RECOVERY AND EVALUATION OF SOMATIC CELLS FROM OVINE AND BOVINE SEMEN FOR USE IN NUCLEAR TRANSFER

A Dissertation

by

JIE LIU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Veterinary Physiology

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Approved by:

Co-Chairs of Committee,	Duane C. Kraemer
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ABSTRACT

Recovery and Evaluation of Somatic Cells from Ovine and Bovine

Semen for Use in Nuclear Transfer. (May 2008)

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Co-Chairs of Advisory Committee, Dr. Duane C. Kraemer
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Somatic cells in semen are a potential source of nuclei for cloning animals by somatic cell nuclear transfer. Culture of the cells from frozen semen, if possible, would be extremely valuable for preservation or restoration of endangered, exotic, and extinct animals when other ways of obtaining somatic cells are unavailable. In the present study, somatic cells isolated from ovine and bovine semen samples were characterized, culture systems were evaluated for attachment and proliferation of these cells, and usefulness of these cells for somatic cell nuclear transfer was determined.

Semen samples were collected from eight rams representing three breeds: Dorper, Suffolk, and Hampshire and nine bulls representing three breeds: Charolais, Brahman, and a crossbred Brahman. Somatic cells were isolated immediately post collection by centrifuging through percoll columns and the epithelial cells were identified by immunofluorescence analysis. Culture systems were evaluated for their ability to support attachment and proliferation of the cells. A supplemented medium composed of DMEM/F12, 10% fetal bovine serum, 10 ng/ml epidermal growth factor,

30 μg/ml bovine pituitary extract, 5 μg/ml insulin, 10 ng/ml cholera toxin, and 50 μg/ml gentamicin significantly improved cell proliferation over sheep fetal fibroblast-conditioned medium, 3T3 cell-conditioned medium, and basic medium ($p<0.05$). Cell proliferation and attachment were further improved when Matrigel-coated culture surfaces were used ($p<0.05$). However, the system was not adequate for obtaining cell growth from frozen semen.

To check the chromosome anomalies, metaphase chromosomal complements of the cells cultured from 4 rams were evaluated. The predominant chromosome number of cells from three of the rams (Dorper 18-month-old; Suffolk 17-month-old; Suffolk 18-month-old) was $2n = 54$, which is the normal modal number for sheep. However, the numbers of chromosomes of cells cultured from the fourth ram (Hampshire, 18-month-old) were near-triploid. These results indicate the need for chromosome analysis of cells before using them for cloning experiments. In our attempts to clone animals, blastocyst stage embryos were successfully produced using epithelial cells cultured from semen of three different bulls. However, no compact morulae or blastocysts were obtained when somatic cells isolated from frozen semen but not cultured were used as donor cells.

DEDICATION

To my heavenly father Liu, Shu-Ren, my mother Yang, Li-Hua, and my sister Liu, Xiao-Hong for their constant love, encouragement, patience, and support.

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Many thanks to my committee members, Dr. Dunne and Dr. Harms, for their suggestions and support throughout the course of this study.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In addition to spermatozoa, human ejaculates contain 10^3 to 10^7 cells of other types (Phillips *et al*. 1978). These cells, often referred to as "round cells", are spermatogenic and non-spermatogenic in origin, including immature early stages of the spermatozoon, epithelial cells, and various major leukocyte subpopulations including granulocytes, monocytes/macrophages, B lymphocytes, helper and suppressor/cytotoxic T lymphocytes (Phillips *et al*. 1978; Belsey *et al*. 1980; Wolff and Anderson 1988; Homyk *et al*. 1990). Very limited similar research has been performed in livestock and none in companion animals.

Cells cultured from ovine (Nel-Themaat *et al*. 2004, 2007) and bovine (Nel-Themaat *et al*. 2005, 2006) semen have been identified as epithelial or epithelial-like cells by the presence of cytokeratin. Cells cultured from human semen have been identified with certainty as epithelial cells, uncontaminated by fibroblasts. This result suggests that semen samples provide a valuable source of purely epithelial cells, isolation of which, otherwise, requires tedious handling and perseverance (Phillips *et al*. 1978).

Although cell growth can be obtained from fresh semen relatively easily, to date, several attempts to culture such cells from frozen semen have been unsuccessful. Culture

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This dissertation follows the style of *Reproduction, Fertility and Development.*

of the cells from frozen semen, if possible, would be extremely valuable for preservation or restoration of endangered, exotic, and extinct animals when other means of obtaining somatic cells are not available. The culture system established in the study promotes proliferation of the cells, has been found to be useful for culturing cells from bovine semen in our laboratory, and may increase the likelihood of obtaining cells from frozen semen.

It has been demonstrated that cells derived from eland semen can serve as karyoplast donors for nuclear transfer (Nel-Themaat *et al*. 2007). Usefulness of the cells cultured from bovine semen was evaluated and blastocyst stage embryos were produced using cells from semen of three different bulls by somatic cell nuclear transfer.

"Round Cells" in Human Semen

Extensive work has been reported as to the types of cells in human semen. People have interest in the various types of cells in human semen mainly because increased levels of the "round cells" have been proven to relate to infection and infertility (Caldamone *et al*. 1980; Berger *et al*. 1982; Maruyama *et al*. 1985; Wolff and Anderson 1988; Arata de Bellabarba *et al*. 2000). Round cells are increased in cases of infertility associated with infection or hormonal alterations of normal spermatogenesis (Jiang *et al*. 2004). The presence of large numbers of granulocytes (> 3 million/ml) can indicate an acute infection in the prostate, the seminal vesicles or the epididymis (male accessory gland infection, MAGI) (Bras *et al*. 1996).

The epithelial cells in human semen have received less attention for culture experiments. So far only one report of culturing cells out of human semen has been

found. In the report (Phillips *et al*. 1978), 21 semen samples were collected from volunteers and 20 samples from the fertility laboratory. Growth of cells into colonies occurred in 55% of the volunteer samples but in 25% of the fertility laboratory samples.

Somatic Cells in Ovine and Bovine Semen — How Much Do We Know?

Several reports have been published indicating that the cells cultured from ovine (Nel-Themaat *et al*. 2004, 2007) and bovine (Nel-Themaat *et al*. 2005, 2006) semen are epithelial cells. In the reports, somatic cells were successfully cultured from fresh and cooled ram semen, and the cells were identified as epithelial cells by the presence of cytokeratin. No cells were derived from frozen-thawed semen.

Although cells can be obtained from fresh ovine and bovine ejaculates, proliferation rate of the cells was low and most of the cells stopped dividing within one or two months. Studies have been performed to improve proliferation of the cells and 3T3 fibroblast co-culture has been found better than serum-supplemented Mininum Essential Medium. However, further studies on conditioned medium or other specific medium may simplify the system and reduce the possibility of 3T3 cell contamination (Nel-Themaat *et al*. 2006).

Chromosomes and Karyotype

Chromosomes of eukaryotes are composed of DNA and proteins (histones and non-histone proteins such as chromosome scaffold, transcription factors, etc). As a genetic material, DNA of the chromosomes determines the features an offspring inherits from parents. Defects in chromosomes may cause changes in certain body processes or functions (Youngerman 2005). There are two basic classes of chromosome aberrations

including numeric and structural. Common abnormalities of chromosome number include aneuploidy, mosaicism, and polyploidy. Structural abnormalities include duplications, deletions, inversions, shifts, fragile sites, etc.

Chromosome counters determine the chromosome number of a cell by counting the number of chromosomes in a metaphase spread (Verma and Babu 1995). Cautions and detailed information about chromosome counting have been described (Barch 1991). Multiple cells must be counted to determine the modal number of chromosomes in each spread of a particular sample. Random loss or gain of chromosomes in slide preparation can cause variations from the modal number among individual cells. The normal modal number for a human is 46, which is termed the diploid complement. It can vary through addition or subtraction of chromosomes in gametogenesis, fertilization, or embryogenesis, which affects the whole constitution of a person. The number can also be altered in certain cells of the body, leading to mosaic cell lines in the newly formed individual. Aneuploidy is defined as cells that have other than the normal diploid complement. If fewer than 45 chromosomes are present in the metaphase, unless there is reason to suspect the presence of cancer cells, it can be assumed that some have been lost in processing and that the metaphase is unsuitable for analysis. Polyploidy is defined as cells that are aneuploid by complete haploid sets of chromosomes. If the total count is 69 the cell is triploid; triploidy occurs rarely and is lethal. A more common occurrence in tissue culture samples is tetraploid cells. These chromosomes are almost certainly the result of tissue culture process and need not be counted or reported, unless all the cells in the sample are tetraploid. In blood culture analysis, tetraploid cells are seen very rarely.

Karyotyping (or chromosome analysis) refers to use of a microscope (and software) to examine the size, shape, and number of chromosomes. Extra, missing, or abnormal positions of chromosome(s) cause problems with body growth, development, and functions (Youngerman 2005).

The formal karyotype is an orderly arrangement of the chromosomes according to international conventions (Barch 1991). The modal number for a sheep has been found to be 54. Three pairs of autosomes are readily identifiable as submetacentric, while the remaining 23 pairs are acrocentric and not easily distinguishable from each other. The X chromosomes are the largest of the acrocentric members, while the Y is a very small and probably submetacentric chromosome (McFee *et al*. 1965). The biarmed autosomes were assigned numbers 1 to 3, the acrocentric autosomes assigned numbers 4 to 26 in order of decreasing length (Ford *et al*. 1980).

Culture of Epithelial Cells

Epithelia are associated with the major functional role of many tissues, and have been a focus of interest in the development of *in vitro* models for studies of carcinogenesis, regulation of cell proliferation and differentiation, and so on, for many years (Freshney 2002). Several media have been developed that are capable of supporting different types of epithelial cells. Among this work, a burst of activity in the development of culture systems for prostate epithelial cells has occurred due to increasing interest in research into the causes of prostatic diseases. To date, there are three main media in use. All three of the media use additives that include insulin, epidermal growth factor (EGF), and bovine pituitary extract (BPE) (Wise 2002). Insulin,

EGF, and BPE are also frequently used in culturing of epithelial cells from tissues other than the prostate (Hackworth *et al*. 1990; Chopra *et al*. 1991; Sime *et al*. 1997). Chopra *et al.* (1996) reported that cholera toxin, as well as insulin, hydrocortisone, EGF, and BPE, were essential for the growth of prostatic epithelial cells as deletion of any one of the factors strongly inhibited their growth.

Swiss mouse 3T3 cells (mitomycin-C treated or lethally-irradiated) are among the most widely used feeder cells for epithelial cell culture. Plating of epithelial-like cells (ELC) obtained from ovine semen on 3T3 feeder layers or co-culture of ELC with feeder cell inserts significantly ($p<0.05$) improve proliferation (Nel-Themaat *et al*. 2006). However, due to the difficulty of separating epithelial cells completely from the feeder cells, it is not recommended to plate the epithelial cells on 3T3 feeder cells if the epithelial cells are to be used for nuclear transfer. 3T3 conditioned medium can be a substitute for plating on feeder cells since epithelial cells and feeder cells do not require contact with each other to have the growth stimulatory effects by the feeder cells (Nel-Themaat *et al*. 2006).

Collagen and matrigel are the other matrices that have been used for epithelial cell culture. They contact epithelial cells and promote their adhesion, migration, differentiation, and morphogenesis (Hadley *et al*. 1990). It has been reported that less than 10% of the human lens epithelial cells attached on the non-coated plate from 30-180 min of culture while 55% of the cells attached on type IV collagen-coated plates at this time period (Oharazawa *et al*. 1999). When human keratinocytes were cultured on

collagen I, collagen IV, or matrigel, both initial attachment and proliferation of the cells were enhanced (Dawson *et al*. 1996).

Cell Cryopreservation

Cryopreservation has been developed and widely used to preserve or maintain cells, microorganisms, embryos, etc., at low temperatures. Even after decades of research, the freezing process remains ill-defined due to the complex phenomena it involves. Since water is the major component of living cells, ice crystal formation and osmotic imbalance occurring during the freezing process have been widely studied (Mazur *et al*. 1972; Farrant 1980).

In a brief review, Mazur (1984) systematically described mechanisms of freezing of living cells. He described and analyzed several chief physical events occurring in cells during freezing. Down to ~-5°C, the cells and their surrounding medium remain unfrozen both because of supercooling (the process of chilling a liquid below its freezing point, without it becoming solid) and the depression of the freezing point by the protective solutes. Between -5°C and ~-15°C, ice forms in the external medium. Because the plasma membrane blocks formation of ice crystals in the cytoplasm, the cell contents remain unfrozen and supercooled. The supercooled water in the cells has a higher chemical potential than that of water in the partly frozen solution outside the cell. In response to this difference in potential, water flows out of the cell and freezes externally. This prevents intracellular freezing and crystal formation.

The subsequent physical events in the cell depend on cooling velocity. If cooling is sufficiently slow, the cell is able to lose water rapidly enough to concentrate the

intracellular solutes, eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. Therefore the cell dehydrates and does not freeze intracellularly. If the cell is cooled too rapidly, it is not able to lose water fast enough to maintain equilibrium; it becomes increasingly supercooled and eventually attains equilibrium by freezing intracellularly, which usually causes extensive cell death (Mazur 1984). It has been suggested that the optimal cooling rate for different types of cells is greatly influenced by the cryoprotectant. When the cells are treated with a very permeate agent, they rapidly restore osmotic balance and are thus able to tolerate faster cooling (Poncet and Leble 2003).

Slow freezing can be injurious. Mazur (1984) discussed the evidence that slow freezing injury may be primarily a consequence of the reduction of liquid surrounding the cells and secondarily a consequence of the increasing solute concentration.

Warming rate has a dramatic effect on cell survival as well. A much higher percentage of cells often survive intracellular freezing when the rate of warming and thawing is high rather than low. Table 1 lists some examples. If rapid warming can "rescue" some of the intracellularly frozen cells, these cells must have been viable before warming was initiated (Mazur 1984). However, when human red blood cells are cooled more slowly than optimum, the response is reversed – survival is considerably higher when slow warming was applied than when rapid warming was applied (Mazur 1984). A similar reversal is also found in early mammalian embryos (Whittingham *et al*. 1979).

Protective additives or cryoprotectants are necessary and important. By increasing the total concentration of all solutes in the system, cryoprotectants reduce the amount of ice formed at any given temperature. Cryoprotectants must also be able to penetrate into the cells and have low toxicity (Pegg 2007). Many compounds have been tested as cryoprotectants, which include serum, sugars, egg yolk, glycerol, DMSO, etc. The choice of a cryoprotectant depends upon the type of cell to be preserved. For many cells, glycerol is the agent of choice because it is usually less toxic than DMSO. However, DMSO is more permeable and is usually the agent of choice for larger, more complex cells (Shah 1983).

Besides cryopreservation agents and protocols derived that are based on the ideas of minimizing the damaging actions of solution effects, extra- and intracellular ice formation, new concepts in cryopreservation solution design have been developed. They focus on the use of an intracellular-type, hypothermic maintenance medium (HypoThermosol) coupled with additives (Caspase I Inhibitor V) that inhibit cryopreservation-induced apoptosis (Baust *et al*. 2000, 2001), membrane stabilizers (trehalose and taurine) and antioxidants (such as catalase and curcumin) (Limaye and Kale 2001; Kanitkar and Bhonde 2008), with results better than standard DMSO-based cryopreservation methods.

Table 1. Examples of "rescue" of rapidly frozen cells by rapid thawing.

Cell Type	Cooling Rate (°C/min)	Survival After Warming		References
		Slowly*	Rapidly†	
Hamster tissue culture cells				
V79	600	5	65	Mazur *et al*. 1972
CHO	100	30‡	75	Griffiths *et al*. 1979 Harris and Griffiths 1977
Mouse and human stem cells	200	0-4	25-30	Leibo *et al*. 1970 McGann *et al*. 1981
Ascites cells	?	0	54	Asahina *et al*. 1970
Human red blood cells	600	8	63	Miller and Mazur 1976
Mouse and rabbit (8-32 cell embryos)	2 step§	0	80	Kasai *et al*. 1981 Landa 1982 Rall 1981 Whittingham *et al.* 1979 Renard and Babinet 1984
Mulberry cells	100	5	60	Sakai and Yoshida 1967
Cultured carrot	2 step§	5	45	Nag and Street 1975
Lily pollen	200	0	100	Nath and Anderson 1975
Yeast	450	10^{-6}	8	Mazur and Schmidt 1968
Neurospora spores	250	8	95	Barnhart and Terry 1971 Leef and Mazur 1978

CHO, Chinese hamster ovary.
*Slow warming was 1-2°C/min except where noted.
†Rapid warming was generally 600-700°C/min except in yeast (10,000°C/min) and embryos (100-200°C/min).
‡Warming was 100°C/min.
§ 0.5°C/min to -40°C, then >100°C/min to -196°C.

(Adapted from Mazur 1984)

Somatic Cell Nuclear Transfer

Nuclear transfer/transplantation is used to produce genetically identical animals by the introduction of a donor cell nucleus into an enucleated metaphase II oocyte or a zygote temporarily arrested in mitosis.

Mammalian Nuclear Transfer – Key Events

Spemann was the first to describe the possibility of reversing the process of cell differentiation and of using more developed cells for nuclear transfer in amphibians (Spemann 1938; Vajta and Gjerris 2006). In order to test whether nuclei of cells from various differentiated parts of the Amphibian embryo remain equivalent or become differentiated, Briggs and King (1952) brought out the idea of transplanting nuclei of differentiated cells into enucleated eggs. Nuclei from advanced blastula cells were transplanted into enucleated eggs of *Rana pipiens* and normal embryos were obtained. Later nuclei of mesoderm and endoderm of late gastrulae (*Rana pipiens*) were transplanted into enucleated eggs. Recombined eggs were able to develop into complete blastulae, but the majority was arrested in various stages of development (King and Briggs 1955). This was explained as due to "intrinsic restrictions in potentiality for differentiation on the part of the nuclei of the late gastrula" (King and Briggs 1955). Although adult frogs were cloned using tadpole gut-cell nuclei in 1962, it was believed that differentiated cells from tadpoles contained "genetic information necessary for the formation and functioning of a normal adult individual", and adult somatic cells had not been used to clone adult frogs (Gurdon 1962; Meissner and Jaenisch 2006). In the following three decades or so, blastomeres and embryonic cells were used in attempts to

clone animals of different species with a common idea that terminally differentiated adult cells were not genetically totipotent or reversible and therefore were not able to make clones. The birth of "dolly" shocked the world by showing that sheep can be cloned using cells derived from mammary epithelium of a 6-year-old ewe (Wilmut *et al*. 1997). However, due to the low efficiency and the fact that most adult tissues contain a small number of adult stem cells, it is possible that the surviving clones may originate from the nuclei of adult stem cells instead of terminally differentiated cells. Therefore in 2002, Hochedlinger and Jaenisch demonstrated that terminally differentiated cells can be successfully reprogrammed to produce adult cloned animals by generating cloned mice using mature B-cell and T-cell nuclei and the mice carrying genetic alterations of the donor nuclei in all tissues (Hochedlinger and Jaenisch 2002a; 2002b). However, after around 10 years of study, SCNT remains inefficient and there still are questions needing to be answered and problems solved.

SCNT Is an Inefficient Process

Even after around 10 years of study and to our knowledge, 16 species being cloned to date (Table 2), SCNT remains an inefficient process, with 0-4% of reconstructed embryos developing into live young (Wilmut *et al*. 2002), and a variety of abnormalities have been observed in some of the resulting offspring (Hill *et al*. 1999; Renard *et al*. 1999). Specific causes of these remain to be identified (Brem and Kuhholzer 2002). Table 3 shows types of cells that have been used in nuclear transfer.

As nuclear transfer technology became more standardized, a series of variables that influence cloning outcomes were being discussed. Eggan and Jaenisch (2002)

described four key parameters of nuclear transfer that influence the development of cloned embryos. The four parameters are: (1) the state and nature of the recipient cytoplast; (2) the cell cycle status of the donor cell; (3) the identity, differentiation state, and developmental potency of the donor cell, and, finally, (4) genetic influences on the successful development of cloned embryos.

(1) The recipient oocyte and its cytoplasm. Table 4 summarizes effects of the recipient cytoplasm on nuclear transfer embryo development. It is obvious that the unactivated, metaphase II (MII) oocyte is a suitable recipient cytoplasm with reasonable efficiencies. Wakayama *et al*. (1998) thoroughly analyzed recipient cytoplasm for nuclear transfer and effects of timing of activation on cloning results. Development to the blastocyst stage of reconstructed oocytes activated one to three hours after nuclear transfer, immediately after nuclear transfer, and one hour before nuclear transfer were 69% (n = 144), 34% (n = 175), and 6% (n = 197), respectively. When enucleated zygotes were used as recipient cytoplasm, blastocyst development was zero. These data indicate that the timing of activation is critical to the outcome of development (Wakayama *et al*. 1998), and that the metaphase environment (maturation/meiosis/mitosis- promoting factor, MPF) is somehow critical for preparing the somatic chromosomes for preimplantation development after nuclear transfer (Eggan and Jaenisch 2002). However, interestingly, cloned goats (Baguisi *et al*. 1999) and sheep (Campbell *et al*. 1996) have been generated by introducing somatic nuclei into activated oocytes,

Table 2. Species cloned by somatic cell nuclear transfer

Year	Species	Donor Cell Type	References
1997	Sheep (Dolly)	Mammary epithelium	Wilmut *et al*. 1997
1998	Cattle	Cumulus and oviductal cells	Kato *et al*. 1998
	Mouse	Cumulus cells	Wakayama *et al*. 1998
1999	Goat	Fetal karyoplasts	Baguisi *et al*. 1999
2000	Pig	Granulosa cells	Polejaeva *et al*. 2000
	Gaur	Adult fibroblasts	Lanza *et al*. 2000
2001	Mouflon	Granulosa cells	Loi *et al*. 2001
2002	Cat	Cumulus or adult fibroblast cells	Shin *et al*. 2002
	Rabbit	Cumulus cells	Chesne *et al*. 2002
2003	Rat	Fetal fibroblasts	Zhou *et al*. 2003
	Mule	Fetal fibroblasts	Woods *et al*. 2003
	Horse	Adult fibroblasts	Galli *et al*. 2003
2005	Dog	Adult fibroblasts	Lee *et al*. 2005
2006	Ferret	Cumulus cells	Li *et al*. 2006a
	Takin	Adult fibroblasts	Li *et al*. 2006b
2007	Buffalo	Fetal fibroblast and granulosa cells	Shi *et al*. 2007

(Adapted from Meissner and Jaenisch 2006)

Table 3. Types of cells used in nuclear transfer

Species	Donor cell age	Donor cell type	% Embryo formed from NT oocytes	Live offspring (% embryos transferred)	Cloning Efficiency
Mouse	Adult	Cumulus	42.1%	7 (23.3%)	5.8%
			52.8%	9 (5.3%)	2.8%
		Fibroblasts	21.8%	5 (1.7%)	0.3%
		Lymphocytes	63.7%	38	?
	Newborn	Sertoli	23.6%	16 (3.6%)	0.9%
	Fetal	Fibroblasts	16.4%	5 (1.1%)	0.2%
		Gonad	55.1%	6 (2.7%)	1.5%
		Neural cells	29.7%	12 (10.3%)	3.1%
Cattle	Adult	Cumulus	39.4%	5 (83.3%)	5%
		Oviduct	39.3%	3 (75%)	2%
		Uterine	?	2 (14.2%)	2%
		Granulosa	69.4%	10 (10%)	1.8%
		Mammary gland	16.1%	1 (25%)	0.4%
			16.8%	2 (4.4%)	0.7%
		Muscle	14.2%	4 (15.3%)	0.8%
		Fibrlblasts	53.3%	1 (6.25%)	1.1%
	Newborn	Fibroblasts	?	2 (12.5%)	1.1%
		Liver	?	2 (20%)	1.3%
		Testis	?	0	0%
	Fetal	Fibroblasts	27.5%	25 (11.3%)	0.9%
		Germ cells	22.7%	1 (3.1%)	0.2%
Pig	Adult	Fibroblasts	?	2 (0.9%)	0.9%
		Granulosa	?	5 (0.8%)	0.1%
	Newborn	Fibroblasts	9.5%	4 (0.9%)	?
	Fetal	Fibroblasts	?	2 (0.3%)	0.2%
Sheep	Adult	Mammary gland	10.5%	1 (3.4%)	0.4%
		Granulosa	30.4%	1	4.3%
	Fetal	Fibroblasts	27.3%	3 (7.5%)	1.7%
Goat	Adult	Granulosa	?	7 (7.3%)	?
		Cumulus	?	3 (1.3%)	0.7%
	Fetal	Fibroblasts	32.1%	5 (13.2%)	3.1%
cat	Adult	Cumulus or Fibroblasts	?	1 (1.1%)	?
Rat	Adult	Cumulus	?	6 (0.6%)	0.3%

(Adapted from http://www.roslin.ac.uk/downloads/webtablesGR.pdf, retrieved in March, 2008)

Table 4. Effects of the recipient cytoplasm on nuclear transfer embryo development

Recipient cytoplasm	Species	Efficiency of in vitro development to morulae/blastocyst	Development after embryo transfer	References
Zygote	Mouse	0	0	McGrath *et al*. 1984 Wakayama *et al*. 2000
	Cow	0	0	Tani *et al*. 2001
MII oocyte, then serial transfer to zygote	Mouse	29%	Adult	Ono *et al*. 2001
	Pig	N/A	Adult	Polejaeva *et al*. 2000
MII oocyte	Mouse	70%	Adult	Wakayama *et al*. 2000
	Cow	65%	Adult	Wells *et al*. 1999
	Sheep	20-40%	Adult	Wilmut *et al*. 1997
	Pig	30%	Adult	Onishi *et al*. 2000
	Goat	N/A	Adult	Baguisi *et al*. 1999
Activated oocyte	Mouse	6%	0	Wakayama *et al*. 2000
	Sheep	12%	Adult	Campbell *et al*. 1996
	Goat	N/A	Adult	Baguisi *et al*. 1999

(Adapted from Eggan and Jaenisch 2002)

suggesting that exposing the donor nucleus to cytoplasm rich in MPF may not be strictly required for the successful development of cloned animals. Further investigation is needed to determine whether this discrepancy reflects species-specific differences in nuclear remodeling, or simply variation in MPF half-life following oocyte activation (Eggan and Jaenisch 2002).

(2) Cell cycle status of the donor cell. High MPF levels in the recipient oocyte cause donor nuclear envelop breakdown and premature chromosome condensation (Fulka *et al*. 1996). Campbell *et al*. (1993) have shown that when bovine embryos are reconstructed by nuclear transfer into MII oocytes at the time of activation, nuclear

envelope breakdown occurs in all donor nuclei, regardless of their cell cycle stage, followed by reformation of the nuclear envelop and DNA synthesis. However, when previously enucleated and activated oocytes are used as recipient cytoplasts, in which MPF activity has been declined, no nuclear envelop breakdown occurs, then replication of donor nuclei depends on the cell cycle stage of the transferred nucleus. Eggan and Jaenisch (2002) compared cloning experiments using a variety of murine cells and demonstrated a clear correlation between the efficiency of blastocyst development and the proportion of donor cells in the G1 phase of the cell cycle. When cumulus (Wakayama *et al*. 1998), Sertoli (Ogura *et al*. 2000), and serum-starved fibroblast cells (Wakayama and Yanagimachi 1999), all primarily in a G1 (2N) state, were used for nuclear transfer, 70%, 65%, and 58% of the reconstructed embryos developed to blastocysts, respectively. In contrast, when rapidly cycling embryonic stem cells were used as nuclear donors, only 10-20% of nuclear transfer embryos completed cleavage development (Wakayama *et al*. 1999; Rideout *et al*. 2000; Eggan *et al*. 2001). Because most embryonic stem cells in a given population are in S phase, this may be the cause of their poor development after nuclear transfer. Consistent with this interpretation, when embryonic stem cells forced into G1 phase of the cell cycle, i.e. by partial serum withdrawal, were used as nuclear donors, blastocyst development was increased (Wakayama *et al*. 1999). These data demonstrated the importance of the nucleus donor cell cycle state to its compatibility with the oocyte cytoplasm. Chromatin structure in the S phase of the cell cycle may be incompatible with this condensation, leading to DNA damage and zygotic arrest. In contrast, chromatin in G1 and G2/M phase seems

compatible with this condensation. However, further evidence has shown that the numeral chromosome content of the donor cell is critical, rather than a particular cell cycle state per se (Wakayama *et al*. 1999).

(3) The inherent developmental potential of the donor cell. Comparing the efficiency of nuclear transfer experiments using both somatic and embryonic cells supports the hypothesis that intrinsic developmental potency of the donor cell plays a role in the cloning outcome, with embryonic stem cells and blastomeres having greater blastocyst and term rates than those of adult somatic cells in mice (Eggan and Jaenisch 2002). The increased developmental efficiency suggests that the embryonic stem cell and blastomere must be either more amenable to, or require less, epigenetic reprogramming than that of somatic cells. Somatic cells have chromatin configurations and DNA methylation levels appropriate for expression of genes necessary for maintenance of their differentiated state. Inability to reprogram gene expression appropriately, followed by depletion of the maternal stores of embryonic proteins and RNAs in the oocyte which are needed to support development of nuclear transfer embryos, could lead to the frequently observed developmental failure (Eggan and Jaenisch 2002).

However, in farm animals such as cattle and sheep, there is no apparent difference in cloning efficiency when the nuclear donors are embryonic (Wells *et al*. 1997; Sims and First 1994) or somatic cells (Wells *et al*. 1999; Wilmut *et al*. 1997). Furthermore, adult hematopoietic stem cells have been shown to result in very low (0-0.7%) birth rates (Inoue *et al*. 2006) in mice. The assumption that tissue-specific stem

cells might serve as efficient donors for nuclear transfer because of the undifferentiated state of their genome may need to be restated, at least for adult hematopoietic stem cells. Despite all efforts, the relation between donor cell differentiation and cloning efficiency remains to be clarified with effective comparison systems (Oback and Wells 2007).

Donor cell type has been another factor of interest that might correlate with cloning efficiency. Cloned animals have been produced by SCNT from organs such as mammary gland, cumulus, oviduct, ear, skin, muscle, liver, tail, and Sertoli cells. However, it is not clear which cell types or cell origins are most successful for animal cloning (Tsunoda and Kato 2002). In a study performed by Kato *et al*. (2000), the developmental potential of enucleated oocytes receiving somatic cells from various tissues of adult, newborn, and fetal cows was systematically examined. The percentage of blastocysts that developed from each of the donor cell types was not significantly different, except for those at the extremes of the range such as female fetal muscle cells (23%) compared with male adult liver cells (53%) ($P < 0.001$). There were no significant differences in the percentages of blastocysts that developed from oocytes containing adult (42%), newborn (37%) or fetal (40%) cell nuclei, or between female (39%) and male (40%) nuclei (Kato *et al*. 2000; Tsunoda and Kato 2002). When cloned blastocysts were transferred to recipient cows, fifty five of 139 recipients were pregnant. The frequency of abortion in the pregnant cows was 27% by day 100, 38% by day 150, and 49% by day 250 of gestation. The remaining pregnant recipients gave birth to 32 calves but 14 of them died around or within 7 days after parturition (Kato *et al*. 2000; Tsunoda and Kato 2002). Due to limited data on transfer of the cloned blastocysts, precise

comparison of the potential to develop to live offspring and peri- or postnatal death of calves was difficult. Two groups in which a relatively large number of cloned balstocysts were transferred were compared. Blastocysts produced by adult female cumulus and oviduct cells had a significantly lower pregnancy rate (28%) than those produced by male skin and ear cells (50%). Significantly more fetuses were aborted from the latter group; therefore, the proportions of calves produced from the two groups were not different. Similar results were found in rabbit somatic cell cloning (Yang *et al*. 2007a).

Applications of Nuclear Transfer Technology

Since its development, nuclear transfer has been widely used to produce genetically identical animals both for research and commercially, albeit the fact that nuclear transfer is inefficient. Nuclear transfer is promising in producing endangered (Lanza *et al*. 2000; Loi *et al*. 2001; Li *et al*. 2006b; Kim *et al*. 2007), exotic (Williams *et al*. 2006), extinct, and transgenic animals (when combined with homologous recombination and/or RNA interference). Transgenic goat fetuses (Golding *et al*. 2006), bovine fetuses (Jang *et al*. 2006), piglets (Dai *et al*. 2002; Hao *et al*. 2006), cats (Yin *et al*. 2007), etc. have been created by transplantation of nuclei of genetically modified donor cells into enucleated oocytes. Nuclear transfer is also a powerful tool to generate patient-specific embryonic stem cells, which hold great promise for the treatment of many human and animal diseases (Yang *et al*. 2007b).

CHAPTER II

CULTURE, IDENTIFICATION, AND EVALUATION OF SOMATIC CELLS FROM FRESH OVINE AND BOVINE SEMEN SAMPLES

Introduction

Extensive work has been done to identify the types of somatic cells in human semen. In addition to spermatozoa, human ejaculates contain 10^3 to 10^7 cells of other types (Phillips *et al*. 1978). These cells, often referred to as "round cells", include immature early stages of the spermatozoon, epithelial cells, and various major leukocyte subpopulations including granulocytes, monocytes/macrophages, B lymphocytes, helper and suppressor/cytotoxic T lymphocytes (Phillips *et al*. 1978; Belsey *et al*. 1980; Wolff and Anderson 1988; Homyk *et al*. 1990). When human ejaculates were plated out and cultured, epithelial cells were obtained (Phillips *et al*. 1978).

Types of somatic cells in semen of livestock animals have received less attention. Cells cultured from ovine (Nel-Themaat *et al*. 2004, 2007) and bovine (Nel-Themaat *et al*. 2005, 2006) semen have been identified as epithelial or epithelial-like cells by the presence of cytokeratin. In the present study, types of the cells cultured from ovine and bovine semen were further identified by differential immunofluorescence analysis which distinguishes epithelial cells (express cytokeratin), mesenchymal cells (express vimentin), and smooth muscle cells (express α-smooth muscle actin) based on differentiation markers that each type of cell expresses.

It has been demonstrated that cells derived from bovine (eland) semen can serve as karyoplast donors for nuclear transfer (Nel-Themaat *et al*. 2007). Somatic cells in semen could be a valuable source of nuclei for nuclear transfer to recover animals of genetic value, especially when other ways of obtaining somatic cells are not available. Chromosome number analysis in the study underlines the need for chromosome analysis of cells before using them for cloning experiments.

Materials and Methods

All procedures involving animals were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Semen Collection

Eight rams and nine bulls were used in the study. Rams included one 18-month-old Dorper, one 17-month-old Suffolk, three 18-month-old Suffolks, one 14-month-old Hampshire, and two 18-month-old Hampshires. Bulls included three one-year-old Charolais, two one-year-old crossbred Brahmans, one five-year-old Brahman, two six-year-old Brahmans, and one eight-year-old Brahman. All of the animals were in good health condition at the time of collection. Fourteen ejaculates were collected from rams by electro-ejaculation or artificial vagina, with one to three samples being collected from each animal. Volumes of ovine ejaculates obtained ranged from 0.5ml to 2ml. Twelve ejaculates were collected from bulls by electro-ejaculation, with one or two samples being collected from each animal. Volumes of bovine ejaculates obtained ranged from 5ml to 20ml.

Isolation and Culture of the Somatic Cells in Semen

Somatic cells were isolated as described (Nel-Themaat *et al*. 2005) with minor modifications. Each semen sample was extended (ram semen) by adding an equal volume of Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) or remained un-extended (bull semen), evenly distributed on top of four 15-ml conical tubes, each of which contained a column of 2.5ml each of 20%, 50%, and 90% Percoll, and centrifuged at 400×g for 20 min. Cells in the 20% layers and top half of the 50% layers were washed 2 to 3 times and resuspended in DMEM/F-12 containing 10% Fetal Bovine Serum (FBS), 200μg/ml gentamycin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and cultured in T-25 or T-75 flasks at 37ºC with 5% CO_2 in humidified air. Medium was changed 24 hr after plating; old medium was kept in new dishes for two more days to give additional time for cell attachment. Medium was then changed at least twice per week. Gentle washing of the culture surfaces to which cells might have attached using culture medium during medium changes helped remove bacteria and fungi. The concentration of gentamycin in the medium was decreased to 150μg/ml, 100μg/ml, and finally 50μg/ml with medium changes. Penicillin, streptomycin, and amphotericin B were gradually excluded from the culture medium.

Immunofluorescence Analysis

Types of cells cultured from ejaculates of 7 rams and 3 bulls were examined by immunofluorescence analysis. The procedures as described (Johnson *et al*. 1999) were applied. Cells from each ejaculate were transferred to a 4-well chamber slide (Nunc,

Naperville, IL) with a concentration of 4×10^4cells/well and incubated at 37 °C with 5% CO_2 in humidified air until the cells reached 60-70 % confluence. Then the slides were fixed in -20ºC methanol for 10 min. Goat serum (Sigma) was added in each well to prevent non-specific binding before first antibodies were added. After a quick rinse in 0.02 M PBS containing 0.03% Tween (PBS/Tween), cells in each of the 4 wells were incubated with mouse anti- IgG (Invitrogen), anti-cytokeratin (Sigma), anti-vimentin (Sigma), or anti-α-smooth muscle actin (Sigma) at 4 ºC overnight. After 3 rinses in PBS/Tween for 10 min each, the slides were incubated with fluorescein-conjugated goat-anti-mouse IgG (Zymed, San Francisco, CA) at room temperature for 1 hour. Two drops of anti-fade (Invitrogen) were added to each slide, which was then gently overlaid with a cover glass to let the anti-fade cover the whole slide. The slides were viewed by a Zeiss Photomicroscope III (Zeiss, Thornwood, NY) equipped with a filter set for fluorescein.

Chromosome Numbers of the Cells

Chromosome numbers of the cells obtained from four rams were analyzed according to the method of Dr. Chowdhary's lab (Texas A&M University, College Station) and as described (Barch 1991; Verma and Babu 1995). Upon reaching 50-60% confluence, cells were incubated with 10μg/ml ethidium bromide solution (FisherBiotech, Fair Lawn, NJ) and 0.1μg/ml demecolcine solution (Sigma) at 37°C for 1.5 hr. Medium was removed from each flask and saved in a pre-labeled centrifuge tube. The flask was washed once using trypsin/EDTA solution (Sigma). The solution was removed and added to the centrifuge tube containing the old medium. One ml of fresh trypsin-EDTA solution was added to the flask, which was incubated at 37°C for 5-10

min. to free the cells from surface of the flask. The contents of the centrifuge tube were added to the flask and mixed. The mixture was centrifuged at 400 g for 5 min. Supernatant was discarded and the cell pellet was dislodged gently by flicking the tube with fingers. Fourteen ml of pre-warmed hypotonic solution (Rainbow Scientific, Inc.) was added slowly to the tube, which was incubated at 37°C for 40 min, and then 0.5 ml of freshly prepared fixative (methanol: glacial acetic acid = 3:1) was added to the tube following incubation. Contents of the tube were mixed gently and centrifuged at 100g for 10 min. Supernatant was discarded. Five ml of freshly prepared fixative was added to the tube. The contents were mixed gently and centrifuged at 100 g for 10 min. The fixation procedure was repeated at least 3 more times. The final cell pellet was resuspended in 1.5 ml of fixative. Metaphase cell preparations were obtained on clean glass slides and examined for quality under a phase contrast microscope. Giemsa (Sigma) stained chromosome preparations were examined at 100X and numbers of chromosomes per cell counted.

Results

Isolation and Culture of the Somatic Cells in Semen

Cell growth was obtained from 11 of the 14 ram ejaculates (7 of the 8 rams) (Table 5). Most of the cell lines stopped dividing within one or two months and then died even thought growth factors were provided in the culture medium. Cell lines obtained from four rams, however, proliferated for more than 2 months (Figure 1). Cell growth was obtained from 7 of the 12 bull ejaculates (5 of the 9 bulls) (Table 5). Cell

Table 5. Number of semen collections from each animal (a) or breed (b) and number of collections with cell growth.

(a)

Species	Animal code	Breed	Age	# of collections	# of collections with cell growth
Ovine	1	Dorper	18 month	3	2
	2	Suffolk	17 month	1	1
	3	Suffolk	18 month	3	3
	4	Suffolk	18 month	1	1
	5	Suffolk	18 month	1	0
	6	Hampshire	14 month	3	2
	7	Hampshire	18 month	1	1
	8	Hampshire	18 month	1	1
Bovine	1	Charolais	1 year	1	1
	2	Charolais	1 year	1	0
	3	Charolais	1 year	1	0
	4	Crossbred Brahman	1 year	1	1
	5	Crossbred Brahman	1 year	2	0
	6	Brahman	5 year	1	0
	7	Brahman	6 year	2	2
	8	Brahman	6 year	1	1
	9	Brahman	8 year	2	2

(b)

Species	Breed	# of collections	# of collections with cell growth
Ovine	Dorper	3	2
	Suffolk	6	5
	Hampshire	5	4
Bovine	Charolais	3	1
	Crossbred Brahman	3	1
	Brahman	6	5

(a) Cell growth was obtained from 11 of the 14 ram ejaculates (7 of the 8 rams) and 7 of the 12 bull ejaculates (5 of the 9 bulls). (b) is a summary of (a), comparison between breeds.

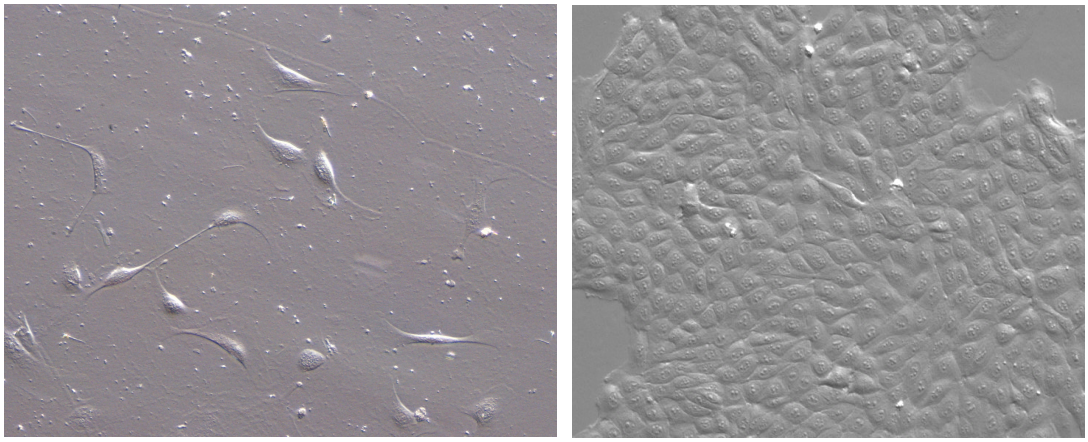

Figure 1. Cells cultured from ovine semen. Left: cells attached at bottom of a culture dish 3-4 days post isolation. Right: a cell colony was formed.

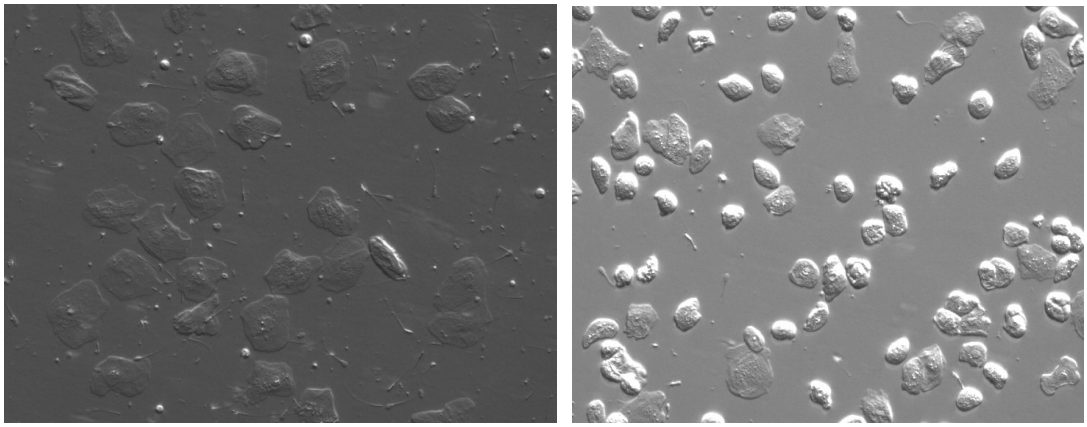

Figure 2. Cells isolated from fresh bovine semen.

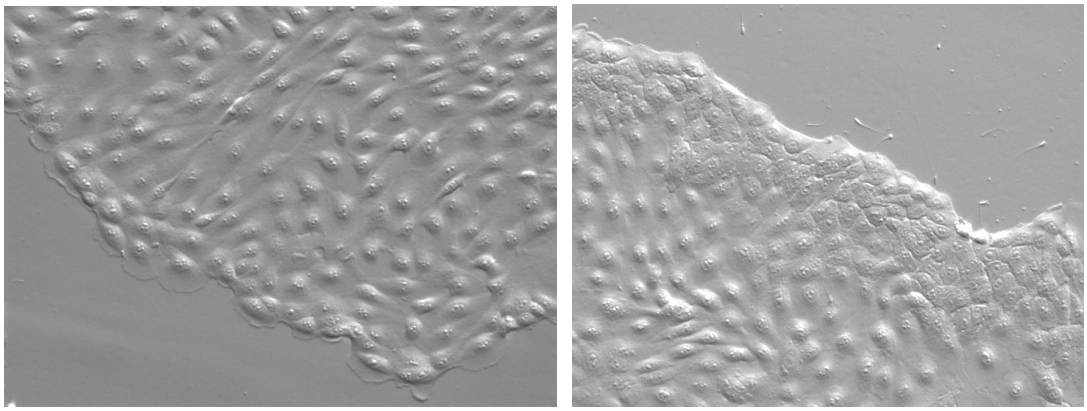

Figure 3. Cells cultured from bovine semen. The cells had classic epithelial morphology.

lines obtained from two bulls stopped dividing within one or two months while cell lines from 3 bulls proliferated for more than 2 months (Figures 2, 3).

Immunofluorescence Analysis

As is shown in Figures 4 and 5, a single cell type was successfully cultured from ovine and bovine ejaculates. These cells had classic cobblestone morphology and expressed cytokeratin indicating they are of epithelial origin. These cells also expressed vimentin so they had undergone and /or were undergoing an epithelial-to-mesenchymal transformation that is often observed in primary cultures of epithelial cells. Little to no α-smooth muscle actin expression was found, so it was not likely that the isolated cells were of vascular origin.

Chromosome Numbers of the Cells

The predominant chromosome number of cells from three of the rams (Dorper 18-month-old; Suffolk 17-month-old; and Suffolk 18-month-old) was 2n = 54, which is the normal modal number for sheep (Figures 6, 7). However, the numbers of chromosomes in cells from the fourth ram (Hampshire, 18-month-old) were near-triploid (Figure 8). Table 6 shows chromosome number distribution of cells obtained from the four rams.

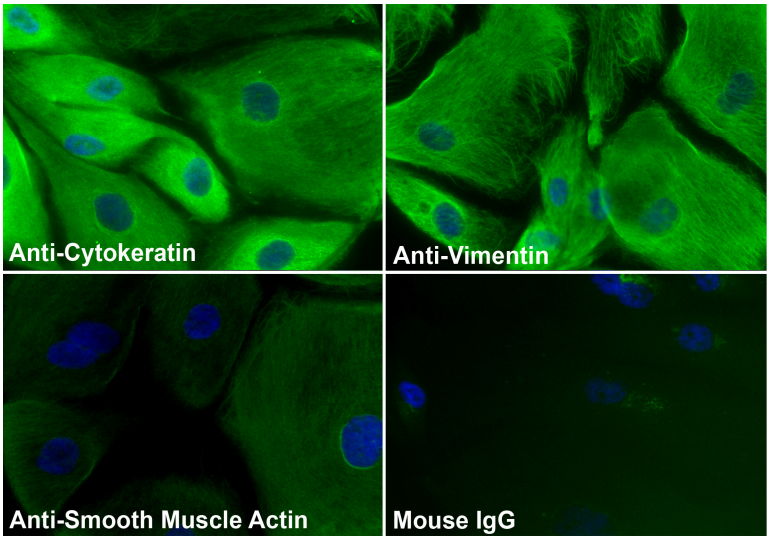

Figure 4. Immunofluorescence analysis of the cells cultured from ovine semen. The cells expressed cytokeratin and vimentin (top row). Little to no α-smooth muscle actin expression was found (bottom left).

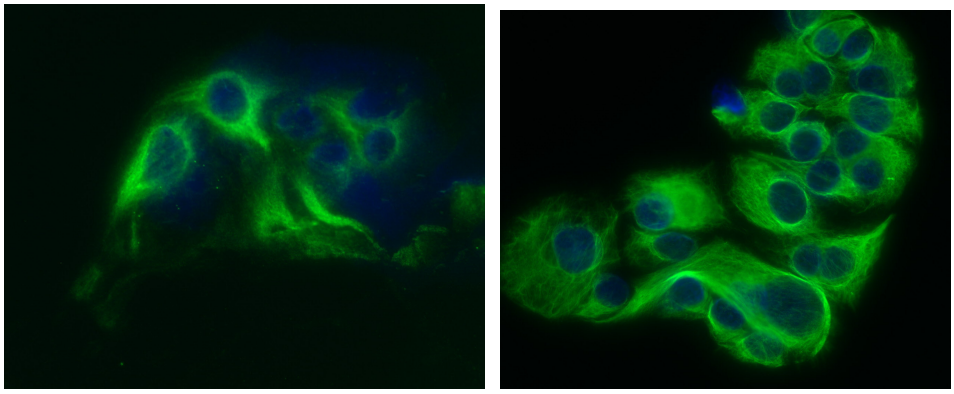

Figure 5. Immunofluorescence analysis of the cells cultured from bovine semen (Left: anti-cytokeratin; Right: anti-vimentin). The cells were both cytokeratin and vimentin positive.

Figure 6. Metaphase chromosomes of a cell cultured from semen of an 18-month-old Dorper (2n = 54).

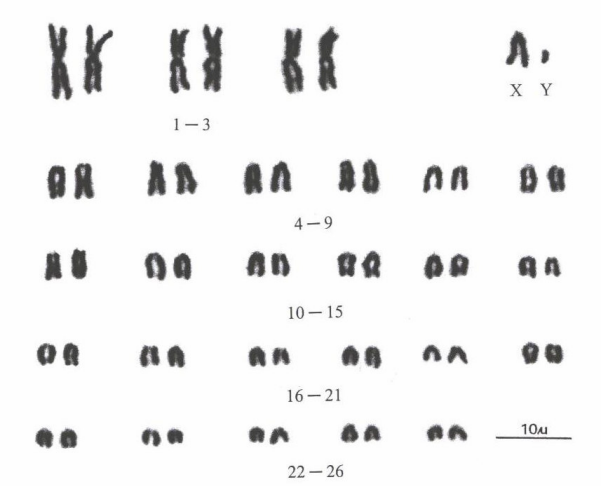

Figure 7. Karyotype of a normal male sheep from a cell cultured from semen of an 18-month-old Dorper (52A + XY).

Figure 8. Metaphase chromosomes of a cell cultured from ejaculate of an 18-month-old Hampshire (87, ~ triploid).

Table 6. Chromosome number distribution of cells cultured from four rams.

Sheep (age)	Chromosome Number					
	< 53	53	54	55	>55	Total
Dorper (18 month)	1	0	9	1	1	12
Suffolk (17 month)	3	0	21	0	2	26
Suffolk (18 month)	1	0	18	1	2	22

Sheep (age)	Chromosome Number										
	<54	54	55-80	81	82	83	84	85	86	>86	Total
Hampshire (18 month)	0	2	2	11	0	1	5	4	4	6	36

Discussion

In the present study, cell lines were successfully cultured from 11 of the 14 ovine ejaculates and 7 of the 12 bovine ejaculates. These cells had classic cobblestone morphology and expressed cytokeratin indicating they are of epithelial origin, which agrees with those previously reported (Nel-Themaat *et al*. 2004, 2007). Epithelial cells, uncontaminated by fibroblasts, were also obtained from human ejaculates, indicating that semen samples provide a valuable source of purely epithelial cells, isolation of which usually is very difficult (Phillips *et al*. 1978).

Somatic cells have been successfully cultured from fresh and cooled ram semen, but not frozen-thawed semen groups (Nel-Themaat *et al*. 2004). Attempts to culture somatic cells from frozen semen in our laboratory have also been unsuccessful. Culture of the cells from frozen semen, if possible, could be extremely valuable for preservation or restoration of endangered, exotic, and extinct animals when other means of obtaining somatic cells are not available, as the cells from semen might prove useful for animal cloning. An optimized culture system might increase the likelihood of obtaining cells from frozen semen, which might serve as nuclear donors for nuclear transfer. This will be the focus of Chapter III.

Metaphase chromosomal complements of the cells cultured from semen of four rams were evaluated. The numbers of chromosomes in cells from one ram was near-triploid, indicating the need for chromosome analysis of cells before using them for cloning experiments. To our knowledge, this is the first attempt to evaluate chromosome numbers of cells obtained from ovine ejaculates. It has been suggested that epithelial

cells become quickly polyploid in culture but the rate of polyploidy does not increase with the life span of the culture (David *et al*. 1981), so it remains to be determined if aneuploidy observed was initiated *in vivo* or due to *in vitro* culture.

The present study demonstrated that epithelial cells can be obtained from fresh ovine and bovine ejaculates with relatively high efficiency, 78.57% (11/14) for ovine and 58.33% (7/12) for bovine. Chromosome number analysis underlined the need for chromosome analysis of cells before using them for cloning experiments. Usefulness of the cells cultured from bovine semen for nuclear transfer will be further evaluated in Chapter IV using cells obtained from semen of three different bulls.

CHAPTER III

EVALUATION OF CULTURE SYSTEMS AND CELL RECOVERY FROM FROZEN SEMEN

Introduction

Somatic cells in semen are a potential source of nuclei for cloning animals by somatic cell nuclear transfer. It has been demonstrated that cells derived from bovine (eland) semen can serve as karyoplast donors for nuclear transfer (Nel-Themaat *et al*. 2007). Chromosome analysis of the cells cultured from ram semen (see Chapter II) revealed that the predominant chromosome number of cells from three of the four rams was diploid and could be useful for cloning.

There have been many cases that animals of genetic value have died and the only material available from these animals is frozen semen. Since freezing of donor cells did not adversely affect cloning efficiency, culture of the cells from frozen semen, if possible, would be extremely valuable for preservation or restoration of endangered, exotic, and extinct animals when other ways of obtaining somatic cells are not available.

Although cell growth can be obtained from fresh semen relatively easily, to date, several attempts to culture such cells from frozen semen have been unsuccessful. The culture system established herein promotes proliferation and attachment of the cells obtained from fresh ovine semen samples, and may increase the likelihood of obtaining cells from frozen semen, which could be used for cloning to recover animals of genetic value.

Materials and Methods

Cell Lines

Cell lines from 4 rams with the highest growth rates were used in the study. All of the cell lines had been cultured in basic medium, i.e. DMEM supplemented with 10% FBS and 50μg/ml gentamycin, for at least two weeks before the trials were performed.

Preparation of Conditioned Medium

Swiss 3T3 fibroblast- and sheep fetal fibroblast (SFF)-conditioned media were chosen to see if SFF-conditioned medium would have beneficial effects over 3T3 fibroblast-conditioned medium due to same cell origin as cells cultured from ovine semen. Swiss 3T3 cells were purchased from www.atcc.org. Two sheep fetuses, both of which were 31mm in crown rump length, were obtained from an abattoir and shipped to our laboratory overnight on ice. SFF were isolated from the fetuses by trypsin digestion. Conditioned media were generated by seeding SFF and Swiss 3T3 cells in basic medium (DMEM/F-12 supplemented with 10% FBS and 50μg/ml gentamycin), respectively, culturing at 37°C with 5% CO_2 in humidified air, and collecting and filtering the media 24 hr after seeding (Kobayashi *et al*. 2005).

Proliferation Test on Cells Cultured in Four Different Media

Upon reaching 80% confluence, cells in each cell line were trypsinized and resuspended in one of the four media: medium 1 (supplemented medium) contained DMEM/F-12 supplemented with 10% FBS, 10ng/ml EGF, 30μg/ml BPE, 5μg/ml insulin, 10ng/ml cholera toxin, and 50μg/ml gentamycin; medium 2 was sheep fetal fibroblast (SFF)-conditioned medium; Medium 3 was Swiss 3T3 fibroblast-conditioned

medium; and medium 4 was basic medium, i.e., DMEM/F-12 supplemented with 10% FBS and 50μg/ml gentamycin. Concentration of the cells in each cell solution was counted using a hemocytometer and the cell solutions were diluted to 1×10^4 cells/ml or 2×10^4 cells/ml. Cells were then seeded on 6-well (1×10^4 cells/ml $\times$3ml/well = 3×10^4 cells/well) or 12-well (2×10^4 cells/ml $\times$0.6ml/well = 1.2 $\times10^4$ cells/well) culture plates (Falcon, Franklin Lakes, NJ). Twenty 6-well plates or ten 12-well plates were seeded each time for each cell line, so that four 6-well plates or two 12-well plates would be counted at each of the five test points with 6 repeats per cell line per treatment. Total numbers of attached cells in each well were counted on day 1, 2, 3, 4, and 5 post seeding using a hemocytometer. Attached cells were detached from the culture plates by trypsinization before counting. Proliferation rate was defined as the percentage of total number of attached cells divided by the total number of seeded cells.

Attachment Test and Proliferation Test on Cells Cultured on Matrigel, Collagen I, and Plastic Surfaces

Twelve-well plates were coated with bovine collagen-1 (10μg/cm^2; BD Biosciences, Bedford, MA), matrigel (38.16 μg/cm^2; BD Biosciences, Bedford, MA), or remained uncoated. Cells from each cell line were resuspended in medium1 (designed medium) following trypsinization. Concentrations of the cell solutions were counted using a hemocytometer and diluted to 3×10^4 cells/ml. Cells from each of the 4 rams were then seeded at a density of 1.8×10^4 cells per well (3×10^4 cells/ml $\times$ 0.6ml/well). Five twelve-well plates were seeded each time for each cell line, so that one plate would be counted at each of the five test points with 4 repeats per cell line per treatment. For the

attachment test, total numbers of attached cells were counted 30, 60, 90, 120, and 150 min post seeding. Floating cells were removed by gently washing twice with 37 ºC PBS (Ca^{++}, Mg^{++} free) before counting. Attached cells were trypsinized and number of cells in each well counted using a hemocytometer. For the proliferation test, total numbers of attached cells were counted each day for 5 days post seeding. The same method of counting stated above was used. Attachment rate and proliferation rate were defined as the percentages of total number of attached cells divided by total number of seeded cells.

Culture of the Cells Found in Frozen Semen

A total of one hundred and twenty ovine semen pellets and one hundred and twenty bovine semen straws were used in the experiment. Since frozen semen is highly diluted, four pellets or straws were thawed each time to increase the likelihood of obtaining somatic cells. Ovine semen pellets were thawed in plastic bags in 37 °C water bath for 1-2 min. Bovine semen straws were placed directly in 37 °C water bath for 1-2 min. The same methods of somatic cell isolation and culture stated in Chapter II were used. Following washing, somatic cells were plated in Matrigel-coated dishes and cultured in the supplemented medium at 37ºC with 5% CO_2 in humidified air.

Statistical Analysis

One way ANOVA, together with Fisher's least significant difference (LSD) were used to compare means of different groups in the proliferation tests and the attachment test. Analysis was performed using SAS 9.1.3 software and differences were considered significant at $p< 0.05$.

Results

Proliferation Test on Cells Cultured in Four Different Media

Figure 9 shows the averaged proliferation rate of cells cultured in each medium at each of the five test points. Cell proliferation was significantly higher in the supplemented medium, SFF-conditioned medium, and 3T3 cell-conditioned medium compared to the basic medium by day 2 of culture ($p<0.05$, $n=24$), and significantly higher in the supplemented medium compared to the SFF-conditioned medium and 3T3 cell-conditioned medium by day 4 of culture ($p<0.05$, $n=24$). No significant difference was found between SFF-conditioned medium and 3T3 cell-conditioned medium.

Proliferation Test and Attachment Test on Cells Cultured on Matrigel, Collagen I, and Plastic Surfaces

Figures 10 and 11 show the averaged proliferation rate and attachment rate of cells cultured on matrigel, collagen I, and plastic surfaces at each of the five test points, respectively. Cell proliferation was significantly higher in culture with the matrigel-coated surface compared to the collagen I-coated surface and plastic surface by day 2 of culture ($p<0.05$, $n=16$; Figure 10). No significant difference was found between the collagen I-coated surface and plastic surface. Cell attachment was significantly higher in culture with the matrigel-coated surface and collagen I-coated surface compared to the plastic surface ($p<0.05$, $n=16$; Figure 11).

Culture of the Cells Found in Frozen Semen

No cell attachment or cell growth was found in frozen thawed semen groups.

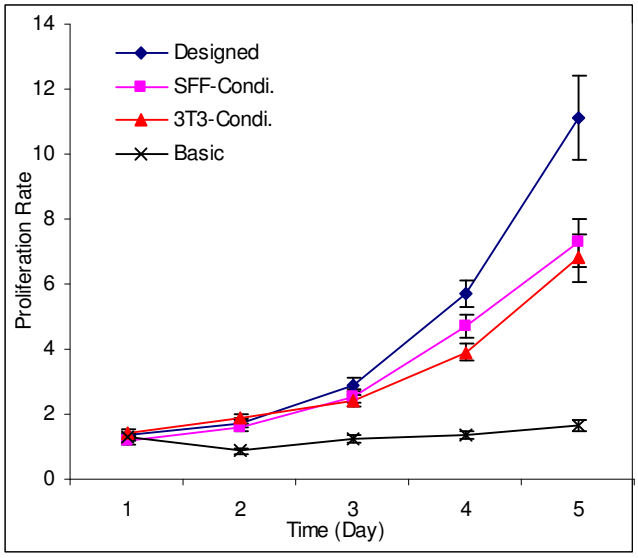

Figure 9. Effects of different media on cell proliferation. Cell proliferation was significantly higher in the supplemented medium, SFF-conditioned medium, and 3T3-conditioned medium compared to the basic medium by day 2 of culture, and significantly higher in the supplemented medium compared to the SFF-conditioned medium and 3T3-conditioned medium by day 4 of culture. ($p<0.05$ Fisher's LSD; $n=24$).

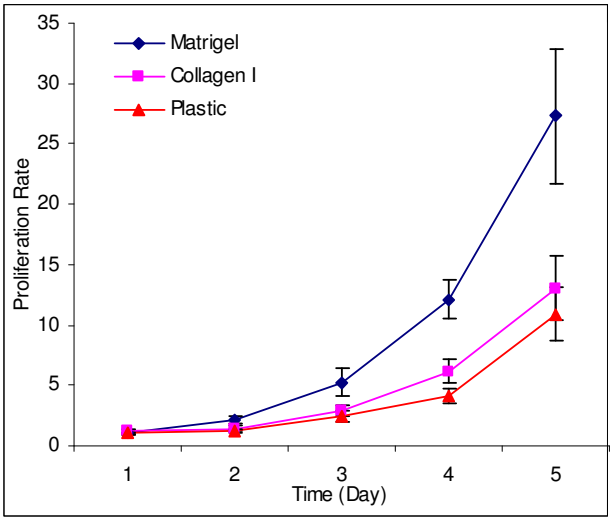

Figure 10. Effects of different surfaces on cell proliferation. Cell proliferation was significantly higher in culture with the matrigel-coated surface compared to the collagen I-coated surface and plastic surface by day 2 of culture. ($p<0.05$ Fisher's LSD; n=16).

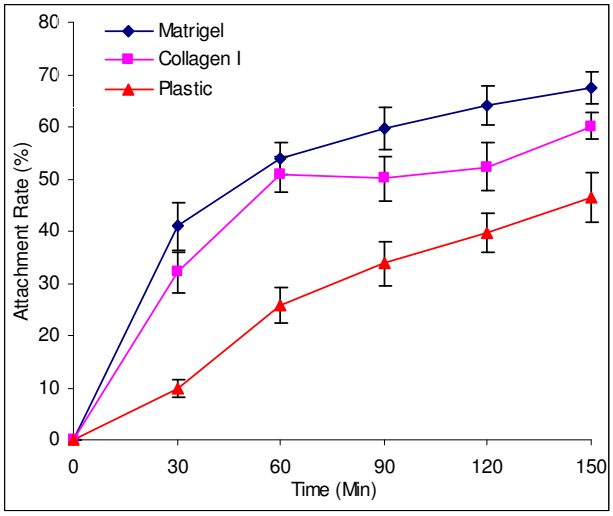

Figure 11. Effects of different surfaces on cell attachment. Cell attachment was significantly higher in culture with the matrigel-coated surface and collagen I-coated surface compared to the plastic surface. ($p<0.05$ Fisher's LSD; $n=16$).

Discussion

Several media have been developed that are capable of supporting different types of epithelial cells (Wise 2002). Insulin, EGF, BPE, etc. are frequently used in culturing prostate epithelial cells and epithelial cells from tissues other than the prostate (Hackworth *et al*. 1990; Chopra *et al*. 1991; Sime *et al*. 1997). Chopra *et al.* (1996) reported that cholera toxin, as well as insulin, hydrocortisone, EGF, and BPE, were essential for the growth of prostatic epithelial cells as deletion of any one of the factors strongly inhibited their growth. Effects of Insulin, EGF, BPE, hydrocortisone, and cholera toxin on the epithelial cells obtained from ovine semen were evaluated and Insulin, EGF, BPE, and cholera toxin were selected for the supplemented medium based on cell proliferation and morphology (unpublished data). More growth factors and hormones may be explored and added and better proliferation rates may be achieved. This is confirmed in the proliferation test on cells cultured on three different surfaces as cell proliferation was significantly higher by day 2 of culture with the matrigel-coated surface (which contains additional growth factors) compared to the other two surfaces.

3T3 fibroblast co-culture has been shown to improve proliferation of the cells obtained from sheep and eland semen (Nel-Themaat *et al*. 2006). However, even if cell inserts are used, 3T3 cell contamination remains a problem as 3T3 cells may migrate through the holes in the inserts and complete separation of epithelial cells from 3T3 cells is almost impossible. This is critical especially when the epithelial cells are used for nuclear transfer. The supplemented medium presented here could be used to circumvent this problem. It also is easy to prepare and simplifies the culture system.

Leukocytes are also present in ejaculates. Further studies are needed to verify the possibility of obtaining leukocyte populations and their usefulness for animal cloning.

The present study describes an effective system for the culture and proliferation of epithelial cells obtained from ovine semen samples. Cell attachment or cell growth was not found in frozen thawed semen groups. Further studies are needed to see how well somatic cells survive the semen freezing process and the viability of somatic cells isolated from frozen-thawed semen. The next Chapter presents evaluation of the somatic cells in bovine semen as potential nucleus donors for nuclear transfer.

CHAPTER IV

USEFULNESS OF THE SOMATIC CELLS IN BOVINE SEMEN FOR NUCLEAR TRANSFER

Introduction

It was not realized until after the birth of Dolly, the first sheep cloned using an adult somatic cell in 1997 (Wilmut *et al*.), that fully differentiated adult cells can be reprogrammed and live clones produced. Even after 10 years of study, SCNT remains an inefficient process, with 0-4% of reconstructed embryos developing into live young (Wilmut *et al*. 2002). Nuclear transfer, however, is a powerful tool and has been widely used to produce genetically identical animals both for research and commercially. Nuclear transfer is also promising in producing endangered (Lanza *et al*. 2000; Loi *et al*. 2001; Li *et al*. 2006b; Kim *et al*. 2007), exotic (Williams *et al*. 2006), extinct, and transgenic animals (when combined with homologous recombination and/or RNA interference) (Dai *et al*. 2002; Golding *et al*. 2006; Jang *et al*. 2006; Hao *et al*. 2006; Yin *et al*. 2007).

Somatic cells in semen are a potential source of nuclei for cloning animals by nuclear transfer. There have been many cases that animals of genetic value have died and the only material available from these animals is frozen semen. If the somatic cells in semen can be used for nuclear transfer, it would be extremely valuable for preservation or restoration of endangered, exotic, and extinct animals, especially when other ways of obtaining somatic cells are not available.

Epithelial cells have been successfully cultured from ovine and bovine ejaculates (see Chapter II). Chromosome analysis revealed that cells cultured from 3 of the 4 rams were diploid, which could be useful for nuclear transfer. In the present study, usefulness of the cells in bovine semen for nuclear transfer was further evaluated using cells cultured from semen of three different bulls and somatic cells isolated from frozen semen and not cultured.

Materials and Methods

Preparation of Donor Cells

Epithelial cells obtained from semen of three different bulls (a 1-year-old Charolais; a 6-year-old Brahman; and an 8-year-old Brahman) which proliferated for more than 2 months were used in the study (see Chapter II). All of the cells were cultured to 90-100% confluence which took 3-4 days. Then the confluent cultures were maintained for another 2-3 days without serum starvation before the cells were used as nuclear donors. Immediately before utilization for nuclear transfer, the cells were trypsinized and resuspended in M199 (Hanks' salts) (Gibco) supplemented with 10% FBS. All of the cells had been cultured *in vitro* for 4 to 14 weeks at the time of nuclear transfer.

Somatic cells isolated from frozen bovine semen but not cultured were also used in this study. The same method of somatic cell isolation stated in Chapter II and III was used, with minor modifications. Twenty to twenty-five straws (0.5 ml/straw) of semen were thawed each time to make sure enough somatic cells were obtained. After isolation, somatic cells were stored at 4 °C in DMEM/F12 supplemented with 10% FBS and 100

µg/ml gentamycin for about 4 hours before being injected into the perivitelline space of enucleated oocytes.

Preparation of Oocytes

Ovaries of mature cows were collected from a local abattoir and transported to the laboratory in warm saline solution within 2-4 hours. Oocytes were released from antral follicles (> 3 mm in diameter) by mincing using a scalpel blade. Compact cumulus-oocyte complexes with evenly distributed cytoplasm and at least 2-3 layers of cumulus cells were selected and matured for 18 hours at 38.5 °C with 5% CO_2 in humidified air. Maturation medium was composed of M199 (Earle's salts) (Gibco) supplemented with 10% FBS, 0.045 unit/ml bFSH (Sioux Biochemical, Sioux Center, Iowa), 1mg/ml estrogen (Sigma), and 50 µg/ml gentamycin. Oocytes transported to our laboratory overnight in maturation medium in a portable incubator with a temperature set at 38 °C were purchased from Ovitra Biotechnology (Hereford, Texas) and used in nuclear transfer when cells isolated from frozen semen and not cultured were donor cells.

Enucleation

Eighteen hours post maturation, cumulus cells were removed by repeated pipetting in M199 medium (Hanks' salts) supplemented with 0.3% hyaluronidase (Sigma) for 2-3 min. Oocytes with good morphology and the presence of a first polar body were selected and stained in M199 (Earle's salts) supplemented with 10% FBS, 5µg/ml Hoechst 33342 (Sigma) and 5µg/ml cytochalasin B (Sigma) for 10-15 min before enucleation. Oocytes were exposed to ultraviolet (UV) light briefly to locate the

metaphase plate and sometimes the first polar body. The metaphase plate and polar body were then gently removed with a micropipette (~ 15µm in outside diameter). Successful enucleation was confirmed by brief exposure of the oocytes to UV light. On average, each oocyte was exposed to UV light for no more than 3 seconds.

Injection and Fusion

For the epithelial cells cultured from bovine semen samples, around 10 cells were loaded into an injection pipette (~ 18µm in outside diameter) each time and a single cell was inserted into the perivitelline space of an enucleated oocyte. Fusion was induced by applying two 1.8-1.9 KV/cm, 20 µsec direct-current pulses delivered by an Eppendorf Multiporator (Eppendorf, North America) in fusion medium that is composed of 0.28M Mannitol (Sigma), 0.1 mM $CaCl_2$ (Sigma), and 0.1mM $MgSO_4$ (Sigma).

For the somatic cells isolated from frozen semen samples, 5-10 cells were loaded in an injection pipette (10-15 µm in outside diameter) each time. Piezo-driven pulses were applied to penetrate through a zona pellucida (Speed 5, Intensity 5), and then the oolemma (Speed 1 or 2, Intensity 1 or 2). A single cell was deposited into the ooplasm and the injection pipette withdrawn (Kimura and Yanagimachi 1995). Epithelial cells cultured from fresh bovine ejaculates were used as positive controls.

Activation

One and one half to two hours post fusion or direct injection, reconstructed couplets were equilibrated in fusion medium, then transferred to a fusion chamber containing a drop of fusion medium. Activation was induced by applying two 0.3 KV/cm, 55 µsec direct-current pulses, followed by incubation in M199 (Earle's salts)

supplemented with 10% FBS, 10 µg/ml cycloheximide (Sigma) and 5 µg/ml cytochalasin B for 5 hr in a humidified 5% CO_2, 5% O_2, and 90% N_2 gas mixture at 38.5 ºC.

Embryo Culture

Embryos were washed three times in M199 (Earle's salts) supplemented with 10% FBS and once or twice in commercially available G1 medium (Vitrolife, Colorado, USA) (Gardner *et al.* 1994) and cultured in G1 medium for 3 days, then transferred to G2 medium (Vitrolife, Colorado, USA) (Gardner *et al.* 1994) and cultured for an additional 7 to 8 days in a humidified 5% CO_2, 5% O_2, and 90% N_2 gas mixture at 38.5 ºC. Cleavage rates and blastocyst development rates were examined on day 3, day 7 and day 10 of *in vitro* culture, respectively.

Results

Table 7 shows the *in vitro* development of embryos cloned using epithelial cells cultured from semen of three different bulls. Blastocyst development rates using cells

from three of the bulls, 1-year-old charolais, 6-year-old Brahman, and 8-year-old Brahman, were 15.9%(18/113), 34.5%(29/84), and 14.4%(13/90) of the fused one cell embryos, respectively. Of these blastocyst stage embryos, 38.9% (7/18), 72.4% (21/29), and 61.5% (8/13) hatched, respectively. Figures 12, 13, 14, and 15 show the *in vitro* matured bovine oocytes before denuding, matured oocytes with first polar bodies, cleaved embryos, and cloned blastocysts, respectively.

Table 8 shows the cleavage and development rates of embryos cloned using somatic cells isolated from frozen semen and fresh semen (positive control). In the frozen semen group, 27.4% (82/299) of recombined embryos were either cleaved or fragmented. Of these embryos, 48 were examined and 42 contained intact donor cells. In the positive control group, blastocyst development rate was 4% (3/74) of the embryos survived piezo injection. Of these 3 blastocyst stage embryos, 66.7% (2/3) hatched. Figure 16 shows the cloned blastocysts from the positive control group.

Table 7. *In vitro* development of embryos cloned using epithelial cells cultured from semen of three different bulls.

Breed	Fused	Compact Morula	Blastocyst	Hatched	Age of cell
Charolais, 1-year-old (CH131)	21	4	3	3	4 weeks
	12	5	2	0	5 weeks
	40	6	4	1	7 weeks
	40	11	9	3	14 weeks
Total	113	26	18	7	
Rates			18/113=15.9%	7/18=38.9%	

Breed	Fused	Compact Morula	Blastocyst	Hatched	Age of cell
Brahman, 6-year-old (BH125)	48	22	20	16	5.5 weeks
	36	11	9	5	6.5 weeks
Total	84	33	29	21	
Rates			29/84=34.5%	21/29=72.4%	

Breed	Fused	Compact Morula	Blastocyst	Hatched	Age of cell
Brahman, 8-year-old (BH030)	32	3	3	2	5 weeks
	35	4	3	2	8 weeks
	23	6	7	4	9 weeks
Total	90	13	13	8	
Rates			13/90=14.4%	8/13=61.5%	

Blastocyst development rates using cells from three of the bulls, 1-year-old charolais, 6-year-old Brahman, and 8-year-old Brahman, were 15.9%(18/113), 34.5%(29/84), and 14.4%(13/90) of the fused one cell embryos, respectively. Of these blastocyst stage embryos, 38.9% (7/18), 72.4% (21/29), and 61.5% (8/13) hatched, respectively.

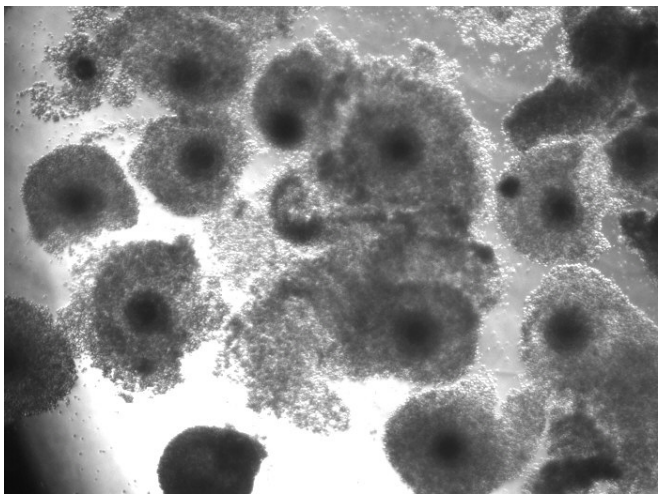

Figure 12. *In vitro* matured bovine oocytes (18 hours in maturation medium). The oocytes had evenly distributed cytoplasm and at least 2-3 layers of cumulus cells. Cumulus cell expansion was obvious after maturation.

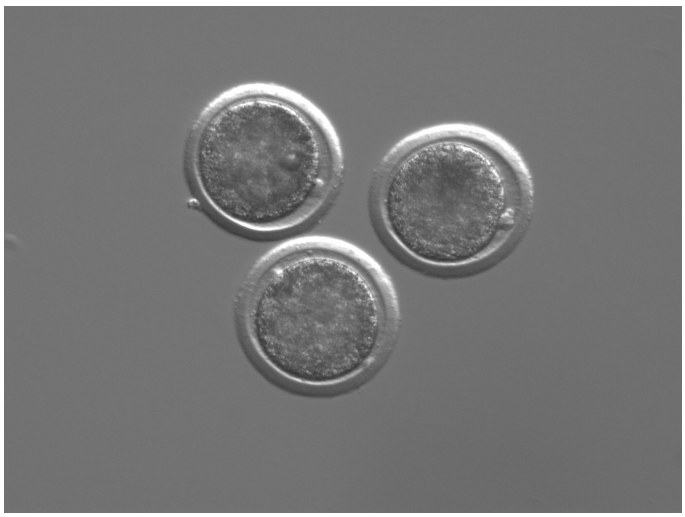

Figure 13. Bovine oocytes with first polar bodies. Cumulus cells had been removed by repeated pipetting in M199 medium (Hanks' salts) supplemented with 0.3% hyaluronidase for 2-3 min.

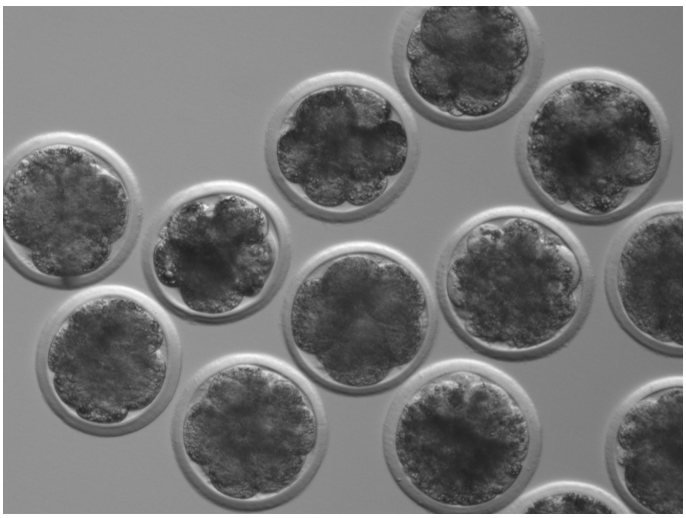

Figure 14. Cloned bovine embryos using epithelial cells cultured from fresh bovine semen (2.5 days in G1 medium).

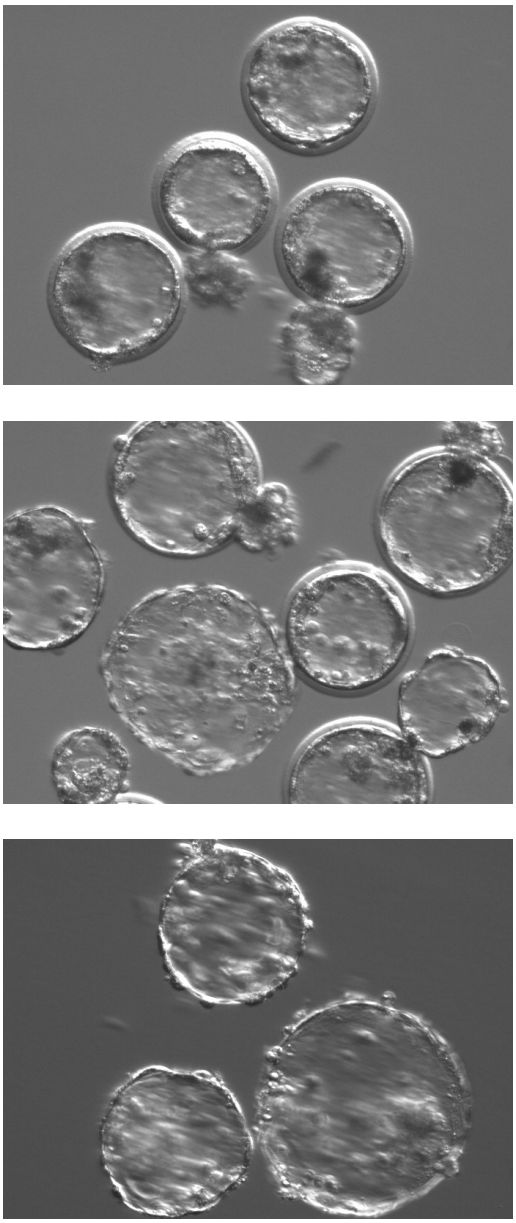

Figure 15. Blastocysts cloned using epithelial cells cultured from fresh bovine semen. Fusion was applied to introduce donor cell nuclei into the oocyte cytoplasm.

Table 8. Cleavage and development rates of bovine embryos cloned using cells isolated from frozen and fresh (positive control) semen samples.

Donor cell source	# survived injection	# cleaved/ fragmented (%)	# not cleaved (%)	Compact morula	Blastocyst
Frozen semen	299	82 (27.4)*	217 (72.6)	0	0
Fresh semen (Brahman, 6-year-old)	74	73 (98.6)	1 (1.4)	5	3

* 42 of the 48 embryos/oocytes tested were fragmented (The injected somatic cells were found intact in/between the blastomeres). Injected somatic cells were not found in the other 6 embryos so it is likely that these 6 embryos cleaved.

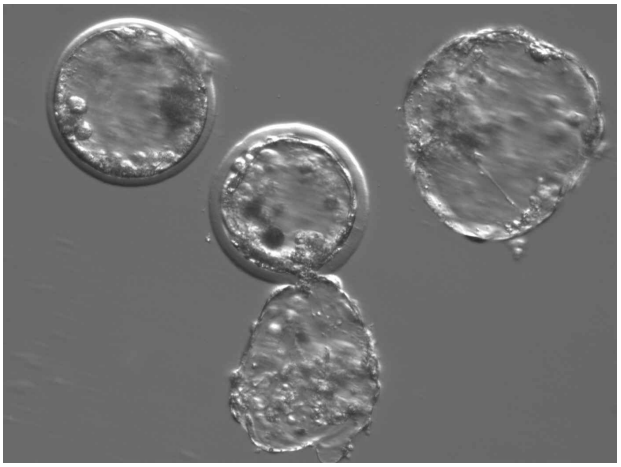

Figure 16. Blastocysts cloned using epithelial cells cultured from fresh bovine semen by piezo injection (positive control).

Discussion

Blastocysts have been cloned using epithelial cells cultured from semen of three different bulls, which agrees with those previously reported (Nel-Themaat *et al*. 2007). In the previous report, two female elands were superovulated and oocytes were collected by transvaginal ultrasound-guided aspiration of mature ovarian follicles. A total of 17 couplets were fused, resulting in two blastocysts and no pregnancy after embryo transfer (Nel-Themaat *et al*. 2007).

Whole cell intracytoplasmic injection was applied to introduce nuclei of somatic cells isolated from frozen semen without culturing, into enucleated oocytes for two reasons: 1) electrofusion did not work well, with a fusion rate of 7.8% (5/64); 2) membranes of the somatic cells isolated from frozen semen were hard and difficult to break by piezo pulses or drawing in and out of the injection pipette. Pigs and Asian yellow goats have been cloned by whole cell intracytoplasmic injection (Lee *et al*. 2003; Chen *et al*. 2007). The present study demonstrated that whole cell intracytoplasmic injection can also be applied to produce cloned bovine blastocysts.

No compact morulae or blastocysts were obtained when somatic cells isolated from frozen semen without culturing were used as donor cells. Less than 10% of viable cells have been detected in somatic cells isolated from frozen semen by propidium iodide. Nuclear transfer itself is an inefficient process, combined with low percentage of viable donor cells, making the process even more inefficient. Maybe one day when a live stain which tells viable cells/nuclei from non-viable cells/nuclei is developed, efficiency of the process could be improved and cloned fetuses and animals can be produced.

However, chromosome analysis of the viable cells in frozen semen may also be needed to determine percentage of the cells having normal chromosomes.

In the present study, hatched blastocysts have been cloned using epithelial cells cultured from semen of three different bulls, but not from somatic cells isolated from frozen semen and not cultured. Further studies are needed to determine the possibility and success rate of obtaining live animals using cells obtained from semen.

CHAPTER V

CONCLUSIONS

In the present study, somatic cells were successfully cultured from 11 of the 14 ovine ejaculates and 7 of the 12 bovine ejaculates. These cells had classic cobblestone morphology and expressed cytokeratin indicating they are of epithelial origin. Chromosome numbers of the cells cultured from one of the four rams were near-triploid, illustrating the need for chromosome analysis of cells if the objective is to use them for cloning experiments.

An effective system for the culture and proliferation of epithelial cells obtained from fresh ovine semen samples was described. However, the system was not adequate for obtaining cell growth from frozen semen. Further studies are needed to find out the possibility of obtaining cell growth from frozen semen. A more advanced culture system may need to be developed.

Blastocyst stage embryos were cloned using epithelial cells cultured from semen of three different bulls. Further studies are needed to determine the possibility and success rate of obtaining live animals. No compact morulae or blastocysts were obtained when somatic cells isolated from frozen semen but not cultured were used as donor cells. Evaluation of embryos indicated problem with membrane breakdown in cells from frozen semen, as such may have inhibited exposure of nucleus to cytoplasm needed for reprogramming. Future experiments should test methods to break membranes of these cells prior to injection.

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