Transgenesis and Germ Cell Engineering in Domestic Animals

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ABSTRACT : Transgenesis is a very powerful tool not only to help understanding the basics of life science but also to improve the efficiency of animal production. Since the first transgenic mouse was born in 1980, rapid development and wide application of this technique have been made in laboratory animals as well as in domestic animals. Although pronuclear injection is the most widely used method and nuclear transfer using somatic cells broadens the choice of making transgenic domestic animals, the demand for precise manipulation of the genome leads to the utilization of gene targeting. To make this technique possible, a pluripotent embryonic cell line such as embryonic stem (ES) cell is required to carry genetic mutation to further generations. However, ES cell, well established in mice, is not available in domestic animals even though many attempt to establish the cell line. An alternate source of pluripotent cells is embryonic germ (EG) cells derived from primordial germ cells (PGCs). To make gene targeting feasible in this cell line, a better culture system would help to minimize the unnecessary loss of cells *in vitro*. In this review, general methods to produce transgenic domestic animals will be mentioned. Also, it will focus on germ cell engineering and methods to improve the establishment of pluripotent embryonic cell lines in domestic animals. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 6 : 910-927)***

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INTRODUCTION

The first transgenic mice by pronuclear injection were produced two decades ago (Gordon et al., 1980) and since then, transgenesis has had a great effect on basic as well as applied bioscience. While mice remain the major species of interest, transgenic domestic animals have recently become a powerful tool with multiple potential applications such as improvement of animal production, expression of valuable proteins in milk, as models of human genetic diseases, and organ sources using xenotransplantation (Wall, 1996).

The method to generate transgenic domestic animals is mainly pronuclear injection. Recently, the emergence of nuclear transfer using somatic cells adds an another way of producing transgenic domestic animals (Wilmut et al., 1997). However, the demand for precise manipulation of the genome, such as complete abolishment or subtle modification of genes, insertion of new genes, and chromosomal translocation, leads to the utilization of homologous recombination. This system requires embryonic stem (ES) cells as a carrier of desired mutations. Another important characteristic of ES cells is that they have ability to develop into any tissue when introduced into blastocysts (Köller and Smithies, 1992). Unfortunately, ES cells with the capability of germline transmission have not yet been isolated from domestic animals. An alternate source of pluripotent stem cells is embryonic germ (EG) cells established from primordial germ cells (PGCs) (Matsui et al., 1992; Resnick et al., 1992). EG cells have similar characteristics to those of ES cells, and more importantly, also have ability to transmit genetic mutations to the next generation by means of germline transmission (Stewart et al., 1994).

Many attempts have been made to make pluripotent ES and/or EG cells from domestic animals using the culture system developed for mouse ES cells (Piedrahita et al., 1999). However, due to differences in requirements for development, there has been no successful generation of ES cells with germline transmission using the mouse culture system. Therefore, if the culture environment were optimized to prevent unnecessary loss of cells during the initial period of culture, the chances of isolating pluripotent cells from domestic animals could be increased.

TRANSGENESIS IN DOMESTIC ANIMALS

The word “transgenesis” refers to the introduction of foreign genetic materials into the chromosome of a host organism. Transgenic technology, originally developed in the mouse system, has had a great impact on research in domestic animals targeted to the improvement of animal production (Wall, 1996). Before the development of this technology, the improvement of animal production had been achieved by genetic selection using conventional breeding strategies. However, this approach has several obstacles such as limitation of genetic variation to select a desired phenotype and requirement for considerable time to fix the genetic changes within a population.

Using transgenic technology, it is possible to achieve rapid and superior genetic change in a population and to

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exchange genetic information among different species. Furthermore, using homologous recombination, subtle manipulation of the genome becomes possible to generate desired genetic mutations (Capecchi, 1994). In conjunction with site specific recombination system, such as Cre-*loxP* system from bacteriophage P1 (Austin et al., 1981) and FRP-*FRT* system from *Saccharomyces cerevisiae* (Andrews et al., 1985), the possibilities for manipulating the genome are beyond our imagination. Unfortunately, there are several drawbacks such as large expense, lack of basic knowledge about the control of gene expression and the physiological effects of transgenes in animals, as well as lack of pluripotent stem cells. However, many researchers have pushed ahead to produce transgenic domestic animals not only for the improvement of animal production, but also for the production of valuable proteins, models for human genetic diseases, and for a source of organs for xenotransplantation. There are several methods available to produce transgenic domestic animals. These include pronuclear injection, nuclear transplantation, and transfer of genetically modified ES cells into host blastocyst.

Pronuclear injection

The first and most widely used method to generate transgenic domestic animals is injection of a foreign gene at the pronuclear stage of embryos. This technique was first developed in *Xenopus* (Gurdon, 1976) and this provided the basis for analogous experiments in mice. The first transgenic mice were produced two decades ago by this method (Gordon et al., 1980). Since then, hundreds of different genes have been introduced into various mouse strains.

Currently, the only reliable method for introducing new genetic material into domestic animals has been gene transfer by pronuclear injection (Wall, 1996). Using a micromanipulator, purified DNA is microinjected into one of the pronuclei of one cell stage embryos, visualized after centrifugation (Wall et al., 1985). Since this technique introduces foreign DNA into the pronucleus of the fertilized embryo, the majority of transgenic animals produced by this method are restricted to a gain-of-function phenotype. With this approach, it is possible to overexpress a gene of interest and produce large quantities of valuable biopharmaceuticals (Houdebine, 1994). Nevertheless, there are several ways to examine the loss of gene function via pronuclear injection, including dominant negative (overexpression of defective subunit of multiple subunit protein or receptor; Peters et al., 1994), antisense RNA, and ribozymes (block a particular mRNA to prevent translation; Larson et al., 1994; Pepin et al., 1992). Although these methods can reduce the expression of certain proteins, there is no complete loss of gene function due to the leaky nature of these systems.

Therefore, most experiments designed to produce transgenic animals by pronuclear injection are focused on the improvement of growth performance and modification of milk composition (Houdebine, 1994; Pursel et al., 1989; Rexroad Jr et al., 1989). Transgenic pigs overexpressing human growth hormone (GH) showed enhanced growth performance, including enhanced growth rate, better feed efficiency, less fat deposition and increase in protein deposition and muscle mass (Pursel et al., 1989). However, overproduction of GH in transgenic pigs resulted in detrimental side effects, such as unbalanced endocrine profiles and metabolism, insufficient thermoregulatory capacity, joint pathology (lameness and arthritis), low libido, infertility, increased susceptibility to pneumonia, and early mortality (Pursel et al., 1989; Rexroad Jr et al., 1989). It was suggested that these abnormalities might be due to failure to control the expression of GH leading to chronic exposure to high levels of GH released in a non-pulsatile manner or inadequate dietary intake and altered metabolic requirements (Pinkert et al., 1991; Miller et al., 1989). Alternative approaches to increasing skeletal muscle mass and/or feed efficiency are through the controlled expression of more downstream growth-related genes other than GH, such as insulin-like growth factor-1 (IGF-1). This has been tried (Pursel et al., 1999), but there was no significant improvement.

Biopharmaceutical proteins can be produced at high levels to provide an alternative to harvest low abundance natural proteins (Houdebine, 1994; Eyestone, 1999). Due to lack of proper post-translational modifications in bacterial systems and expensive maintenance using mammalian cell culture, the use of animals as a “bioreactor” is the most efficient way to obtain marketable amounts of most human-like proteins (Houdebine, 1994; Eyestone, 1999). Therefore, domestic animals have been engineered with transgenes containing milk protein gene regulatory elements to secrete the protein of interest into the milk. The high protein content in milk and the large volume produced allows for the continuous removal of proteins in a non-invasive way (Wilmut et al., 1991; Houdebine, 1994). Several biopharmaceutical proteins are in the process of commercialization, for example antithrombin III (AT III) (Edmund et al., 1998), tissue plasminogen activator (tPA) (Ebert et al., 1994), human α_1-antitrypsin (Wright et al., 1991), human factor VIII (Paleyanda et al., 1997), and human protein C (Velander et al., 1992).

Although pronuclear injection is relatively easy and well-established, there are major drawbacks. These include; 1) low efficiency of generating transgenic animals. The percentage of pronuclear injected embryos that can develop into transgenic animals varied from 0.3% to 4.0% for pigs, 0.1% to 4.4% for sheep and 0.7% to 3.2% for cattle (Wall, 1996; Wilmut and Clark, 1991). Also the microinjection itself causes some degree of embryo lethality; 2) the

insertional inactivation and/or positional effects of a transgene. Since the incoming DNA undergoes random insertion, it may affect a critical gene which could affect embryonic development (insertional inactivation) or result in an unpredictable level of transgene expression due to neighboring environment (positional effect); 3) variable copy numbers of the transgene are present due to formation of head to tail concatamers of the transgene; 4) mosacism may result due to late integration of the transgene after one or more cell divisions. Therefore, all founder animals are different and the efficiency of transmission of the transgene may be reduced.

Nuclear transplantation

Another method to make transgenic domestic animals is nuclear transfer using embryonic or adult somatic cells (Prather et al., 1989; Wilmut et al., 1997). This technique was originally developed in amphibia to study cellular differentiation (for review see DiBeradino, 1997). McGrath and Solter (1984) initiated development of technology for mammalian cloning by transfer of donor cells into the enucleated recipient oocyte by either direct injection or electrofusion. In mammals, sheep (Willadeson, 1986) were first cloned with this technique, but shortly after cattle (Prather et al., 1987), rabbit (Stice and Robl. 1988), and pigs (Prather et al., 1989) were also successfully cloned (for review see Wolf et al., 1998). In these early studies, the donor nuclei came mostly from either a blastomere of early stage embryos or from an embryonic cell line. However, the choice of the donor cells has widened after the successful cloning of sheep using nuclei from differentiated embryonic cells and adult somatic cells (Campbell et al., 1996; Wilmut et al., 1997). Using same technique, live birth from cattle (Cibeli et al., 1998) and goats (Baguisi et al., 1999) have been achieved using fetal fibroblasts either in quiescent or non- quiescent state. Different technique was used to clone mice using cumulus cells (Wakayama et al., 1998). That is distinctive nonfusion method in which donor nuclei were selectively introduced into enucleated oocytes by piezo-actuated microinjection. By this method, pig was cloned using fetal fibroblast (Onishi et al., 2000). However, first cloned pigs from somatic cells (granulosa cells) were made using double nuclear transfer procedure which is transfer of karyoplasts developed from first nuclear transfer to cytoplasm of normally fertilized and enucleated pronuclear stage zygotes (Polejaeva et al., 2000).

Successful development of embryos reconstructed by nuclear transfer depends on a variety of factors (Campbell, 1999). Most important is the coordination between cell cycles of donor and recipient cells to maintain correct ploidy after nuclear transfer. Since the donor nucleus is reprogrammed by the recipient cytoplasm, it undergoes massive changes. These changes include reduction or cessation of transcription, changes in nuclear structure (nuclear lamins), chromatin structure, nucleolar morphology and stage-specific protein synthesis (Campbell and Wilmut, 1997). The incidence of chromosomal damage and aneuploidy in embryos reconstructed from nuclei in S or G_2 phase depends on the level of maturation/meiosis/mitosis-promoting factor (MPF) activity in the recipient cytoplasm (Campbell et al., 1996). The use of donor nuclei induced to enter the quiescent state (G_0) by serum starvation were successful because they could re-enter the cell cycle, thus allowing reprogramming of the nucleus after transfer into recipient (Campbell et al., 1996; Wilmut et al., 1997). The general parameters for nuclear transfer in domestic animals are, however, the use of donor cells in quiescent state (G_0) induced by serum starvation and post-activated metaphase II oocytes after fusion. The time of embryonic genome activation may also affect nuclear reprogramming and consequently the development of clones to term (Stice et al., 1998). Nuclear transfer by fusion of donor cells with recipient oocytes introduces both donor genetic and cytoplasmic materials. A high karyoplast-cytoplast volume ratio may interfere with the development of bovine nuclear transfer embryos (Zakhartchenko et al., 1997)

After cloning of sheep with differentiated somatic cells (Wilmut et al., 1997), other species such as cattle, goats and mice were successfully cloned using a similar method (Schnieke et al., 1997; Cibelli et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999). Since the nucleus from donor cells that is transferred to the enucleated oocyte is reprogrammed and determines further development of the embryo, it could be an ideal method for making transgenic animals if a stable transfected cell line was used (Schnieke et al., 1997; Baguisi et al., 1999). Using this approach, mosacism, one of the major problems associated with pronuclear injection, could be overcome since all cells of the resulting animal contain the modification and transmit it to next generation. However, it is very difficult to perform precise modification of genes of interest, such as knock-out and knock-in, in differentiated somatic cells when compared to embryonic cell lines such as embryonic stem cells (Piedrahita et al., 1999; Capecchi, 2000). Several obstacles remain to be overcome such as improving nuclear reprogramming, solving imprinting disorders, correcting mal- or mis-communication between donor nucleus and recipient cytoplasm, understanding large offspring syndrome, and reducing the high abortion and death rates for cloned animals (Piedrahita et al., 1999; Capecchi, 2000).

Recently, a large number of studies are carrying out in the field of biotechnology using nuclear transfer. Cell replacement therapy or therapeutic cloning is considered as a promising way to cure the degenerative human diseases, such as diabetes and Parkinson's disease. This approach consists of the nuclear transplantation and stem cell

technology. Since the shortage of the organs for transplantation or the intrinsic problems in immune rejection, autologous source of cell or tissues is preferred. Therefore, generation of embryonic stem cells from nuclear transplantation using patient's cells will overcome the problems mentioned above (Kind and Colman, 1999).

Gene targeting via homologous recombination

Another powerful method to produce genetically modified animals is gene targeting using embryonic cell lines. Gene targeting is the transfer of a gene into a host cell which is later incorporated into the genome by homologous recombination (Thomas and Capecchi, 1987; Capecchi, 1994). The first gene targeting in mammalian cells was reported by Smithies et al. (1985) who modified the β-globin gene in EJ bladder carcinoma cells. After this, the first chimeric mice were born by ES cells (Robertson et al., 1986; Gossler et al., 1986). However, the first gene targeting in ES cells by site-directed inactivation of the hypoxanthine phosphribosyl transferase (HPRT) gene using both replacement and insertion type vectors, was reported by Thomas and Cappechi in 1987. Since then, there have been many reports on gene targeting by homologous recombination in the mouse and over 300 different knockout mouse lines have been produced (Brandon et al., 1995).

Gene targeting via homologous recombination is enhanced by a region of uninterrupted homology with the targeting vector and by the creating double strand breaks within the targeting vector DNA (Hasty et al., 1991, 1994; Köller and Smithies, 1992) The gene targeting construct is usually introduced into cells by electroporation. The linearized targeting vector in the nucleus is recognized by the cell as damaged and is repaired by either random insertion or insertion into the homologous site. The alignment of the targeting vector with its homologous sequence in the target gene and a recombination process between targeting vector and endogenous gene allow the incoming DNA to replace the endogenous gene or insert in its homologous region (Köller and Smithies, 1992; Hasty et al., 1994).

A targeting DNA construct consists of the region of homology to the gene being targeted and selectable markers (Capecchi, 1994). The positive selection marker is for detection of the insertional event of the targeting vector and is also used to inactivate the gene of interest by placement in a coding region. The negative selection marker reduces the frequency of random insertion events. The most common marker for positive selection is the neomycin resistance gene and thymidine kinase gene for the negative selection (Smithies et al. 1985; Thomas and Capecchi, 1987).

There are two kinds of targeting construct: insertional and replacement type. An insertional type or o-type vector is linearized in the region of homology and a single cross-over reaction inserts the whole targeting vector in the region of homology resulting in heteroduplex DNA (Hasty et al., 1991, 1994). In this type of construct, the selectable markers can be placed either within the non-homologous region or a region of homology. A replacement type or Ω-type vector is the most common type of targeting vector (Köller and Smithies, 1992; Hasty et al., 1994). This vector is linearized outside the region of homology and requires a double crossover reaction to replace the endogenous gene with the homologous targeting vector. Thus, to inactivate the gene of interest, the selectable marker must be placed in the region of homology.

In addition to conventional gene targeting, there are several other strategies in gene targeting experiments such as "In and Out" (Smithies et al., 1985; Valancius and Smithies , 1991) or "Hit and Run" (Hasty et al., 1991), "Tag and Exchange" (Askew et al., 1993), and "Plug and Socket" (Detloff et al., 1994). All of these strategies are designed to introduce small mutations in a site-directed manner using two rounds of homologous recombination events. Insertion type vectors use the "In and Out" or "Hit and Run" strategy while replacement type vectors utilize the last two methods. "Tag and Exchange" and "Plug and Socket" also make it possible to use the same locus to introduce further modifications (Askew et al., 1993; Detloff et al., 1994). In conjunction with a site-specific recombination system such as Cre (causes recombination)-loxP (locus of crossing-over(×)) from bacteriophage P1 (Austin et al., 1981) or FLP (flipase) -FRT (FLP recombination target) from *Saccharomyces cerevisiae* (Andrews et al., 1985), it is possible to modify a wider range of genes in various ways (Sauer and Handerson, 1988; Kuhn et al., 1995; Araki et al., 1997). Site-specific recombination involves a recombinase (Cre or FLP) enzyme that recognizes and binds two DNA recognition sequences (lo×P or FRT) inducing a recombination reaction, either excision or insertion depending on whether it is an intramolecular or intermolecular event, respectively (Sauer and Handerson, 1988).

However, the efficiency of homologous recombination is extremely low, yielding approximately 1 target per 10^6 events (Piedrahita et al., 1992). Therefore, this technique is not suitable for use on embryos. Only one study reported an attempt to correct a deletion in a major histocompatibility gene by gene targeting using pronuclear injection (Brinster et al., 1989). Therefore, gene targeting strategy requires pluripotential embryonic cell lines, which can proliferate indefinitely *in vitro* while retaining the ability to develop into any type of organ when introduced into host blastocysts.

GERM CELL ENGINEERING IN DOMESTIC ANIMALS

Embryonic stem cells

In the 1980's, embryonic stem (ES) cells were first established from preimplantion murine embryos (Evans and Kaufman, 1981; Martin, 1981). Mouse ES cells are derived from the inner cell mass (ICM) of an expanded blastocyst at 3.5 days post-coitum, or delayed blastocysts collected 4-6 days after ovariectomy. First, the ICM is isolated by immunosurgery to remove trophoblast cells. After several days in culture, the cells from the ICM form a colony that can be expanded by disaggregating and re-seeding on mitosis-inactivated feeder cells (for review see Hogan et al., 1994; Robertson, 1987). ES cells can be isolated only from permissive strains of mice, 129/SV or 129/Ola, to obtain totipotent cells (Robertson, 1987; Nicholas et al., 1990; Pease et al., 1990). A feeder layer is generally required to isolate ES cells and to support their successive passages (Suemori et al., 1987). The main role of feeder cells is probably to provide growth factors necessary for proliferation and inhibition of differentiation. The principal inhibitory factor is leukemia inhibitory factor (LIF), as LIF-defective fibroblasts cannot maintain undifferentiated ES cells (Stewart et al., 1992) and LIF in the medium can support ES cells without feeder cells. (Pease et al., 1990; Suemori et al., 1987). LIF is a pleitrophic cytokine that acts through the gp130 pathway (Yosida et al., 1994), which is common to related cytokines such as ciliary neurotrophic factor (CNTF) (Conover et al., 1993), oncostatin M (OSM) (Rose et al., 1994), and interleukin-6 (IL-6) (Nicholas et al., 1994). Each of these cytokines can maintain the pluripotentiality of ES cells.

To prevent spontaneous differentiation, ES cells must be maintained by repeated passages on feeder layers, usually non-proliferative mitomycin-C treated or irradiated fibroblast (STO cells or primary mouse embryonic fibroblast). ES cells can also be maintained less effectively without feeder layer on gelatin or extracellular matrix substrate in conditioned medium or in LIF-supplemented medium (for review see Wiles, 1993). The culture media used contain fetal bovine serum (FBS), so is not well characterized and is susceptible to variation from batch to batch. The presence of differentiation factors such as retinoids in FBS may explain the spontaneous differentiation of ES cells (Tamura et al., 1990).

ES cells appear as small aggregated and unpolarized cells forming islands on the feeder cells (Evans and Kaufman, 1981; Martin, 1981; Abbondanzo et al., 1993). ES cells show a high nucleo-cytoplasmic ratio and large nucleoli indicating active transcription and a correlative high protein synthesis, at least relevant to active cell proliferation. ES cells express cell markers that can be used to characterize undifferentiated versus differentiated ES cells. A common marker for the undifferentiated state is alkaline phosphatase (Wobus et al., 1984) which is equivalent to non-specific alkaline phosphatase of the inner cell mass of the mouse blastocyst. Other markers generally correspond to carbohydrate residues of membrane proteins including ECMA-7 (Kemler et al., 1980) and SSEA-1 (Solter and Knowles, 1980). The germline specific transcription factor, Oct-4, is also a reliable marker for undifferentiated embryonic cells and ES cells (Pesce and Schöler, 2000). Each of these markers is down-regulated upon differentiation of ES cells.

Since ES cells are a pluripotent cell line, under specific conditions, they can be induced to differentiate into cells of multiple lineages *in vitro* (Palacios et al., 1995). The conditions required to induce differentiation may be a high number of passages, absence of LIF and/or feeder cells, or the addition of differentiation factors such as retinoic acid (RA), or dimethyl sulfoxide (DMSO). When ES cells are cultured at high cell density on a non-adhesive surface, they form round embryoid bodies showing many similarities to embryo development *in vivo* (Doetschman et al., 1985). The embryoid bodies develop an outer layer of endoderm-like cells and eventually a central cavity, resulting in a cystic embryoid body. When these cells are allowed to attach again and form outgrowths, embryoid bodies can give rise to differentiated tissues such as myocardium, blood islands, and hematopoietic stem cells (Palacios et al., 1995; Doetschman et al., 1985). *In vitro* differentiation of ES cells appears to be a promising model for the study of factors involved in the establishment of cell lineage and to develop cell replacement therapy for certain human diseases such as Parkinson's (Stocum, 1998).

ES cells can also differentiate in vivo. When ES cells or embryoid bodies are implanted into immunodeficient mice, highly differentiated tissues can be obtained (Chen and Kosco, 1993). More importantly, ES cells can be injected into a morula or into the cavity of an expanded blastocyst, giving rise to chimeric mice in which ES cells take part in the development of all types of tissue including the germ line (Robertson et al., 1986). ES cells are also able to contribute to the complete generation of embryos using tetraploid blastocysts as host (Nagy et al., 1993); however, the procedure is very inef ficient and not routinely feasible. The ability of ES cells to proliferate indefinitely and maintain pluripotentiality makes them the best carrier cells to perform gene targeting by homologous recombination. Therefore, the establishment of ES cell lines in domestic animals would improve the production of transgenic animals and overcome the problems associated with pronuclear injection.

Isolation of ES cells have been attempted rat (Iannaccone et al., 1994), mink (Sukoyan et al., 1993), rabbit (Giles et al., 1993), hamster (Doetschman et al.,

1989; Piedrahita et al., 1990a), primate (Thomson et al., 1995), sheep (Piedrahita et al., 1990b; Handyside et al., 1987), cattle (Evans et al., 1990; Strelchenko and Stice, 1994; Stice et al., 1996), pig (Piedrahita et al., 1990b, 1990c; Notarianni et al., 1990; Gerfen and Wheeler, 1995; Talbot et al., 1993a; Moore and Piedrahita, 1997) and human (Shamblott et al., 1998). A wide range of pluripotentiality has been demonstrated for ES cells from each of these species, but only in the mouse have germline chimeras been produced (Robertson et al., 1986). Porcine ES-like cells have been derived from early pig embryos, but lose their pluripotency over time in culture (Moore and Piedrahita, 1997). Although chimeras have been produced from freshly isolated porcine inner cell mass injected into host blastocysts, the ability of chimera production is lost after culture of porcine ICM *in vitro* (Anderson et al., 1994). This may be due to improper culture conditions and/or a requirement for species-specific growth factors. Therefore, further improvements in culture conditions are required to isolate pluripotent stem cells from pigs. It has been shown that heterologous cytokines, for example human LIF, do not inhibit differentiation of porcine ICM and are unlikely to assist in porcine ES cell isolation (Moore and Piedrahita, 1997). Future progress in the isolation of pluripotent ES cells from domestic animals may depend on the appropriate manipulation of the LIF signal transduction pathway using homologous cytokines.

Primordial germ cells and embryonic germ cells

In general, current techniques available for the generation of transgenic domestic animals have problems associated with random insertion of foreign DNA into the chromosome (Wall, 1996). While isolation of ES cell lines has been attempted in various species (Piedrahita et al., 1999), germline chimeras have been produced only in the mouse (Robertson et al., 1986). An alternative source of pluripotent stem cells are embryonic germ (EG) cells (Matsui et al., 1992; Resnick et al., 1992) derived from primordial germ cells (PGCs) which are precursors of male and female germ cells in the developing mouse fetus (for review see Buehr, 1997; Bendel-Stenzel et al., 1998).

In mice, PGCs are identified by the expression of tissue nonspecific alkaline phosphatase (TNAP) at 7.0 days post-coitum (d.p.c.) (Ginsburg et al., 1990). The PGCs are first detected in the extraembryonic mesoderm posterior to the primitive streak and migrate towards the developing embryonic gonad over the next five days of embryonic development (Chiquoine, 1954). When PGCs are first identified at 7.0-7.5 d.p.c., they are localized to the region of the forming hindgut. By 9.5 d.p.c., PGCs begin to leave the hindgut and migrate through the dorsal mesentery before reaching their target. By 11.5 d.p.c., almost all PGCs have reached the urogenital ridge. During migration, PGCs actively proliferate and increase in cell number about 300-fold (Tam and Snow, 1981) from 50-100 cells in 8.5 d.p.c. to 25,000-30,000 at 12.5 d.p.c. in the fetal gonad, with a division time of 16 h. Morphological differences are visible by 12.5 d.p.c. with germ cells arranged in cords in the male and a random array in the female gonad. At 13 d.p.c. male germ cells enter mitotic arrest while the female PGCs begin to enter meiosis and to form oogonia which arrest at the end of meiotic prophase I. After birth, male germ cells resume mitosis forming the mitotic stem cells of the male testis, the spermatogonia. It is these cells that give rise to meiotic derivatives that will undergo spermatogenesis to form mature sperm. In the ovary after birth, the oogonia resume meiosis in waves giving rise to fully mature oocytes (Buehr, 1997; Bendel-Stenzel et al., 1998).

PGCs express a number of markers other than TNAP, for example, stage specific embryonic antigen-1 (SSEA-1), F9, EMA-1, Oct-4 and *c-kit* that identify both germ cells and epiblast cells (Buehr, 1997; Bendel-Stenzel et al., 1998). The function of TNAP is unclear and Oct-4 is a transcription factor that is involved in maintenance of totipotentiality of the cells (Pesce and Schöler, 2000). c-kit encodes for a stem cell factor receptor that is important in survival and proliferation of PGCs in vitro (Donovan, 1994). These markers gradually disappear from the area of the epiblast not involved in the formation of the germ line, possibly indicating the loss of totipotency in these cells.

PGCs can be isolated by treating the gonads with trypsin-EDTA and/or mechanical dissociation of the gonads. Further purification to isolate PGCs from somatic cells can be done using percoll gradients (DeFelici and McLaren, 1982). An alternative method to isolate PGCs is immunological affinity separation using monoclonal antibodies to cell surface markers of PGCs (McCarrey et al., 1987). This methodology allows a high degree of PGC purification, although the overall yield is low.

The ability of PGCs to survive in culture depends on their embryonic age. PGCs that have reached the gonads (11.5 d.p.c. and older) survive for a limited time in culture in the absence of a feeder layer, whereas PGCs before colonization require feeder cells to survive. Even the development of an *in vitro* culture system over the past 10 years, PGCs proliferate and survive only up to one week. (Dolci et al., 1991). PGC survival is best when they are cultured on a confluent monolayer of mouse feeder cells, such as STO, Sl/Sl^4 m220 or TM_4. However, in general, PGCs proliferate as long in culture as they would in the embryo. PGC survival and proliferation is supported by the feeder cells because they produce membrane-bound stem cell factor (SCF) and LIF as well as other factors that have not been characterized (Dolci et al., 1991; Godin et al., 1991). Addition of exogenous growth factors, such as LIF and soluble SCF, can stimulate PGC proliferation in culture

without extending their survival (Dolci et al., 1991; Godin et al., 1991; Donovan, 1994).

Even though the survival and proliferation is limited in culture, PGCs can be effectively immortalized to embryonic germ (EG) cells. EG cells can be established with the mixture of growth factors, LIF, SCF and basic fibroblast growth factor (bFGF), and co-culture with confluent feeder cells (Matsui et al., 1992; Resnick et al., 1992). Initially, the PGCs form small colonies of approximately 8-10 cells as a monolayer on top of the feeder cells. After 7-8 days, these colonies begin to form multilayered clumps, similar in morphology to mouse ES cells. When the colonies are dissociated by trypsinization and replated on a fresh feeder cells, they form new colonies that can be passaged indefinitely (Matsui et al., 1992; Resnick et al., 1992). They also have similar biochemical characteristics as mouse ES cells, such as expression of AP, SSEA-1 and Oct 4 (Matsui et al., 1992; Resnick et al., 1992; Pease and Schöler, 2000). They can be induced to differentiate into several cell types such as fibroblast, endoderm, endothelial cells, and muscle cells *in vitro* as well as give rise to embryoid bodies. In vivo, they develop into teratocarcinomas when injected into nude mice (Matsui et al., 1992; Resnick et al., 1992). More importantly, when introduced into blastocysts, EG cells can contribute to the formation of chimeras including the germ line (Stewart et al., 1994).

Pig (Takagi et al., 1997), cattle (Lavoir et al., 1994), rabbit (Moens et al., 1996) and rat (Kemper and Peters, 1987) PGCs have been collected and characterized by their morphology and immunohistochemistry. Porcine PGCs can be identified as early as Day 18 by anti-SSEA-1 antibody and several lectins including DBA (*Dolichos biforus* agglutinin) and LTA (*Lotus tetragonolobus* agglutinin) (Takagi et al., 1997). By light and electron microscopic analyses (Pelliniemi, 1975, 1976), they also exhibit the same morphological characteristics as mouse PGCs. PGCs from cattle, rabbit and rat show similar characteristics to mouse PGCs. However, there are some species-specific characteristics of PGCs, such as the cytoplasmic vesicles of cattle (Lavoir et al., 1994), dense vesicles in pig (Pelliniemi, 1975, 1976), and lipid droplets of rabbit PGCs (Moens et al., 1996).

Establishment of EG cells from domestic animals has been attempted. Porcine EG cell lines were isolated by several groups (Shim et al., 1997; Piedrahita et al., 1998; Müeller et al., 1999; Lee et al., 2000ab). The morphology of the porcine EG colony resembled mouse ES cells and were AP and SSEA-1 positive indicating that they maintained pluripotential characteristics (Lee et al., 2000ab). For the establishment of EG cells from porcine PGCs, a mixture of growth factors and a feeder layer is necessary (Dolci et al., 1991; Piedrahita et al., 1998; Lee et al., 2000a b). However, porcine EG cells were also isolated successfully using a feeder layer and porcine LIF only (Shim et al., 1997), or only a feeder layer expressing porcine membrane bound SCF (Müeller et al., 1999). Further experiments to determine the optimal culture system showed that feeder cells should express membrane bound SCF and cocktail of cytokines (LIF, SCF and bFGF) is needed to establish porcine EG cells with good quality during initial period of the culture (Lee et al., 2000a). Genetic transformation of porcine EG cells was performed, and they expressed a marker gene, green fluorescent proteins (GFP) (Piedrahita et al., 1998; Lee et al., 2000b). More importantly, porcine EG cells could contribute to chimera development when introduced into host blastocysts (Shim et al., 1997; Piedrahita et al., 1998; Müeller et al., 1999). The isolation of putative bovine EG cells has been reported (Strelchenko, 1996; Lee et al., 2000c), and human EG cells were established recently (Shamblott et al., 1998). Putative EG cells from other mammalian species, such as goat, rabbit and rat were isolated and characterized (Lee et al., 2000c; Kühholzer et al., 2000). Using the same culture system to culture the porcine EG cell, EG cells from cattle, rabbit, goat and rat showed typical morphology and were stained for alkaline phosphatase, indicating that mammalian PGCs show common physiological and biochemical behavior *in vitro* (Lee et al., 2000c). In addition to porcine EG cells, EG cells from cattle, rabbit and rat were able to be genetically transformed using marker gene, which opened the possibility of genetic modifications in these species using genetically manipulated EG cells (Lee et al., 2000c).

Apoptosis : When pluripotential ES and/or EG cells from domestic animals are isolated, they might be used for gene targeting to manipulate the genes of interest. However, in case of EG cell establishment, there is a significant amount of PGC loss during the initial period of culture reducing the number of viable cells, thus exacerbating the problem of low efficiency of homologous recombination.

Cell death is generally categorized into one of two forms, necrosis and apoptosis (Kerr, 1971). Necrosis and apoptosis are readily distinguishable from one another based on morphological and biochemical characteristics. Necrosis is a non-physiological death resulting from severe and sudden injury, and is characterized by the death of cell groups, loss of membrane integrity and lysosomal leakage which causes the cell to swell and lyse, followed by the propagation of a non-specific inflammatory response (Cohen, 1996).

Apoptosis, in contrast, is a physiological process of cell death that occurs naturally in eukaryotic multicellular organisms. Also, this process is an integral component of in vivo development and *in vitro* culture of PGCs. Apoptosis, also called “programmed cell death” or “cell suicide”, has been associated with remodeling of tissue during development, maintaining homeostasis, removal of

senescent cells, deletion of cells that have genetic damage beyond repair, and as a defense mechanism against viral infection and the emergence of cancer (Kerr et al., 1972; Cohen, 1996). The elimination of unwanted cells by apoptosis occurs through a genetic program within the target cell. Death by apoptosis is characterized by distinct morphological properties. These include death to single cells rather than massive death of an entire population, blebbing of the membrane with no loss of integrity, cell shrinkage up to one-third its normal size, and partial autodigestion of intracellular components with the cell splitting into plasma membrane enclosed vesicles called apoptotic bodies. The nucleus also shrinks and the chromatin becomes marginated and compacts into dense masses. In vivo, there is a change in localization of phosphatidylserine, from inside to outside of the plasma membrane bilayer which serves to mark apoptotic bodies for elimination. Neighboring cells or macrophages quickly phagocytose the apoptotic bodies, so there is no inflammatory response as no cellular contents are released (Kerr et al., 1972; Cohen, 1996).

Biochemically, apoptosis is characterized by non-random nucleosomal breakage of DNA into 50 to 300 kb intervals which are later cleaved into multiples of 200 bp fragments (Wyllie, 1980). These fragments are detected as the characteristic DNA ladder upon gel electrophoresis and serve as the basis to detect apoptotic cells by means of the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling) assay (Gavrieli et al., 1992). Apoptosis is induced by physiological stimuli and is a highly regulated process with synthetic and activation steps. Therefore, there is an energy requirement due to de novo transcription and macromolecular synthesis (Cohen, 1996).

A cascade of proteases has an important role in the progression of apoptosis. This group of intracellular proteases called caspases (cysteine proteases that cleave proteins after aspartate residues) are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis (Thornberry and Lazebnik, 1998). Caspases, of which fourteen have been identified in mammals, are present as inactive pro-enzymes that are activated by proteolytic cleavage. Caspases 3 (effector), 8, and 9 (initiator) are situated at pivotal junctions in apoptotic pathways. Caspase 8 initiates disassembly in response to extracellular apoptosis-inducing ligands and is activated in a complex associated with the cytoplasmic death domain of many cell surface receptors for the ligands (Ashkenazi and Dixit, 1998). Caspase 9 activates disassembly in response to agents or insults causing an increase in oxidative stress that triggers the release of cytochrome *c* from mitochondria (Liu et al., 1996; Green and Reed, 1998). Apoptosis is activated when extra-mitochondrial cytochrome *c* is complexed with the apoptotic protease activating factor 1 (APAF-1) (Li et al., 1997). Caspase 3 appears to amplify caspases 8 and 9 initiation signals into a full-range commitment to disassembly (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). Caspases 8 and 9 activate caspase 3 by proteolytic cleavage and caspase 3 then cleaves vital cellular proteins or other caspases (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998).

The regulation of apoptosis consists of a very complex set of interactions among various gene products. Members of the bcl-2 family are key regulators of apoptosis (for review see Adams and Cory, 1998). Bcl-2 is a mammalian homologue of the anti-apoptotic gene product, Ced-9, of *C. elegans* and first identified at the t (14:18) translocation breakpoint present in follicular lymphomas (Pegoraro et al., 1984). They are divided into two groups, anti-apoptotic (Bcl-2 Bcl-x_L, Bcl-w, Mcl-1, A1), which are similar to Bcl-2 and promote cell survival by inhibiting adapters needed for activation of caspases, and pro-apoptotic (Bcl-x_S, Bax, Bak, Bok, Bik, Blk, Bad, Bid), which are distant relatives and promote apoptosis through displacing the adapters from the pro-survival proteins. They are membrane-bound proteins in mitochondria, endoplasmic reticulum, and nuclear membranes (Hockenbery et al., 1990). Bcl-2 prevents apoptosis induced by a range of factors, including oxidative stress, and may prevent the release of cytochrome *c* and apoptosis inducing factor (AIF) from mitochondria (Yang and Korsmeyer, 1996). Both pro- and anti-apoptotic members of the Bcl-2 family can heterodimerize and titrate one another's function, suggesting that their relative concentration may act as a switch to determines the cell's fate to undergo apoptosis (Oltvai et al., 1993).

Germ cell apoptosis : The apoptotic process is also involved in germ cell development during the prenatal and postnatal period (Coucouvanis et al., 1993; Hsueh et al., 1996). Normal development of both female and male gonads is characterized by massive cell death. More than 99% of ovarian follicles present in early life are destined to undergo apoptosis. In the testis, up to 75% of male germ cells also undergo apoptosis, perhaps as a mechanism to delete superfluous or defective germ cells (Hsueh et al., 1996). The Fas system is a key regulator of germ cell apoptosis in the testis (Lee et al., 1997) and mouse male germ cells degenerate autonomously by Fas-induced apoptosis during the fetal period (Wang et al., 1998). Ovarian cell apoptosis is responsible for the demise of 99.9% of follicles, and the mechanism underlying follicular atresia is apoptosis (McGee et al., 1998). In addition, apoptosis in germ cells is required for normal gametogenesis (Rodriguez et al., 1997). Transgenic mice expressing high levels of the Bcl-x_L or Bcl-2 proteins in male germ cells show a highly abnormal spermatogenesis accompanied by sterility. This appears to result from the prevention of an early and massive wave of apoptosis in the

testis, which occurs among germinal cells during the first round of spermatogenesis. The intracellular balance of Bcl-x_L and Bax proteins plays a critical role in the control of apoptosis in germ cell development. The apoptotic wave appears necessary for normal mature spermatogenesis to develop, probably because it maintains a critical cell number ratio between germinal stage cells and Sertoli cells (Rodriguez et al., 1997). Hence, apoptosis could play a useful role in avoiding uncontrolled germ cell proliferation and eliminating misplaced germ cells which might develop into germ cell tumors.

Flow cytometric analysis showed that the mechanism of death of the mouse prenatal germ cells during early development is apoptosis (Coucouvanis et al., 1993) When isolated mouse PGCs were cultured for 4-6 hours, 20-60% of PGCs showed typical apoptotic features (Pesce et al., 1993; DeFelici and Pesce, 1994). Transmission and scanning electron microscopic analyses showed chromatin condensation, organelle crowding due to a decrease in cell size, and eventually formation of apoptotic bodies (Pesce et al., 1994). Also, PGCs in culture accumulated high levels of the enzyme tissue transglutaminase, which is induced during apoptosis, as well as extensive degradation of DNA (Pesce et al., 1993).

Porcine PGCs cultured *in vitro* also showed the typical characteristics of apoptosis (Lee et al., 2000b,d). While freshly isolated porcine PGCs had normal morphology, porcine PGCs cultured in suspension had the characteristic morphology of cells undergoing apoptosis. At beginning, PGCs had lost some volume and underwent massive chromatin condensation. PGCs also showed membrane boiling and blebbing, and cytoplasmic vacuoles in the organelle-crowded cytoplasm. Then, the nucleus showed distinct chromatin condensation and fragmentation, and apoptotic bodies, sealed with membrane and containing cellular organelles, had begun to form at one side of the cell. Finally, the PGCs had fully degenerated into apoptotic bodies, without loss of intracellular contents, a distinct difference between apoptosis and necrosis (Lee et al., 2000b). Apoptosis in porcine PGCs was also evaluated using the TUNEL assay which detects internucleosomal DNA double-strand breaks, one of the earliest events of the apoptotic process. Consistent with morphological observations, FACS analysis showed increase in TUNEL positive cells as in advance of culture *in vitro*, with signal from fluorescein being specific to apoptotic nuclei (Lee et al., 2000bd).

Growth factors and extracellular matrix in primordial germ cell apoptosis : To prevent apoptotic death of PGCs *in vitro*, it is important to know which factor(s) is (are) responsible for this event. Apoptosis can be triggered by various factors, including growth factor deprivation, loss of contact with extracellular matrix, reactive oxygen species, binding of a death ligand, UV and γ-irradiation, and viral infection.

Growth factors, such as leukemia inhibitory factor (LIF) and stem cell factor (SCF), are indispensable for maintaining viability and proliferation of mouse PGCs *in vitro* (DeFelici and Pesce, 1994; Donovan, 1994). LIF is a well known growth factor required to maintain an undifferentiated state and stimulate proliferation in mouse ES cells. In addition, LIF can promote survival and to stimulate proliferation (Matsui et al., 1991) through the gp130 pathway in mouse PGCs (Koshimizu et al., 1996). EG cells can also be obtained by adding OSM or the IL-6/sIL-6R complex in place of LIF (Koshimizu et al., 1996). Soluble stem cell factor (SCF), which is the product of the *Steel* locus, can also support PGC survival and, together with LIF, stimulate proliferation (Matsui et al., 1991). When LIF and SCF are present in the culture medium, apoptotic PGCs during the early period of culture are reduced significantly, indicating a critical role for these two cytokines on the survival of PGCs *in vitro* (Pesce et al., 1993). However, without feeder cells, LIF and SCF are unable to prevent further apoptotic death of PGCs. Membrane bound SCF, a splicing variant of SCF expressed by the feeder layer, is absolutely required for survival of PGCs which express the receptor for SCF, c-kit (Matsui et al., 1991). This is one reason why PGCs can survive and proliferate longer in the presence of a feeder layer, such as STO, TM_4 and *Sl*/*S4*[4] m220 cells, which all express membrane bound SCF (Pesce et al., 1997). Another growth factor required for the establishment of mouse embryonic germ (EG) cells, is basic fibroblast growth factor (bFGF). Without bFGF, even PGCs cultured on feeder cells supplemented with LIF and SCF can survive and proliferate only for a limited time. The exact mechanism of bFGF action is not well known. However, a possible role for bFGF is inhibition of *c-kit* transcript downregulation in PGCs *in vitro* and maintenance of high levels of c-kit in PGCs and thus their long-term proliferation (Donovan, 1994). Recent studies (Resnick et al., 1998) found that mouse PGCs express the receptor for bFGF, suggesting a direct mitogenic action of bFGF on PGCs. In previous studies, porcine PGCs were cultured successfully with a mixture of LIF, SCF and bFGF on feeder layers and established an EG-like cell line (Lee et al., 2000a, b). However, Shim et al. (1997) could maintain porcine PGCs in culture with a feeder layer and porcine LIF only, and Müeller et al. (1999) used only feeder cells expressing porcine membrane-bound SCF to culture porcine PGCs.

PGCs are migratory cells and interact with surrounding somatic cells during migration via interaction between integrins and extracelluar matrix (Garcia-Castro et al., 1997), both of which are involved in survival and apoptosis

(Meredith et al., 1993; Boudreau et al., 1995). Since adhesion molecules are involved in signal transduction events that regulate cell motility, proliferation, differentiation and survival, PGC adhesion is crucial for their survival and proliferation. Fibronectin and laminin are expressed along the path of PGC migration and are involved in adhesion of PGCs (Garcia-Castro et al., 1997; DeFelici et al., 1997). c-kit receptor expressed by the PGCs can also increase adhesion to somatic cells expressing membrane-bound SCF. This interaction was significantly reduced by antibodies directed against c-kit receptor or SCF, as well as by soluble SCF. Therefore, *in vivo* migration and proliferation of PGCs may occur only when they contact somatic cells expressing SCF. This offers a possible mechanism for control of the number and position of PGCs based on the elimination of PGCs that stray from the migratory route (DeFelici et al., 1997).

Growth factors are indispensable for the survival of PGCs as well as establishment of EG cells in mouse (Matsui et al., 1992; Resnick et al., 1992; Donovan, 1994) and this requirement was confirmed in porcine PGCs (Lee and Piedrahita, 2000a). Since the growth factors required for *in vitro* culture of murine PGCs are highly conserved among mammals (LIF: Willson et al., 1992; SCF: Zhang and Anthony, 1994; bFGF: Bechtner et al., 1993), they increased the number of AP-positive porcine PGC-derived colonies. In addition, bFGF might stimulate the proliferation of porcine PGCs, suggesting a direct mitogenic effect of bFGF on PGCs (Resnick et al., 1998; Lee and Piedrahita, 2000a). However, bFGF might also promote proliferation of porcine PGCs indirectly through its action on the feeder cells. In contrast, Durcova-Hills et al. (1998) reported that membrane-bound form of porcine SCF and LIF efficiently stimulated survival and proliferation of porcine PGCs *in vitro* over 5 days of culture but bFGF together with SCF and LIF had no significant increase in the PGC number. Moreover, Shim and Anderson (1998) reported that none of the growth factors (LIF, SCF, and bFGF) used in their study enhanced *in vitro* growth of porcine PGCs when they were cultured up to 5 days. However, all three growth factors, LIF, SCF and bFGF, are required in the aspect of increasing cell survival and further establishment of healthy undifferentiated porcine EG cells (Lee and Piedrahita, 2000a).

Feeder cells expressing membrane-bound SCF are critical for survival of porcine PGCs and establishment of porcine EG cells (Lee and Piedrahita, 2000a). In mice, EG cells can be isolated using feeder cells such as STO, TM_4 and *Sl*/*Sl*^{4} m220, all of which express membrane-bound SCF (Matsui et al., 1992; Resnick et al., 1992). These feeder cells known to express membrane-bound SCF were capable of supporting the survival and growth of porcine PGCs at a high frequency. This suggests that, as in mice, porcine PGCs require membrane-bound SCF for maximal survival *in vitro* (Lee and Piedrahita, 2000a).

Inhibition of apoptosis by protease inhibitors and antioxidants : During apoptosis, various enzymes are activated including caspases which cause specific cleavage of proteins leading to the cascade of activation and deactivation of other proteolytic enzymes responsible for degradation of cellular and chromatin structures (Thornberry and Lazebnik, 1998). Cysteine, aspartic, and serine proteases inhibitors can inhibit cell death under certain conditions (Chow et al., 1995; Kumar and Harvey, 1995). Therefore, this effect could result from the inhibition of enzymes involved in apoptosis (Stefanis et al., 1997). This raises the possibility that other non-caspase proteases may also be part of the apoptotic pathway. Therefore, protease inhibitors with a wide range of capacities to inhibit various proteases might be more effective than specific protease inhibitors.

α_2-macroglobulin (MAC) is a large homotetrameric glycoprotein found at high concentrations in plasma (Sottrup-Jenson, 1987) and classically described as a broad range protease inhibitor (Gonias et al., 1994). Two forms of MAC can be identified by their electrophoretic mobility. The ‘slow’ or ‘native’ form can interact with all four classes of proteases (Barret and Starkey, 1973). The ‘fast’ or ‘activated’ form is already bound to proteases and inhibits superoxide anion production by activated macrophages (Hoffman et al., 1983). Thus, MAC may work through either non-specific inhibition of proteases activated during apoptosis or by reducing oxidative stress generated by superoxide anion production. Interestingly, MAC can also bind and transport cytokines into the cell (Borth and Luger, 1989) to regulate cellular growth and physiology. MAC can bind to transforming growth factor-β1 (TGF-β1), TGF-β2, platelet-derived growth factor BB, nerve growth factor-β and interleukin-1β (Gonias et al., 1994). All of these except TGF-β2 have a higher affinity for activated MAC, but, since there is a large excess of native MAC in plasma and serum-supplemented medium, the native form of MAC is responsible for cytokine carrier activity in biological systems (Gonias et al., 1994). However, not all cytokines can bind to MAC. Therefore, MAC may alter the balance of cytokines within the microenvironment surrounding responsive cells. Furthermore, the MAC gene contains a LIF-responsive element that is transcriptionally activated by LIF in ES cells (Hocke et al., 1995).

Another factor involved in induction of apoptosis is oxidative stress mediated by reactive oxygen species (ROS) (Buttke and Sandstrom, 1994). ROS are natural by-products of cellular metabolism and enzyme processes, and are a group of compounds consisting of both radical and non-radical species that may or may not contain oxygen (Davies, 1998). ROS has detrimental effects on cells by interacting

with proteins, lipids, and DNA. They cause the oxidation of amino acid side chains, the formation of covalently linked protein aggregates and fragmented polypeptides (Stadtman, 1998). In addition, they react with both bases and sugars in DNA chains (Dizdaroglu, 1993), and cause lipid oxidation (Bors et al., 1990). Therefore, ROS have been linked to cell death, both apoptosis and necrosis, depending on the relative concentration of ROS (Davies, 1998). High levels of ROS cause necrosis while milder amounts of ROS cause apoptosis. These compounds are also involved in signal transduction (Slater et al., 1995), resulting in the activation of transcription factors such as NF-κB and AP-1 (Meyer et al., 1993), and subsequent gene expression which might be implicated in cell death and in signaling pathways for cytokines (Dugan et al., 1997). Oxidative stress is also one of the factors involved in apoptosis in rat testicular germ cells and mouse embryonic stem cells (Castro-Obregón and Covarrubias, 1996; Ikeda et al., 1998). The apoptotic program was activated in ES cells either by removal of the reducing agent β_2-mercaptoethanol (BME) or by the addition of retinoic acid. In both cases, the level of ROS was increased (Castro-Obregón and Covarrubias, 1996).

Several antioxidants, which prevent the harmful effects generated by oxidative stress, have been shown to prevent apoptosis by many stimuli that result in the elevation of ROS levels in cells (Briehl et al., 1997). For example, apoptosis caused by a UV-irradiated human luekemia cell line was significantly reduced by the various antioxidants (Verhaegen et al., 1995). N-acetylcysteine (NAC) is a thiol-containing antioxidant that prevents apoptosis induced by ROS in a variety of cell types. NAC may rescue cells from apoptosis by scavenging free radicals or by promoting the uptake of extracellular cystine and synthesis of the cellular antioxidant, glutathione, a potent inhibitor of oxidative stress and apoptosis (Issels et al., 1988). NAC can block the expression of proteins and the activation of proteins that are stimulated by oxidative stress (Bergelson et al., 1994; Huot et al., 1995). Also, NAC can inhibit apoptosis in human male germ cells *in vitro* (Erkkilä et al., 1998). Glutathione (GSH) is present at a 1-10 mM concentration in most cells and some data suggest intracellular depletion of reduced GSH during apoptosis (Bai et al., 1994). Reduced GSH serves as a storage and transport form of cysteine, protects cells from toxicants, functions as a redox buffer, and protects cells from ROS. GSH protects cells from ROS directly and by serving as a substrate for GSH peroxidase, resulting in the oxidation of GSH. Then GSH reductase regenerates GSH from oxidized GSH using NADPH. GSH can be synthesized de novo by the γ-glutamyl pathway to recycle extracellular GSH (Meister, 1973). However, some cells can import GSH from the extracellular space (Bai et al., 1994). Therefore, a supply of additional GSH in medium could reduce oxidative stress and prevent apoptosis of cells. Butylated hydroxyanisole (BHA) is a synthetic phenolic antioxidant that decreases apoptosis by decreasing the intrinsic oxidative stress in leukemia cells (Verhaegen et al., 1995; Lotem et al., 1996). N-t-butyl-α-phenylnitrone (BPN) is known as selenosubstilin and mimics the antioxidant enzyme glutathione peroxidase (Peterson et al., 1995). Along with cimetidine (CIM), an antioxidant and known suppressor of gastric acid secretion, BPN also could suppress apoptosis induced by oxidative stress (Lotem et al., 1996).

Molecular events governing apoptosis in cultured porcine PGCs are not yet known. Results from several studies suggest the involvement of products of the Bcl-2 gene family in apoptosis especially that induced by oxidative stress (Peterson et al., 1995; Yang and Korsmeyer, 1996). Bcl-2 also inhibits retinoic acid-induced apoptosis during neural differentiation of embryonal stem cells (Okazawa et al., 1996). Mammalian heat shock protein 27 (hsp 27) plays a role as a switch between differentiation and apoptosis in mouse ES cells (Mehlen et al., 1997). The underexpression of hsp27 attenuates cell growth arrest, and induces differentiation and eventually apoptosis in ES cells, while overexpression enhances the differentiation-mediated decrease in rate of ES cell proliferation (Okazawa et al., 1996).

The effect of protease inhibitors and antioxidants on *in vitro* survival and proliferation of PGCs from domestic animals, especially porcine, were assessed (Lee et al., 2000b). While the culture system provided all known growth factors required to support survival and proliferation of PGCs *in vitro*, rapid loss of porcine PGCs indicated that apoptosis was still occurring (Lee et al., 2000bd). To determine whether apoptosis inhibitors rescue *in vitro* cultured porcine PGCs, effects of α_2-macroglobulin (MAC), a broad range protease inhibitor and known cytokine carrier, and N-acetylcysteine (NAC), an antioxidant were evaluated. Both MAC and NAC increased the number of PGC colonies surviving after 8-10 days of culture, and, when combined, had a slight additive effect. In the experiment testing the effects of other protease inhibitors, only MAC was effective on survival of porcine PGCs. Since MAC is a cytokine carrier (Borth and Luger, 1989; Gonias et al., 1994), and PGCs and EG cells in culture are dependent on cytokines (DeFelici and Pesce, 1994; Donovan, 1994), the beneficial effect of MAC may be due to its ability to facilitate the activity of cytokines present in the media to increase survival and proliferation of PGCs in culture (Lee et al., 2000b).

In case of antioxidants, all antioxidants tested were effective at increasing the number of AP-positive colonies suggesting that by reducing the oxidative stress and

apoptosis, there was a beneficial effect on porcine PGCs (Lee et al., 2000b). These results suggest that oxidative stress is an inducer of apoptosis and that antioxidants suppress apoptosis in porcine PGCs. In experiments with mouse ES cells, which have similar characteristics to PGCs, apoptosis induced by increasing ROS could be suppressed by the addition of antioxidants such as NAC and GSH to culture medium (Piedrahita et al., unpublished).

Further experiment with feeder-free culture system and quantitative *in situ* TUNEL assay showed that the beneficial effect of both molecules, protease inhibitor and antioxidant, on the survival of porcine PGCs in culture, was direct inhibition of apoptosis of porcine PGCs *in vitro* (Lee et al., 2000b). The beneficial effect of MAC on PGCs requires a constant supply of MAC and cytokines in the medium. NAC, on the other hand, was equally effective in the presence and absence of growth factors although the response was greater in the presence of growth factors. Furthermore, the beneficial effects of MAC and GSH on survival of porcine PGCs resulted from the inhibition of apoptosis during the early culture period, as confirmed by using quantitative *in situ* TUNEL assay (Lee et al., 2000b).

CONCLUSIONS

Production of genetically modified domestic animals has a huge potential on improving animal production as well as providing valuable products and information to human medicine. Despite the rapid development in the production of transgenic domestic animals via pronuclear injection and nuclear transfer, the precise modification of the genome using pluripotential ES cells is not readily available due to the absence of gene targeting- and/or germ line-competent ES cells in domestic animals. EG cells, an alternate source of pluripotential embryonic cells, in domestic animals could be used in generation of transgenic domestic animals. Since the low efficiency nature of gene targeting, to make gene targeting feasible in this cell line, a better culture system would help to minimize the unnecessary loss of cells *in vitro*.

Since the one of possible cause of cell loss in porcine PGCs is apoptosis, apoptosis inhibitors, such as protease inhibitors and antioxidants, could reduce the apoptosis in EG cells thus increasing cell survival *in vitro*. This should increase the probability of gene targeting in porcine PGCs by increasing the number of cells available for homologous recombination. Recent studies with bovine nuclear transfer (Zakhartchenkp et al., 1999) indicated that germ cells could be reprogrammed and drive subsequent development of the transferred embryo. Also, apoptosis inhibitors could reduce apoptotic cells in serum deprived porcine embryonic fibroblasts (Lee and Piedrahita, 2001). Therefore, suppression of apoptosis of PGCs in culture could also improve the quality of donor cells for nuclear transplantation.

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