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Characterization of Apoptosis in Porcine Primordial Germ Cells *In Vitro*

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ABSTRACT

When porcine primordial germ cells (PGCs) isolated from the genital ridge and placed in culture to establish EG cells, a large proportion of PGCs are lost during the early period of culture. To characterize the *in vitro* death of porcine PGCs, PGCs were cultured in suspension, and apoptosis analyzed using a fluorescent activated cell sorter-based DNA fragmentation assay. The results from flow cytometric analysis showed an increase in apoptosis in cultured cells. However, the cells isolated from the genital ridges are a mixture of PGCs and somatic cells. To detect apoptotic signals specific from porcine PGCs, quantitative TUNEL assay was performed at different time of culture (0~24 h). The proportion of apoptotic porcine PGCs determined by double staining with alkaline phosphatase activity and *in situ* TUNEL assay increased as the time of culture progressed and continued at least 24 h. These results demonstrate that one of the causes of loss of porcine PGCs *in vitro* is apoptosis.

(Key words : Porcine PGCs, Apoptosis, TUNEL assay)

I. INTRODUCTION

Apoptosis is a natural physiological process of cell death in eukaryotic multicellular organisms (Kerr, 1971). This process, 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 change 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 is initiated through a genetic program within the target cell. Apoptosis is characterized by morphological features such as cell shrinkage, chromatin condensation, membrane blebbing, and disintegration into apoptotic bodies (Cohen, 1996). In addition, there are several biochemical characteristics including internucleosomal DNA cleavage (Wyllie, 1980; Gavrieli et al., 1992), release of cytochrome c from mitochondria into the cytoplasm (Liu et al., 1996), a cascade of caspase reactions (Thornberry and Lezebnik, 1998), and translocation of phosphatidylserine from inner to outer plasma membrane (Fadok et al., 1992). Apoptosis differs from necrosis in that it does not elicit an immune response since the apoptotic bodies are disposed of by phagocytosis (Cohen, 1996).

Primordial germ cells (PGCs) are the precursors of germ cells found in developing gonads of fetus,

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and origin of one of the pluripotential embryonic cell lines, embryonic germ (EG) cells. (Matsui et al., 1992; Resnick et al., 1992). In domestic animals, EG cells derived from culture of PGCs may be an alternate choice of cells for precise genetic modification of the genome through homologous recombination.

In mice, apoptosis is one of the causes of the *in vivo* and *in vitro* loss of PGCs (Coucouvanis et al., 1993; Hsueh et al., 1996). In a previous studies, techniques were developed whereby porcine PGCs were isolated and cultured *in vitro* successfully (Shim et al., 1997; Piedrahita et al., 1998; Mueller et al., 1999). Since pluripotential EG-like cells from porcine PGCs are available, the next step would be gene targeting in this cell line. However, there is a high level of PGC loss during the initial period of culture, exacerbating the problem of low efficiency of homologous recombination by reducing number of cells available. Based upon studies with mouse PGCs, the possible cause of this loss may be apoptosis (Pesce et al., 1993; Pesce and DeFelici. 1994).

This study was carried out to characterize the *in vitro* death of porcine PGCs by DNA fragmentation assay. Also, quantitative analysis of apoptotic porcine PGCs were performed using both alkaline phosphatase staining and *in situ* TUNEL assay.

Ⅱ. MATERIALS AND METHODS

1. Collection of Porcine Primordial Germ Cells (PGCs)

Porcine fetuses from crossbred gilts were collected by hysterectomy between Days 25 and 30 of pregnancy (estrus=Day 0). Fetuses were washed in PBS supplemented with BSA (0.4%, Sigma, USA) and penicillin/streptomycin (1%, Gibco BRL, USA) (PBS/BSA/PS). Urogenital ridges, detected as longitudinal protrusions along the medial mesonephric surface, were isolated and washed in PBS/BSA/PS followed by several washes in PES medium [50:50 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F10 medium supplemented with 15% fetal bovine serum (FBS), L-glutamine (2mM), β-mercaptoethanol (0.1mM), 1% MEM non-essential amino acids and 1% penicillin/streptomycin] before PGC collection. All media and supplements were purchased from Gibco BRL. The FBS, selected for its ability to maintain mouse ES cells, was obtained from Summit Biotechnology (USA).

Primordial germ cells (PGCs) were isolated by incubation of the genital ridge in 0.25% trypsin/ 1 mM EDTA solution (Gibco BRL) followed by gentle pipetting or by mechanical dissociation of genital ridges into small fragments followed by gentle pipetting. After tissue disruption, cells were centrifuged for 5 min at 50g to remove tissue debris and clumps. The supernatant containing most of the single cells was collected and centrifuged at 200g for 10 min.

2. *In Vitro* Culture of Porcine PGCs

PGCs were resuspended with PES medium without growth factors. Resuspended cells were placed in suspension culture in a humidified environment of 5% CO_2 in air at 38℃ for 6 h. Occasionally, the cells were agitated to prevent the formation of clumps. The PGCs were collected by centrifugation and rinsed with PBS before further analysis.

3. Analysis of Apoptosis in Cultured PGCs by Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-end Labeling (TUNEL) Assay

After culture, cells were fixed and stained according to protocols with the APO-BRDU kit (Phoenix Flow Systems, USA). Briefly, cells were fixed in 1% paraformaldehyde in PBS on ice for 15

minutes, washed in PBS, fixed in 70% ethanol in PBS and stored at −20℃ until use. After removing the fixative, cells were washed twice and suspended in 50 μl of labeling solution containing Br-dUTP (bromolated deoxyuridine triphosphate) and TdT (terminal deoxynucleotidyl transferase) for 60 minutes at 37℃. TdT catalyzes the addition of Br-dUTP to the 3'-OH ends of DNA strand breaks in a template independent manner. To determine base line fluorescence, half of each sample was incubated without TdT. After rinsing, cells were incubated with an anti-BrdU monoclonal antibody conjugated to fluorescein for 30 minutes in the dark at room temperature; apoptotic cells which contain many free 3'-OH ends are intensely labeled, while non-apoptotic cells whose DNA is largely intact have little Br-dUTP incorporation and remain unlabeled. Following counterstaining with a propidium iodide/RNase A solution for 30 minutes in the dark, which allows simultaneous analysis of cell cycle position and apoptosis, cells were analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson, USA). Fluorescence emissions were measured at 582±21nm for propidium iodide and 530±15 nm for fluorescein, without spectral compensation, after excitation at 488 nm by an argon-ion laser. For analysis, gates were set so that less than 2% of samples incubated without TdT were positive. Any event above this gate was deemed TUNEL positive for samples incubated with TdT. After staining, cells were plated on slide glass and observed for apoptotic cells under the fluorescent microscope.

4. Quantitative Analysis of Apoptosis in Porcine PGCs

To analyze the apoptosis in porcine PGCs quantitatively, both *in situ* TUNEL assay and alkaline phosphatase (AP) staining were performed simultaneously. Briefly, about 1,000 freshly isolated cells were cultured for 6, 12, and 24 h in PES medium without feeder cells in a chambered slide (NalgeNunc Int., USA) coated with poly-L-lysine. After culture, cells were fixed and stained for AP to identify PGCs from other somatic cells. The AP activity was determined as described previously (Piedrahita et al., 1998), after slides were rinsed twice in PBS and fixed in 4% formaldehyde in PBS for 15 min at room temperature. Fixed cells were washed twice with PBS and stained in naphthol AS-MX phosphate (200 μg/ml; Sigma) and Fast Red TR salt (1 mg/ml; Sigma) in 100 mM Tris buffer, pH 8.2 for 30 min at room temperature. Washing slides in PBS terminated staining. Specificity of AP activity was determined by staining in the presence of the AP inhibitor, tetramisole (0.5mM; Sigma).

Apoptotic cells were then detected by TUNEL assay using ApopTag Kit (Intergen, USA). Briefly, cells were treated with equilibration buffer and stained in labeling solution containing digoxigenin-dNTP and Tdt for 60 min at 37℃. After termination of the reaction and several washes, cells were incubated with an anti-digoxigenin antibody conjugated to fluorescein for 30 min at room temperature. After a wash with PBS, slides were counterstained for 30 min at room temperature and mounted using mounting medium containing 0.5～1.0 μg/ml propium iodide (Antifade™; Oncor, USA). Slides were then viewed using fluorescence microscopy (excitation 488 nm and emission 520 nm). At least 300 AP-positive cells were counted and the proportion of apoptotic PGCs that were both AP positive and fluorescent was determined. For statistical analysis, experiments were replicated three times. The means were tested for homogeneity of variance, and analyzed by ANOVA. Mean separation was accomplished by Fisher's protected least significant difference (LSD) using Super-ANOVA software (Abacus Concepts, USA). The level of significance was set at $p<0.05$.

Ⅲ. RESULTS

When porcine PGCs were isolated and cultured, large numbers of cells were found unattached in the early period of the culture. In a preliminary experiment, a large portion of these floating cells were AP-positive when collected. These cells were also not able to exclude trypan blue dye indicating that they were dead. Therefore, large numbers of porcine PGCs were lost during the initial period of culture.

Apoptosis in porcine PGCs was evaluated using the TUNEL assay which detects internucleosomal DNA double strand breaks, resulting from the activated endonucleases at early stage of the apoptotic process. To determine the rate of initial apoptosis, freshly isolated cells from genital ridge were cultured in suspension for 6 hr. TUNEL-stained cells were then observed under the fluorescent microscope. TUNEL staining failed to detect any chromosomal damage immediately after isolation of cells (Fig. 1A). However, TUNEL positive cells increased after culture (Fig. 1B), with signals from fluorescein being specific to apoptotic nuclei. The proportion of apoptotic cells was determined in TUNEL-stained cells using flow cytometer Results indicated that apoptotic cells were increased from 2.92 % to 7.78 % after 6 h of culture in suspension (Fig. 1C, D). Also, before and after culture, most of the cells isolated were G_0/G_1 stage according to propidium iodide (PI) counter-staining (Fig. 1C, D).

The drawback of the FACS analysis is that cells isolated from genital ridges are not pure PGCs. They also include somatic cells, such as epithelial cells, fibroblasts, and blood cells. AP staining right after PGC isolation indicates that only 50～70 % of cells are AP-positive. Therefore, apoptotic signals detected from previous experiments could come from PGCs or contaminating somatic cells.

To determine the exact proportion of apoptotic PGCs during culture, freshly isolated cells were cultured for 0, 6, 12 and 24 h, stained for AP activity first to detect PGCs and then performed *in situ* TUNEL assay to detect apoptotic cells. Therefore, both AP and TUNEL-positive cells are apoptotic PGCs (Fig. 2). The proportion of apoptotic PGCs were calculated from TUNEL positive cells from at least 300 AP-positive cells. As in Fig. 3, apoptotic porcine PGCs were only 4.7 % at 0 h. However, as the time of culture increase, the proportion of apoptotic porcine PGCs was significantly increased up to 57.8 % at 24 h ($p< 0.05$). The proportion of apoptotic porcine PGCs was not increased more than 60～70% when cultured up to 72 h (data not shown).

Ⅳ. DISCUSSION

Results from the previous reports (Lee et al., 2000) and the present study indicated that a large proportion of PGCs die soon after culture *in vitro*. Apoptosis is implicated in germ cell development during both the prenatal and postnatal periods. In mice, the mechanism of death of prenatal germ cells during early development is apoptosis according to the result of flow cytometric analysis (Coucouvanis et al., 1993). Coucouvanis et al. (1993) measured sub G_1 fraction of cells, less than 2N genetic content representing apoptotic cells, to measure *in vivo* rate of apoptosis in mice. Furthermore, for the functional spermatogenesis, an early massive wave of germ cell apoptosis is required (Rodriguez et al., 1997). Ovarian cell apoptosis is responsible for atresia of 99.9% of follicles (Hsueh et al., 1996; McGee et al., 1998). Therefore, apoptosis could play an important role in avoiding uncontrolled germ cell proliferation and in eliminating misplaced germ cells which might be harm to the host. Subsequent amount of mouse

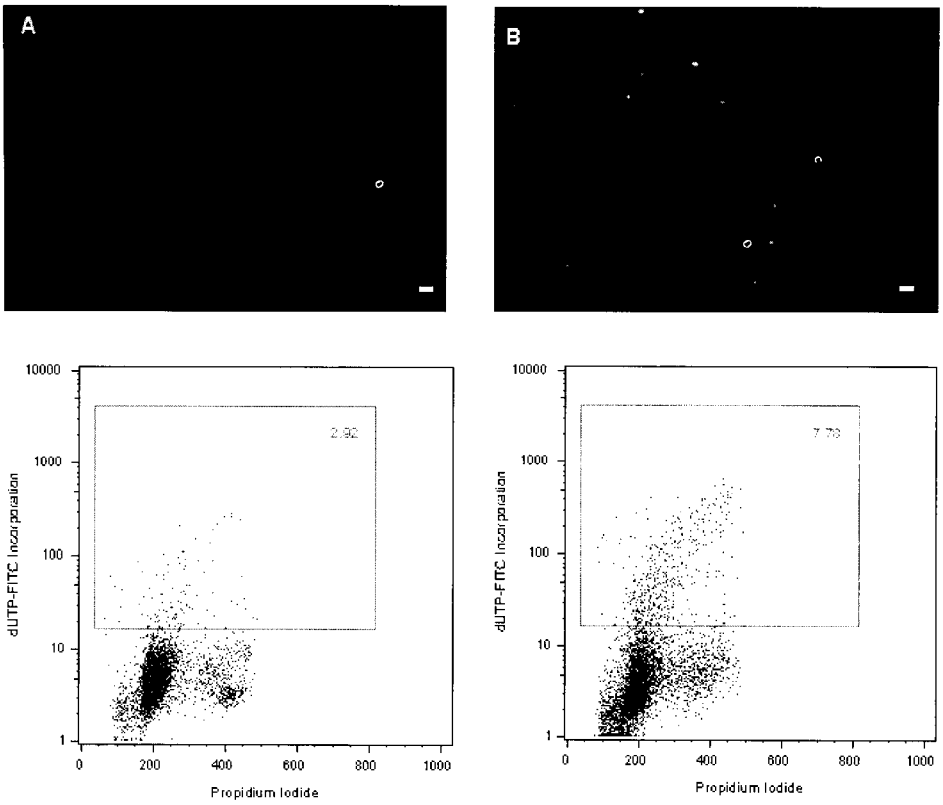

Fig. 1. Analysis of cultured porcine PGCs by TUNEL assay. A) Freshly isolated porcine PGCs stained by TUNEL. B) Porcine PGCs cultured in suspension for 6 h and stained by TUNEL. Bar=100 μM. Flow cytometric analysis of porcine PGCs C) before and D) after culture for 6 hrs in suspension. X-axis indicates TUNEL-specific staining and Y-axis propidium iodide (PI) counter-staining.

PGCs (20~60%) showed typical apoptotic features when they placed in culture for 4~6 h (Pesce et al., 1993). In previous study, cultured porcine PGCs showed typical morphology of apoptotic cells determined by the transmission electron microscopy and *in situ* TUNEL assay (Lee et al., 2000). Also, by quantitative TUNEL assay used in this study, porcine PGC showed 36.3% and 59.6% apoptosis at 6 h and 12 h of culture without growth factors and feeder cells (Lee et al., 2000).

This experiment is an extension of avprevious study (Lee et al., 2000) to show the logical steps determining the apoptosis in porcine PGCs and also to determine the rate of apoptosis right after the isolation and after 24 h of culture. Cells isolated from the genital ridge showed increase in apoptotic cells when cultured in suspension for 6 h (Fig. 1). Also, the proportion of apoptotic porcine PGCs (Fig. 2) were increased significantly ($p<0.05$) as the time of culture increased (Fig. 3). As mentioned previously, the proportion of apoptotic porcine PGCs did not increase after 24 h. The possible explanation is that after 24 h porcine PGCs died not through the apoptosis but through the necrosis which the TUNEL assay could not detect. It has been shown that if there are excessive and

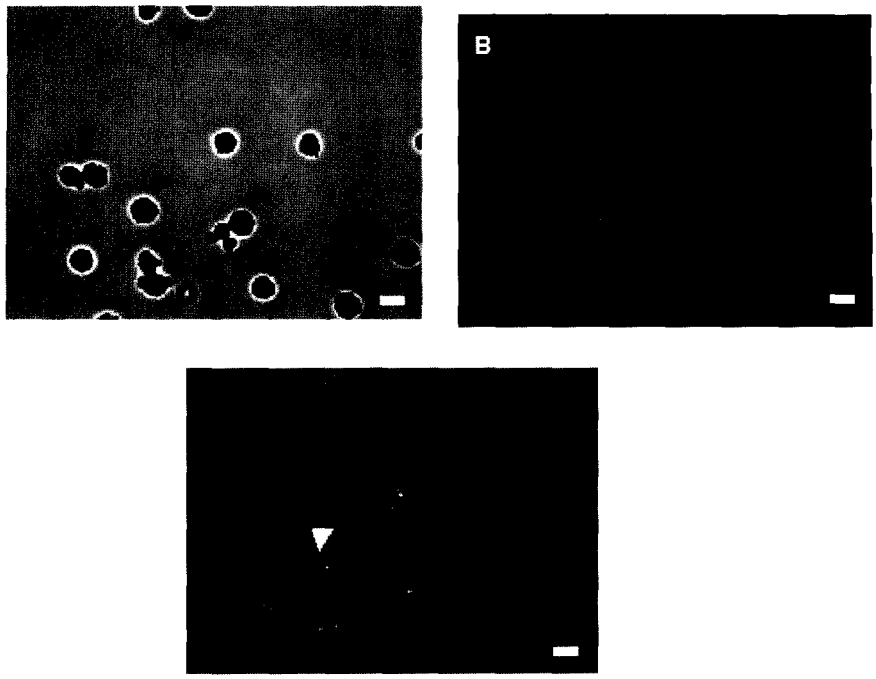

Fig. 2. Identification of apoptotic porcine PGCs by double staining with alkaline phosphatase and *in situ* TUNEL assay. Isolated porcine PGCs were cultured for 6 h in the absence of feeder layers. After culture, cells were fixed, stained for AP, and analyzed for apoptotic cells by *in situ* TUNEL assay. A) Cells under transmitted light. Note that AP-positive PGCs (▼) are present along with somatic cells. AP staining is identified by its dark coloration. B) Same cells under the UV (488 nm) light to identify apoptotic cells by their fluorescent signal. C) Same cells under both fluorescent and normal light. Note that the fluorescent signals are specifically located in the apoptotic nuclei. Apoptotic porcine PGCs can be identified by darker coloration from AP staining and bright signal from apoptotic nuclei (▽), while apoptotic somatic cells show only the signal from TUNEL staining (▼). Bar=10 μM A-C.

continuous apoptotic stimuli present, cells tend to die through necrosis rather than apoptosis (Davies, 1998). The overall finding of the present study was that porcine PGCs in culture *in vitro* undergo rapid apoptosis especially in the early period of culture. This finding is in agreement with the fact that the fate of cells to either proliferate or undergo apoptosis is determined rapidly during the early period of apoptotic stimuli and beyond a certain point during apoptosis, the ongoing apoptotic procedure can not be reversed (Cohen, 1996).

Apoptosis can be triggered in various ways, including growth factor deprivation, loss of contact with extracellular matrix, oxidative stress due to increase in reactive oxygen species, binding of death ligand, UV and γ-radiation, and viral infection. Since the environment of PGCs was changed upon isolation from the gonad, improper growth factors or cytokines in the culture medium might cause apoptosis. Growth factors, in particular, are indispensable for maintaining survival and proliferation of mouse PGCs *in vitro* (DeFelici and Pesce, 1994; Donovan, 1994). In mouse PGCs, LIF can promote survival and stimulate proliferation

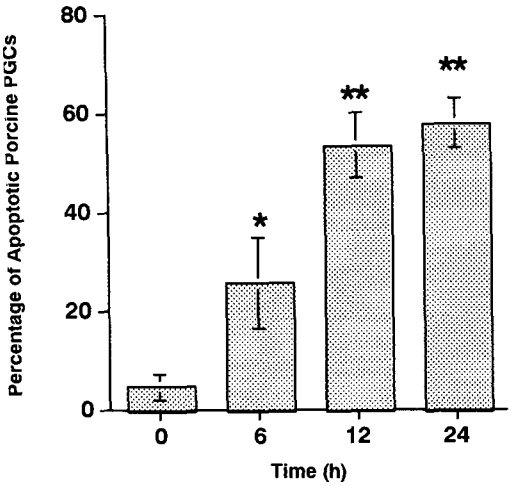

Fig. 3. Quantification of apoptotic porcine PGCs during early period of culture *in vitro*. Isolated PGCs were cultured on chamber slides coated with poly-L-lysine for 0, 6, 12, and 24 h in PES medium. At each time, cells were fixed, stained for AP and for apoptotic cells by the TUNEL assay as described in Materials and Methods. The proportion of apoptotic PGCs, both AP-positive and fluorescent, was calculated from counting at least 300 AP-positive cells. Each experiment was carried out in duplicated wells, and numbers are the means ± SD of three experiments.

(Matsui et al., 1992; Resnick et al., 1992) through activation of the gp130 pathway (Koshimizu et al., 1996). Soluble SCF, the product of the Steel locus, can also support PGC survival and, with LIF, stimulate proliferation (Matsui et al., 1991). Furthermore, membrane bound SCF, a splicing variant of SCF expressed by the feeder cells, is absolutely required for survival of PGCs (Matsui et al., 1991; Pesce et al., 1997). Thus, PGCs can survive and proliferate longer in the presence of feeder cells such as STO, TM_4 and S_l/S_{l4} m220 cells, all of which express membrane bound SCF. Growth factors, LIF, SCF and bFGF, are also indispensable for the *in vitro* survival of porcine PGCs (Lee et al., 2000) and the establishment of EG-like cells (Lee and Piedrahita, in press).

In addition, PGCs are migratory cells and interact with surrounding somatic cells during migration via interaction between integrins and extracellular matrix (Garcia-Castro et al., 1997), which are involved in survival and apoptosis (Meredith et al., 1993; Boudreau et al., 1995). Therefore, PGC adhesion is crucial for their survival and proliferation. Fibronectin and laminin are expressed in the path of PGC migration and involved in adhesion of PGCs (Garcia-Castro et al., 1997; DeFelici et al., 1998). In experiments not described here, porcine PGCs were cultured for 24 h on various adhesion molecules, such as poly-L-lysine, gelatin, and fibronectin, and stained with TUNEL assay. Analysis of apoptosis using flow cytometry indicated that with the exception of poly-L-lysine, none of the treatments rescued the cells from apoptosis (unpublished data).

During apoptosis, many proteases are activated in a cascade way. Caspases are located upstream of this cascade event. Using specific or broad range protease inhibitors to inhibit the activity of caspases, apoptosis can be reversed. Proteases inhibitors of cysteine, aspartic, and serine proteases have been shown to inhibit cell death (Chow et al., 1995; Kumar and Harvey, 1995), perhaps due to inhibition of proteases involved in apoptosis (Thornberry and Lezebnik, 1998; Stefanis et al., 1997). Oxidative stress generated by reactive oxygen species (ROS) is the most common stimulus causing apoptosis. ROS can be produced in many ways including those mentioned above. Oxidative stress is one of the factors involved in apoptosis in rat testicular germ cells and mouse embryonic stem cells (Ikeda et al., 1999; Castro-Obregon and Covarrubias, 1996). Antioxidants, scavengers of oxygen radicals, could reduce oxidative stress and

eventually inhibit apoptosis by removing apoptotic stimuli (Briehl et al., 1997; Verhaegen et al., 1995). NAC, a thiol-containing antioxidant, can inhibit apoptosis in human male germ cells *in vitro* (Erkkilä et al., 1998). 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). Also, α_2-macroglobulin, a protease inhibitor and cytokine carrier, and antioxidants have beneficial effects on survival of porcine PGCs *in vitro* (Lee et al., 2000).

In conclusion, there is a large loss of porcine PGCs in the early period of culture and, as demonstrated by DNA fragmentation assay, apoptosis is the cause of this loss. This early loss of PGCs *in vitro* could affect the efficiency of homologous recombination. Therefore, the inhibition of apoptosis in PGCs *in vitro* could increase the number for cells available for homologous recombination and also increase the efficiency of gene targeting.

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요 약

체외 돼지 원시 생식세포의 Apoptosis 특성 규명

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돼지 원시 생식세포를 미성숙 성선에서 분리하고 체외 배양하여 EG 세포를 얻으려 할 경우, 상당수의 세포들이 배양초기에 손실을 입게 된다. 이러한 돼지 원시 생식세포의 체외 손실 원인을 규명하고자, 미성숙 성선에서 분리된 세포를 부유 배양을 하고 FACS (fluorescent activated cell sorter)를 이용한 DNA 절편 분석법으로 apoptosis를 관찰한 결과 체외 배양된 처리구에서 apoptosis가 증가되었다. 그러나, 미성숙 성선에서 분리된 세포는 원시 생식세포와 체세포가 혼합된 세포들이므로, apoptosis가 일어난 돼지 원시 생식세포를 다른 체세포들로부터 구분하기 위하여 0 시간부터 24 시간까지 배양된 세포를 대상으로 정량 TUNEL 분석을 시행하였다. 이 결과, alkaline phosphatase 활성과 *in situ* TUNEL 분석을 통하여 apoptosis가 일어난 돼지 원시 생식세포가 시간이 경과함에 따라 증가되었다. 이러한 결과들을 종합하여 볼 때 apoptosis가 돼지 원시 생식세포의 체외 손실의 원인 중 하나임을 규명하였다.

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