lines would be tested for their ability to form germ-line chimeras; however, because of the large effort and expense in conducting these studies, relatively few cell lines can be thoroughly tested in a reasonable amount of time.

How can you facilitate testing for ES-like cell contribution to developing embryos?

A fast and easy method used to determine an ES-like cell lines ability to contribute to the developing embryo was performed in cattle embryos (1,12). Cattle ES-like cell membranes were labeled with a membrane specific fluorescent marker. Ten to 20 labeled, disaggregated cells were injected into the perivitelline space of 8-16 cell stage host embryos. After two to three days in culture, the host embryos were observed for labeled ES-like cell contribution to the ICM. ES-like cell contribution in host embryos ranged from little or none, to visibly high contribution. In most of the embryos (73%) some ES-like cell contribution to the ICM could be detected. These experiments serve as rapid initial tests suggesting that ES-like cells can contribute to the developing ICM. This is a starting point for more informative chimera experiments examining ES-like cell contribution to the developing fetus and offspring.

Determining chimerism in embryos and offspring using transgenic markers.

Fluorescent labeling of cell membranes works very well for short term embryo experiments, but for longer term studies (i.e. chimeric fetuses or offspring) the marker is lost due to dilution during growth. For longer term studies, transgene reporter genes or genome specific DNA microsatellite markers are two methods of tracking cell contribution to the fetus or offspring. The beta-
galactosidase gene (beta-gal) is extensively used to study contribution of mouse ES cells in chimera experiments. Similarly, numerous DNA microsatellite markers have been found in the porcine genome. Either method could be employed in determining pig ES-like cell contribution to developing chimeric fetuses and resulting offspring. However, a transgenic reporter gene is ideal because of ease of detection while also demonstrating that these cells can be genetically engineered. Transgenic ES cells contribution was detected in all three germ layers in 35 day bovine fetuses, including the primordial germ cells (12). The final proof that these cell lines are indeed ES cells will occur when the transgenic ES cells contribution can be detected in the progeny of the chimeric animals.

Cloned large domestic animals

There are several advantages to using nuclear transfer instead of chimera techniques. The nuclear transfer procedure can immediately test totipotency of the cells in question whereas, the chimera strategy requires testing offspring derived from chimeric individuals. In cattle, testing totipotency via chimeras is a long process, since a generation interval is nearly three years. Obviously, the elimination of one generation through cloning would be an important barrier to overcome. Embryonic or any cell line (fetal or adult) that can be easily cultured, genetically manipulate and be used to produce a transgenic animals would have tremendous utility. This is where cloning and transgenic merge.

There are several review articles that have explain the nuclear transfer procedure and nuclear reprogramming of donor nuclei( recent review, 13). In short, over the last several years considerable amount of research has focused on using cloning to test the in vivo potency of cultured embryonic cells or ES-like cell lines (14) and embryonic cell lines (1,15). This work was initiated because ICM cells, the cells which give rise to ES cells, produced live offspring when they were used as nuclear donors in the nuclear transfer procedure (16,17). However, it is now known that differentiated sheep cells can be used to produce cloned sheep (18).

Commercial uses of animal cloning technology

"Dollymania" has spread across the world, but lost in the excitement are the practical uses of cloning technology in animal agriculture and uses of cloned animals for biomedical purposes. Cloning of farm animals has been research and developed since 1987 when the first cloned lamb was born (19). However, Dolly was the product of an adult cell (18). This was an unexpected finding and suggests that the plasticity of adult sheep cells is greater than anyone had ever thought.

The idea of cloning genetically superior adult farm animals seems to have great potential for improved agriculture. This may not be true for some cases, since cloning only multiplies animals but does not contribute in itself to genetic progress. In the dairy industry, the idea of cloning progeny tested bulls has some appeal. However, by the time the clone of that bull is of reproductive age the genetics may be out of date. Progeny tested bulls are nearly five years old when they reach the AI companies lineup. These bulls are often replaced in less than three years by younger bulls having better "proofs". Therefore by the time a clone could be uses for AI,
nearly two years have passed. By this time some of the younger bull genetics may have exceeded the genetics of that clone which is seven years old now. In addition, the cloning efficiency using adult sheep cells appears to be significantly lower for than when embryonic donor cells are cloned (18). These two factors should be considered when determining the merit of cloning adult animals.

The cloning application that may be more useful for agricultural is the ability to efficiently produce a large number of identical offspring derived from a particular mating. Therefore, cloning using an embryonic cell lines derived from that mating may be more attractive (1,20). Selected mating are production tested while at the same time cloned and multiplied faster than conventional breeding. After several clonal lines are tested, the best lines are multiplied further. In the dairy industry, female clones with known production potential could be produced and then sold to producers. In the poultry industry fewer multiplier generations might be needed, thus reducing the time needed to get the genetics to market and possibly reducing production costs.

Animal cloning for use in the production of transgenic animals will most likely be first used in the biomedical industry, where certain isolated and sequenced genes are known to be useful. There are too many genes to list but they come under two specific and different applications. One is xenotransplantation. Certain genes, if added or removed from the pig's genome, will reduce the ability the human immune system to reject a transplanted pig organ. These genes are known and effective (reviewed in 21). There are currently over 200,000 people on the waiting list for organ transplants and the demand is expected to increase dramatically in the future. The supply of human organs is very limited. Cloning combined with transgenics in pigs could be the answer to the shortage of human organs for transplantation. The second area is the production of pharmaceutical proteins in the milk of animals (reviewed in 22). Two proteins produced in sheep and goats' milk are now in human clinical trials to test efficacy and safety. As the quantity of protein and its complexity increase there will be continued need for alternative production methods. The production of these proteins in milk and potentially avian eggs is an alternative.

The ES cell, cloning and transgenics procedures are only part answer in future attempts to improve animal agriculture. The other part is finding the right gene to be expressed in certain tissues at specific times during the growth and development of the animal. This area is progressing and with the efforts being put forward in animal genomics we will have improved animal production and disease resistance early the next millennium.

References