THE DEVELOPMENT AND CHARACTERIZATION OF A SOMATIC CELL LINE FOR FELINE NUCLEAR TRANSFER

A Senior Honors Thesis

By

SARAH ADRIANNE HUTCHISON

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

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RESEARCH FELLOWS

April 2000

Group: Cell Biology I

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ABSTRACT

Nuclear transfer is fast becoming an alternative method for reproduction, and it is useful in producing genetically identical animals. This study was designed to develop and characterize a cell line which may be possible to use in nuclear transfer in felines. Cells which are useful for nuclear transfer must be synchronized with the oocyte being fused in order to prevent possible aneuploidy due to high MPF in oocytes arrested in metaphase II. Granulosa cells were collected from cat ovaries and grown in culture. After a few passages, cells were analyzed by using flow cytometry (FACS) to evaluate their stage in the cell cycle and their ploidy. Cells from passages up to passage 2 were analyzed as well as freshly collected granulosa cells. Also, serum-starved cells and cells from passage 1 were compared. It was found that the freshly collected cells had the highest percentage of cells in G0/G1 (89%), suggesting they may be useful for nuclear transfer. However, cells in culture exhibited the highest number of cells in G0/G1 at passage 2. Also, serum starved cells were significantly more synchronized in G0/G1 than regularly fed cells, as expected (85% compared to 70%). It appears as though cells in passage 2 which have been synchronized using serum-starvation are the best candidates for nuclear transfer. Serum-starved cells from passage 2 are currently being used in nuclear transfer.

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INTRODUCTION

Purpose: The purpose of this project is to develop and characterize a somatic cell line which can be used for feline nuclear transfer.

Hypothesis: The nuclear transfer of a somatic cell to an enucleated oocyte will result in an embryo reaching the blastocyst stage in the domestic feline.

Nuclear transfer has been performed in numerous mammalian species, including cattle[8,21,25,26,30,33], rabbits[18,35], sheep[4,8,31,32], mice[13], and pigs[20,22]. However, it has yet to be performed with felines, with whom a few other reproductive techniques have been performed successfully. These include embryo transfer[15], superovulation[28], embryo recovery[28], in vitro fertilization[2], embryo growth in vitro[28], and transplantation[28]. Nuclear transfer simply entails the removal of the nucleus from a mature oocyte (referred to as the cytoplast) followed by the fusion of a diploid cell (referred to as the karyoplast) with the oocyte. After fusion is induced by an electric pulse, cleavage and formation of an embryo result. Upon reaching the blastocyst stage, the embryo can be transferred to a recipient mother, who will carry it to term. Because all chromosomal DNA came from the cell donor, the newborn animal should be genetically identical to the donor of the karyoplast.

Several possible benefits may result from feline nuclear transfer. In particular, numerous genotypically identical animals are useful for research. The lack of genetic variability among these animals helps to develop control groups with the use of fewer test subjects. Cats are especially useful in several areas of biomedical research for humans. In particular, they are useful for research in the areas of HIV and neurological disorders.

Another advantage of nuclear transfer in the domestic feline is the possibility of serving as a model for other feline species. Endangered feline species might benefit from this technology. While the use of nuclear transfer to propagate endangered species may reduce genetic variability, many endangered feline species reproduce from a rather small gene pool as it is. Through the use of selection with nuclear transfer, animals with traits which will aid them in the wild may be proliferated.

Granulosa cells were cultured in this experiment. The cells were characterized by observing the number of embryos resulting from nuclear transfer and a method of flow cytometry called Fluorescence-Activated Cell Sorter (FACS) to observe ploidy and cell cycle stage. Flow cytometry has previously been shown to be a good method for characterization of granulosa cells in cattle Blondin, et al., 1996)[1].

BACKGROUND

Granulosa Cell Characterization

Granulosa cells have previously been cultured in humans[6,11,19], cattle[1,5,15,16,17,30], pigs[9], mice[10,13,23,29], and rats[14]. Granulosa cells have, in fact, been extensively cultured in cattle for numerous purposes. They have also been shown to display some unusual properties across different species. In particular, Evagelatou, Peterson, and Cooke (1997) used FACS to analyze human granulosa cells. They were interested in assessing the percentage of ovarian cells collected which were actually leukocytes. They found that between 15-60% of granulosa cells collected were leukocytes, which was a curious result[11]. However, Spanel-Borowski and Ricken (1997) had reported earlier that year that bovine granulosa cell cultures contained macrophage-like cells after day 10 of culture. These cells were identified as macrophages through immunolabeling for CD14, CD18, or CD45 (all surface molecules for different varieties of macrophage cells). They hypothesized that ovarian macrophages may be involved in basal membrane degradation after follicular rupture or possibly angiogenesis during follicle/corpus luteum development[24]. Evagelatou, et al. (1997) found that macrophages most likely interact with ovarian cells through several possible methods, such as cytokine secretion in order to increase 11beta-hydroxysteroid dehydrogenase activity (involved in ovarian steroidogenesis)[11].

How can one cell type suddenly develop into another as reported by Spanel-Borowski, et al. (1997)? Lavranos, O'Leary, and Rodgers (1999) gave evidence that bovine granulosa cells exhibit properties of stem cells and tumor cells, which may indicate why they have been useful in nuclear transfer[17]. Like Spanel-Borowski, et al. (1997), they reported that granulosa cells can divide without anchorage – a classic stem cell property. They also observed that follicular granulosa cells can divide uninhibited by contact with each other. To test the hypothesis that granulosa cells arise from a population of stem cells, they examined cell cycles and telomerase expression in granulosa cells from follicles in various stages of development. Telomerase is an enzyme which prevents shortening of chromosome ends (telomeres) over time during cell division. It is a key element which allows stem cells and cancer cells to divide indefinitely. They found that telomerase RNA was detected in growing follicles but not in primordial follicles. They also found that telomerase activity was increasingly higher in granulosa cells taken from follicles at earlier stages of development.

Earlier the same team had shown that division of these anchorage-independent granulosa cells which exhibited stem cell properties were stimulated by insulin-like growth factors (IGF) as well as inhibited by insulin-like growth factor binding protein 1 (IGFBP-1)[16]. IGF-1 receptors were also shown to be present on human granulosa cells by De Neubourg, et al. (1998)[6]. Thus, it seems possible that the presence of these IGF-1 receptors indicates similar development of granulosa cells from stem cell-like cells to the bovine development reported by Spanel-Borowski, et al. (1997).

Other groups have characterized granulosa cells, many reporting sub-populations of the cells and differentiation during follicular development. Prior to ovulation, cumulus cells release hyaluronic acid in response to gonadotropins which causes them to form a mucus-like matrix. This process, termed mucification, distinguishes cumulus and mural granulosa cells. Mural granulosa cells ultimately become luteal cells[23].

Schuetz, et al. (1996) found that mouse cumulus granulosa cells have alterations in their cell cycle during expansion and mucification[23]. Kerketze, et al. (1996) observed several different sub-populations of granulosa cells in developing rat follicles using flow cytometry and lectin binding. They found two populations of granulosa cells which they referred to as "small" and "large." Not surprisingly judging from other characterization of granulosa cells, they found that granulosa cells exhibited cellular heterogeneity in rat follicles, different ratios of which exist depending on the stage of follicular development and location within the follicle[14]. Duda, Gasinska, and Gregoraszczuik (1999) separated porcine granulosa cells into two subpopulations based on the amount of binding among cells. The two populations, weakly associated and tightly bound, were analyzed using flow cytometry. They found that the tightly bound subpopulation had lost mitotic potential and were more differentiated (thus making them less desirable for nuclear transfer). They also observed more apoptosis among the weakly-associated, less differentiated subpopulation[9].

Mural granulosa cells and cumulus granulosa cells have been shown by Eppig., et al. (1997) to differ somewhat in structure and function as well. They hypothesized that murine oocytes helped to establish granulosa cell heterogeneity in pre-ovulatory

follicles. They distinguished mural granulosa cells phenotypically on the basis of leutenizing hormone receptors, which are present on mural granulosa cells but not on cumulus granulosa cells. They found that after removing the oocytes from cumulus-oocyte complexes that cumulus granulosa cells began to express LH receptors. They concluded that oocytes suppress LH receptor expression through paracrine signals and thus are responsible for granulosa cell differentiation into cumulus complexes[10].

Schuetz, et al. (1996) also observed cumulus cell differentiation in response to exposure to various hormones, including FSH, DBcAMP, and EGF in vitro. They found that both FSH and DBcAMP suppressed S phase in cumulus granulosa cells and retarded their proliferation. They also noted that these hormones promoted secretion of hyaluronidase and subsequent mucification of cumulus cells[23]. Their data support the theory that DBcAMP somehow controls cumulus cell mucification through mediation of FSH.

Nuclear Transfer

Earlier experiments involving nuclear transfer involved the use of blastomeres as karyoplasts. Willadsen (1986) first used blastomeres for nuclear transfer in sheep, resulting in several live births[31]. Prather, et al. (1987) used blastomeres to successfully produce calves[21]. Also, Prather, Sims, and First (1989) produced pigs with this procedure as well[22]. Several groups began using later-stage embryonic cells for nuclear transfer in different mammalian species. Inner cell mass cells, in particular, were used successfully. These cells belong to one of two cell types found in blastocyst-stage

embryos. Thus, they were more differentiated than those cells which had previously been used for the procedure. Sims and First (1993) used cultured inner cell mass cells for nuclear transfer in cattle, which resulted in several live births. Wilmut, et al. (1996) also used inner cell mass cells successfully in sheep. Several other groups tried to use ICM cells for nuclear transfer in cattle and pigs, but were unsuccessful[27].

The turning point in nuclear transfer technology was when Wilmut, et al. (1997) reported the live birth of Dolly, a sheep produced from nuclear transfer using a mammary epithelial cell as the karyoplast[32]. This paper was the first instance of a differentiated somatic cell being used for nuclear transfer. It had previously been thought that differentiated cells could not be used in nuclear transfer because of DNA modifications, such as DNA methylation histone binding, and chromosome shortening due to lack of telomerase activity.

Wilmut overcame the difficulties of using a differentiated karyoplast through cell cycle synchrony[7]. The cell cycle consists of four phases: G1, S, G2, and M. G1 is a growth phase in which cells are diploid prior to DNA synthesis. The S-phase is the phase in which DNA synthesis takes place. Cells in S-phase vary from 2N to 4N depending upon the stage of replication. Once DNA synthesis is complete, cells enter G2, another growth phase in which DNA has been fully replicated and in which the cells are 4N. Cells then proceed into mitosis, in which DNA is segregated and the cells divide[12].

Maturation Promoting Factor (MPF) is found in the cytoplasm during mitosis and is known to be the cause of several mitosis-related events. These include nuclear

envelope breakdown, chromosome condensation, mitotic spindle formation, and transcription inhibition[3]. MPF is composed of a cyclin (cyclin B) and a polypeptide (cdc2). Cyclins act within the cell cycle to ensure that each step is completed before progression to the next step of the cycle. MPF activity itself is controlled by two separate factors: cdc25 and weel. Active cdc25 is a protein which promotes the transition from G2 to M. Active weel prevents the transition from G2 to M. MPF is activated by a predominance of cdc25, which occurs at late G2. If MPF is present in the cell under any circumstances, it will cause the events of mitosis regardless of which stage of the cell cycle the cell exists. Therefore, in nuclear transfer, the cytoplast and the karyoplast must be in synchrony or aneuploidy could result[12].

Two methods are possible to ensure cell cycle synchrony. In one method, the cytoplast can be activated before fusion with the karyoplast in order to reduce MPF within the cytoplast, allowing the karyoplast to undergo normal mitosis without interference due to mixed messages from the cytoplast. In another method, the karyoplast's cell cycle can be controlled through the use of serum starvation. Oocytes are arrested and Metaphase II of meiosis until fertilization occurs. Karyoplasts which exist in G1 of the cell cycle may be fused with oocytes at MII, and normal development will ensue[3]. Wilmut, et al. (1997), however, induced the cells to enter G0 through serum starvation. G0 is referred to as the "quiescent" state of cells which never enter G1. Wilmut suggests that these cells are more easily reprogrammed in the cytoplasm of the egg because they are in an inactive state[32].

Quiescence occurs when a cell is subjected to unfavorable conditions. There exists a critical point in G1 phase when a cell is committed to enter S phase. However, it may exit the cell cycle in the event of unfavorable conditions. This occurrence has been documented in vivo for somatic cell types that are prepared for differentiation. Basically, the cell maintains viability while refraining from activities leading to division[12].

Use of Granulosa Cells in Nuclear Transfer

Granulosa cells have been used successfully in nuclear transfer in several species, bovines and mice in particular. Wells, Misica, and Tervit (1999) cultured bovine mural granulosa cells which were later used for nuclear transfer[30]. However, they recloned using embryonic blastomeres resulting from nuclear transfer with mural granulosa cells. They obtained blastocysts with significantly more embryos fused with recloned embryonic blastomeres than those fused with the granulosa cells. Of the embryos transferred, 10% derived from mural granulosa cells survived to day 180 of pregnancy, while none survived to day 100 from the recloned group. Ten calves were delivered from the recipients of the embryos constructed with mural granulosa cells.

Colles and Barnes (1994) had less success using cumulus granulosa cells in bovine nuclear transfer[5]. Judging from the success ratios of nuclear transfer using cumulus vs. mural granulosa cells as well as the variation in differentiation in granulosa cells which has been observed, it is likely mural granulosa cells are less differentiated

than cumulus granulosa cells, although Wakayama, Zuccotti, Johnson, and Yanagimachi used cumulus cells for nuclear transfer successfully in mice (1996)[29].

Kato, et al. (1999) in Nara, Japan compared the development of embryos produced by nuclear transfer with follicular epithelial cells (preovulatory granulosa cells) and cumulus granulosa cells in mice[13]. They used serial nuclear transfer in which embryos were allowed to develop to the two-cell stage and then blastomeres were taken and used in a second nuclear transfer. Their success rate was significantly higher with the folliculuar epithelial cells than with the cumulus granulosa cells. With follicular epithelial cells used as karyoplasts, 34% of the nuclear transfer developed into blastocysts, and four living fetuses (25% of those transferred) resulted from transfers by day 10.5. However, those with cumulus granulosa cells numbered 20%, and none transferred developed into fetuses. Schuetz, et al. (1996) reported that significantly fewer cumulus cells in mice were in S phase compared to pre-ovulatory granulosa cells, indicating the cells were more rapidly dividing and in the process of differentiating[23]. They also observed that an inverse relationship exists between proliferation and differentiation, leading them to conclude that cumulus cells in Graafian follicles are more differentiated. They contended that the fact that cumulus granulosa cells have a limited lifespan in vivo lends to the idea that they are terminally differentiated. Thus, it is likely that follicular epithelial cells (pre-ovulatory cumulus cells) were more successful in nuclear transfer than cumulus granulosa cells because of their less differentiated state.

MATERIALS AND METHODS

Cell Collection:

Ovaries were collected from a spay and neuter clinic, and granulosa cells were retrieved by aspirating follicles with a 25 gauge needle. Cells were centrifuged and washed once in Dulbecco's Modified Eagle's Medium (DMEM/F12 medium; Gibco 10565-018) supplemented with 1.0 mM pyruvate, 10% Fetal Bovine Serum (FBS; Hyclone SH 30070-02), and penicillin/streptomycin (1% P/S; Gibco 15140-122).

Cell Cultures:

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM/F12; Gibco 10565-018) supplemented with 1.0 mM pyruvate, 10% Fetal Bovine Serum (FBS; Hyclone SH 30070-02), and penicillin/streptomycin (1% P/S; Gibco 15140-122). Cultures were maintained for four days in an incubator with a temperature of 38.5°C and a humidified atmosphere with 5% carbon dioxide in air. Cells were rinsed in $Ca^{++}Mg^{++}$ free Dulbecco's Phosphate-Buffered Saline (PBS; Gibco 14190-151) and exposed to 10x trypsin-EDTA (Sigma T4174) for 30 seconds to release the cells from the culture flask and then diluted with 25% FBS in culture medium. One million cells/mL were cryopreserved at day 4 of early passages in 10% dimethyl sulfoxide (DMSO; Sigma D5879) in DMEM/F12 by cooling at -1°C/min to -80°C. Frozen cells were thawed and cultured 1 day prior to experimental use. Granulosa cells were serum-starved one day prior to nuclear transfer and also one day prior to serum-starved cell cycle analysis.

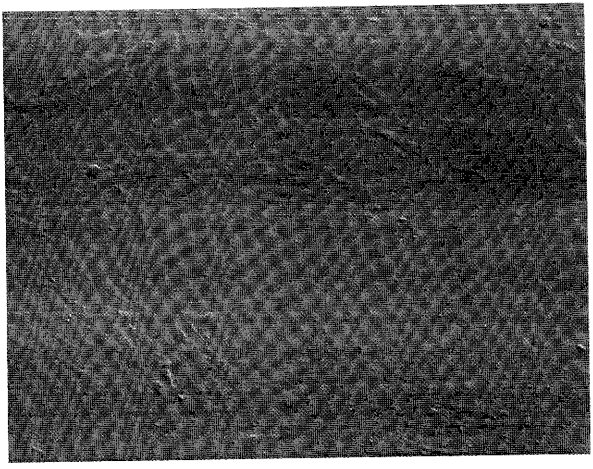


Figure 1. Morphology of feline granulosa cells at passage 1.

Serum-starved cells were kept in culture medium containing 0.5% FBS. Figure 1 shows cell morphology of feline granulosa cells in culture.

FACS:

Cells were released from the flask with 10x trypsin-EDTA and diluted with 25% FBS in DMEM/F12. One million cells were washed in 5 mL of Dulbecco's Phosphate Buffered Saline (PBS; Gibco 14287-098). Cells were then resuspended in 500μL of PBS and fixed by the drop-wise addition of 4.5 mL 70% ethanol (-20°C) while gently vortexing and stored overnight at -20°C. Fixed cells were centrifuged to pellet and washed once in PBS. Cells were then resuspended in propidium iodide/ Triton X-100 staining solution with RNase A (PI/RNase Staining Buffer; Phoenix ABPR19) and protected from light until analysis. DNA analysis was performed using a FACScan flow cytometer (Roger Smith, Texas A&M University) emitting a 488-nm argon ion laser line. Cellular debris and aggregated nuclei or clumps were omitted from the analysis through use of gating, which is based on pulse area vs. pulse width. Data were shown as a histogram based upon the intensity of fluorescence.

Oocyte Collection and Culture:

Ovaries were collected from a spay and neuter clinic, and oocytes were retrieved from the ovaries using light microscopy. The ovaries were sliced with a scalpel blade and then minced laterally. Only Grade I Cumulus-Oocyte Complexes (those with

uniformly dark cytoplasm, and eccentrically located germinal vesicle, and five or more cumulus oophorus cell layers) were used. Usable oocytes were collected in a 10mm dish of TL HEPES (100mM NaCl (Sigma S5886), 3.1 mM KCl (Sigma P5405), 25 mM NaHCO3 (Sigma S5761), 0.29 mM NaH2PO4•H2O (Sigma S9638), 10 mM HEPES (Sigma H3375), 21.6 mM Na Lactate (Sigma L4263), 1 μL/mL phenol red (Sigma P0290), 2.1 mM CaCl2•2H2O (Sigma C7902), 0.5 mM MgCl2•6H2O (Sigma M2393), and 3 mg/mL bovine serum albumin fraction V (BSAV; Sigma A9647)) and evaluated for quality. Oocytes were washed three times in TL HEPES and then matured in 50 μL drops of culture medium under mineral oil (Sigma M8410). Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Gibco 11971-025) containing 0.026g pyruvate, 0.292 g L-glutamine, 0.4% BSA (Sigma), penicillin/streptomycin (1% P/S; Gibco 15140-122), 2μg luteinizing hormone (Sioux Biochemical 720), 2μg follicle stimulating hormone (Sioux Biochemical 710), and 2μg of estradiol. Oocytes were cultured for 24 h in a 38.5°C humidified incubator with 5% CO_2 in air[34].

Nuclear Transfer

Matured oocytes are being exposed to 0.2% hyaluronidase (Sigma H3506) for 5 minutes and then vortexed for 30 seconds. Finally, they are being exposed to 0.5% pronase (Sigma P6911) for 2 minutes. After removal of the cumulus cells, oocytes are being placed in 5 μg/mL cytocholasin B (Sigma C6762) and 5 μg/mL Hoescht 33342 (Sigma B2261) for 20 minutes. Oocytes are then being enucleated using a beveled glass pipette and an inverted microscope. UV light is being used to check for the presence of

DNA and ensure enucleation. A somatic cell is being placed within the perivitelline space of each oocyte[7].

Fusion

Each unit formed is being immersed in 25μg/mL phytohemagluttanin (Sigma L9132) and then Zimmerman's cell fusion medium (ZCFM; 0.28M sucrose (Sigma), 0.5mM $Mg(C_2H_3O_2)\bullet 4H_2O$ (Sigma), 0.1 mM $Ca(C_2H_3O_2)_2$ (Sigma), 1.0 mM K_2HPO_4 (Mallinckrodt), 0.1 mM glutathione (Baker), 0.01 mg/mL BSAV). After equilibration, the units are then being fused in a 3.2 mm fusion chamber containing ZCFM. Electric pulses of 1.5 kV/cm and 220 microsecond-volts will be generated. The fused units are being rinsed in phytohemagluttanin and placed in M199E containing 10% FBS and 1% P/S.

Activation

Units are being activated for 2-5 hours after fusion by exposure to TL HEPES supplemented with 0.001 mg/mL bovine serum albumin – fatty acid free (BSAFAF; Sigma), 1% P/S, and 5 μM ionomycin (CalBiochem). They are then being washed with TL HEPES containing 30 mg/mL BSAFAF and 1% P/S for 4 minutes. Fused units are being washed again with TL HEPES containing 3 mg/mL BSA V with 1% P/S.

Embryo Development

Embryos will be grown for 7 days in M-199 with 10% FBS, 0.36g/L of pyruvate, and 1% P/S. They will be incubated at 38.5°C at 5%CO_2. Light microscopy will be used to detect blastocyst formation. Ratios of blastocyst formation to cells fused will be calculated and reported.

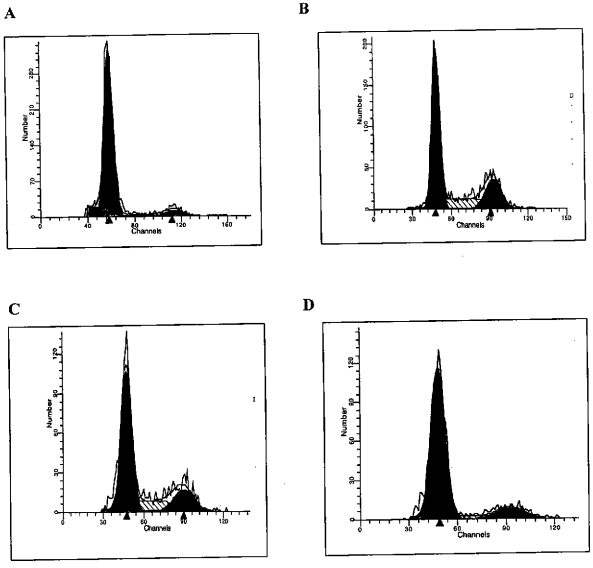


Figure 2. Representative cell cycle analyses from freshly collected feline granulosa cells (A), primary cell line (B), passage 1 (C), and passage 2 (D). The data show a frequency distribution of the fluorescence intensity (channels) of the nuclei recorded at the wavelength of propidium iodide (575 nm).

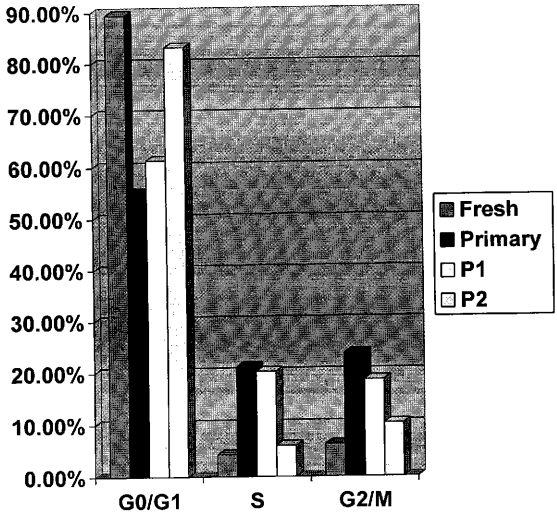


Figure 3. A comparison of FACS results of feline granulosa cells analyzed from freshly collected cells, the primary cell line, passage 1, and passage 2. A significantly larger proportion of freshly collected cells and cells from the second passage were found to be in G0/G1.

RESULTS

Cell Cycle Analyses

Flow cytometry (FACS) was used to determine proportions of cells at various stages of growth and ploidy. Cells from different passages up to passage 2 as well as freshly collected cells were compared. Also, cells within the same passage were divided into serum-starved and regularly fed cultures and compared.

The results from cells in different passages as well as freshly collected cells are shown in Figure 2. Figure 3 shows a comparison of the data. Data from Figure 3 show that cells with the highest percentage in G0/G1 (around 89%) were those in the freshly collected cells. Only approximately 4% were in S phase, while around 6% were in G2/M.

Cells in culture displayed significantly different results until P2. Those collected from the primary cell line were found to have around 55% in G0/G1 while showing 21% in S-phase and 24% in G2/M phases. Also, cells from the first passage had close to 61% in G0/G1, 20% in S-phase, and round 19% in G2/M. However, cells from the second passage had around 83% in G0/G1, around 6% in S-phase, and around 10% in G2/M.

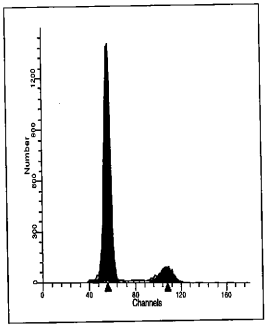
Serum-starved cells from the first passage were compared to regularly fed cells. Results from this comparison are shown in Figure 4. Serum-starved cells showed significantly more cells in G0/G1 (around 85%), as expected. Only approximately 5% of serum-starved cells were in S-phase, and around 10% were in G2/M. Regularly fed

cells showed 70% in G0/G1, 14% in S, and around 16% in G2/M. No cells analyzed exhibited aneuploidy.

Nuclear Transfer

Cells from passage 2 are being serum-starved and used in nuclear transfer. Cells from P2 and not earlier passages are more likely to result in blastocyst formation judging from the cell cycle analysis. Serum-starvation will aid in sending cells into G0/G1 and thus will increase the likelihood of blastocyst formation using granulosa cells.

A



B

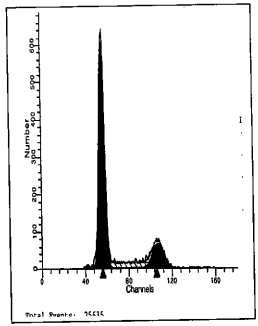


Figure 4. Representative cell cycle analyses from serum starved cells from passage 1 (A) and regularly fed cells from passage 1 (B).

CONCLUSIONS

Judging from the comparison of freshly collected cells to cultured granulosa cells, it is apparent why Wakayama, et al. (1998) achieved higher rates of embryos which developed to term using freshly collected cumulus granulosa cells in nuclear transfer[29]. A higher proportion of freshly collected granulosa cells were found to be in G0/G1 than serum-starved cells or cells from any passage up to passage 2. Thus, freshly collected cells were more likely to be synchronized and as a result more useful for nuclear transfer. However, in culture, it is probably better for cells in passage 2 to be used for nuclear transfer after being subjected to serum starvation based on cell cycle analyses. Feline granulosa cells from passage four slowed to almost no division and began to exhibit signs of aneuploidy (such as multiple nuclei) as well as unusual morphology, thus indicating that feline granulosa cells in culture for over a month are not suitable for nuclear transfer.

It is likely that cells collected were a heterogenous population of granulosa cells in various stages of differentiation. Separation based upon location of cells within the follicles was difficult due to the small size of the follicles themselves. However, it is also likely that after a couple passages the cells became a homogenous population because of subjection to the same conditions and lack of hormonal control though interaction with oocytes.

Because granulosa cells exhibit stem cell properties in other species[11,17], it is likely they have been useful in nuclear transfer because they are less differentiated than

other somatic cells. The next step for characterization of feline granulosa cells after nuclear transfer may be to detect stem cell properties such as telomerase activity. Feline granulosa cells may be tested for telomerase to show whether they are similar to other species in the same respect. If telomerase activity is found, it can be concluded that feline granulosa cells do display stem cell properties. This analysis will help to determine their potential to be used successfully in nuclear transfer.

REFERENCES

[1]Blondin, P., Dufour, M., and Dirard, M. Analysis of atresia in bovine follicles using different methods: flow cytometry, enzyme-linked immunosorbent assay, and classic histology. Biology of Reproduction 1996; 54:631-637.

[2]Bowen, R.A. Fertilization in vitro of feline ova by spermatozoa from the ductus deferens. Biology of Reproduction 1997; 17:144-147.

[3]Campbell, K.H.S. Nuclear equivalence, nuclear transfer, and the cell cycle. Cloning 1999; 1(1):3-15.

[4]Campbell, K.H.S., McWhir, J., RitchieW.A., and Wilmut, I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996; 380:64-66.

[5]Collas, P., and Barnes F.L. Nuclear transplantation by microinjection of inner cell mass cells and granulosa cell nuclei. Molecular Reproduction and Development 1994; 38:264-267.

[6]De Neubourg, D., Robins, A., Fishel, S., and Delbeke, L. Quantification of insulin-like growth factor I receptors on granulosa cells with flow cytometry after follicular stimulation. Human Reproduction 1998; 13(1):161-164.

[7]De Sousa, P.A., Winger, Q., Hill, J.R., Jones, K., Watson, A.J., and Westhusin, M.E. Reprogramming of Fibroblast Nuclei after transfer into bovine oocytes. Cloning 1999; 1(1):63-69.

[8]Dominko, T., Mitalipova, M., Haley, B., Beyhan, Z., Memili, E., McKusick, B., and First, N.L. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. Biology of Reproduction 1999; 60:1496-1502.

[9]Duda, M., Gasinska, A., and Gregoraszczuk, E.L. Flow cytometric cell cycle analysis of two subpopulations of porcine granulosa cells. Experimental & Clinical Endocrinology & Diabetes 1999; 107(3):203-207.

[10]Eppig, J.J., Wigglesworth, K., Pendola, F., and Hirao, Y. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biology of Reproduction 1997; 56:976-984.

[11]Evagelatou, M., Peterson, S.L., and Cooke, B.A. Leukocytes modulate 11-beta-hydroxysteroid dehydrogenase (11beta-HSD) activity in human granulosa-lutein cell cultures. Molecular and Cellular Endocrinology 1997; 133(2):81-88.

[12]Jones, K. DNA hypomethylation of karyoplasts for bovine nuclear transfer 1999.

[13]Kato, Y., Yabuuchi, A., Motosugi, N., and Tsunoda, Y. Developmental potential of mouse follicular epithelial cells and cumulus cells after nuclear transfer. Biology of Reproduction 1999; 61:1110-1114.

[14]Kerketze, K., Blaschuk O.W., and Farookhi, R. Cellular heterogeneity in the membrana granulosa of developing rat follicles: assessment by flow cytometry and lectin binding. Endocrinology 1996; 137(7):3089-3099.

[15]Kraemer, D.C., Kinney, G.M., and Schriver, M.D. Embryo transfer in dogs and cats. Embryo Transfer and In Vitro Fertilisation 1982; 2:223-233.

[16]Lavranos, T.C., O'Leary, P.C., and Rodgers, R.J. Effects of insulin-like growth factors and binding protein 1 on bovine granulosa cell division in anchorage-independent culture. Journal of Reproduction and Fertility 1996; 107:221-228.

[17]Lavranos, T.C., Mathis, J.M., Latham, S.E., Kalionis, B., Shay, J.W., and Rodgers, R.J. Evidence for ovarian granulosa stem cells: telomerase activity and localization of the telomerase ribonucleic acid component in bovine ovarian follicles. Biology of Reproduction 1999; 61:358-366.

[18]Mitalipov, S.M., White, K.L., Farrar, V.R., Morrey, J., and Reed, W.A. Development of nuclear transfer and parthenogenetic rabbit embryos activated with inositol 1,4,5-triphosphate. Biology of Reproduction 1999; 60:821-827.

[19]Neubourg, D.D., Robins, A., Fishel, S., and Gibbon, L. Flow cytometric analysis of granulosa cells from follicular fluid after follicular stimulation. Human Reproduction 1996; 11(10):2211-2214.

[20]Ouhibi, N., Fulka, J. Jr., Kanka, J., and Moor, R.M. Nuclear transplantation of ectodermal cells in pig oocytes: ultrastructure and radiography. Molecular Reproduction and Development 1996; 44:533-539.

[21]Prather, R.S., Barnes, F.L., Sims, M.M., Robl, J.M., Eyestone, W.H., and First, N.L. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biology of Reproduction 1987; 37:859-866.

[22]Prather, R.S., Sims, M.M., and First, N.L. Nuclear transplantation in early pig embryos. Biology of Reproduction 1989; 41:414-418.

[23]Schuetz, A.W., Whittingham, D.G., and Snowden, R. Alterations in the cell cycle of mouse cumulus granulosa cells during expansion and mucification in vivo and in vitro. Reproduction, Fertility, and Development 1996; 8:935-943.

[24]Spanel-Borowski, K. and Ricken, A.M. Evidence for the maintenance of macrophage-like cells in long-term bovine granulosa cell cultures. Cell & Tissue Research 1997; 288(3):529-538.

[25]Stice, S.L., Strelchenko, N.S., Keefer, C.L., and Matthews, L. Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. Biology of Reproduction 1996; 54:100-110.

[26]Tanaka, H. and Kanagawa, H. Influence of combined activation treatments on the success of bovine nuclear transfer using young or aged oocytes. Animal Reproduction Science 1997; 49:113-123.

[27]Van Stekelenburg-Hamers, A.E.P., Van Achterberg, T.A.E., Rebel, H.G., Flechon, J.E., Campbell, K.H.S., Weima, S.M., and Mummery, C.L. Isolation and characterization of permanent cell lines from inner cell mass cells of bovine blastocysts. Molecular Reproduction and Development 1995; 40:444-454.

[28]Verstegen, J.P., Onclin, K., Silva, L.D.M., Donnay, I., Mettens, P., and Ectors, F. Superovulation and embryo culture in vitro following treatment with ultra-pure follicle-stimulating hormone in cats. Journal of Reproduction and Fertility 1993; 47:209-218.

[29]Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R., and Yanagimachi, R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394:369-373.

[30]Wells, D.N., Misica, P.M., and Tervit, H.R. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biology of Reproduction 1999; 60:996-1005.

[31]Willadsen, S.M. Nuclear transplantation in sheep embryos. Nature 1986; 320:63-65.

[32]Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H.S. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810-813.

[33]Wolfe, B.A. and Kraemer, D.C. Methods in bovine nuclear transfer. Theriogenology 1992; 37(1):5-13.

[34]Wood, T.C. and Wildt, D.E. Effect of quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize, and develop into blastocysts in vitro. Journal of Reproduction and Fertility 1997; 110:355-360.

[35]Yang, X., Jiang, S., Kovacs, A., and Foote, R.H. Nuclear Totipotency of cultured rabbit morulae to support full-term development following nuclear transfer. Biology of Reproduction 1992; 47:636-643.

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