OCT-4 EXPRESSION IN EQUINE EMBRYONIC CELLS

A Thesis

by

HEATHER DARBY HARDING

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

MASTER OF SCIENCE

December 2005

Major Subject: Veterinary Physiology

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Approved by:

Chair of Committee,	Katrin Hinrichs
Committee Members,	Young-Ho Choi
	Mark Westhusin
	Bert Binas
Head of Department,	Glen Laine

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ABSTRACT

Oct-4 Expression in Equine Embryonic Cells. (December 2005)

Heather Darby Harding, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Katrin Hinrichs

The Oct-4 transcription factor is believed to co-regulate early embryonic development of mammals due to the correlation of its presence with the maintenance of pluripotency. It is commonly used as a marker for the identification of embryonic stem (ES) cells for this reason. Until 1999, Oct-4 studies were limited to in vivo-produced embryos; equine embryos have not been studied for their Oct-4 expression patterns. In addition, equine stem-like cells (defined by marker expression, induced differentiation, passage survival, and morphology) have recently been isolated from in vivo-produced embryos, but no work has been performed in horses to isolate ES cells from in vitro-produced embryos.

This study investigated the expression of Oct-4 transcription factor using immunocytochemistry in 42 in vitro-produced embryos aged 1-10 days and in 5 in vivo-produced blastocysts aged 7-10 days. Effective conditions for rapid establishment of a feeder layer of equine fetal fibroblasts were established, and this feeder layer was used to grow isolated equine inner cell mass (ICM) cells from in vitro-produced embryos. The expression of Oct-4 was examined in resultant cell growths.

In vitro-produced embryos less than 6 days of age showed variable staining within blastomeres of the same embryo, and the peak of variability correlated with

maternal-zygotic transition. After Oct-4 staining of in vitro-produced blastocysts, no cells could be identified as an ICM based on a difference in fluorescent intensity from the other cells of the blasyocysts. However, in vitro-produced blastocysts that were subsequently cultured in vivo contained a presumptive ICM, visible based on greater fluorescent intensity of Oct-4 stain. The trophoblast of all blastocysts also stained positively for Oct-4 protein. Fibroblasts were successfully isolated from equine feti. Treatment with 20 µg/ml of Mitomycin C arrested cell growth without causing excessive death. Fibroblasts were inactivated and frozen, then thawed as needed to establish a confluent monolayer for ICM isolation overnight. ICMs from in vitro-produced embryos formed outgrowths, but none that could be identified morphologically as ES cells. Outgrowth cells contained about 20% Oct-4 expressing cells in sporadic groupings. Assuming appropriate binding of the Oct-4 antibody, Oct-4 expressing cells (potentially indicating pluripotency) are found throughout the embryo in early development and in the feeder layer after co-culture.

This thesis is dedicated to my husband, who was both my inspiration to begin on this journey and my strength throughout.

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CHAPTER I

INTRODUCTION

POU transcription factors are defined by their region of homology, originally found in mammalian transcription factors Pit-1, Oct-1, Oct-2, and nematode regulatory protein Unc-86 [1]. The POU domain of these factors and of more recently identified members of the POU family binds a specific octamer motif (ATGCAAAT) in the promoter or enhancer regions and regulates target gene expression [2]. This DNA sequence is identical among genes, whether they are expressed ubiquitously throughout the organism or in a tissue-specific manner, and regulation is managed by the presence of different POU transcription factors in different cell types for tissue-specific expression [3]. Oct-4 (also called Oct-3, Oct-3/4, POU5F1, OTF3, and NF-A3 depending on species) is a POU transcription factor involved in regulating early embryonic development and cell differentiation, and was first investigated in the mouse [4].

The POU region of these transcription factors contains a conserved POU-specific domain and a conserved homeodomain separated by a variable region; the POU-specific domain is further divided into 2 distinct subdomains with high homology [5]. Comparison of these POU-specific subdomains and the homeodomains allows classification of POU family members into 5 distinct classes, with Oct-4 representing the fifth and most recently identified class [5-7]. POU proteins differ in the contribution of

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This thesis follows the style and format of Biology of Reproduction.

their specific domains to the binding of the octamer motif, but the homeodomain is necessary for DNA binding in all transcription factors of this family [5]. There is no evidence to suggest that the POU-specific domain can bind the octamer motif without the presence of the homeodomain.

Oct-4 has been studied extensively in embryonic pluripotency research, including spatial and temporal characterization of Oct-4 in embryos and identification of embryonic stem and stem-like cells [8, 9]. Oct-4 protein has not been studied in horses, and has been used in only 1 published instance (in the form of RT-PCR) as a marker for the pluripotency of equine ES-like cells [10]. The Oct-4 transcription factor has been studied extensively, however, in early embryos of mice [9, 11-16], swine [16], cattle [16-19], rats [20], rhesus monkeys [15], and humans [21]. There are a variety of expression patterns evident in the early embryos of these species, and patterns seem to be fairly species-specific. Rodent and rhesus monkey in vivo-produced embryos express Oct-4 protein at later stages of early development (beginning at the 5-cell and 16-cell stages, respectively), and it is isolated to the ICM at the hatched blastocyst stage. At this stage, mRNA expression patterns are identical to protein expression patterns. In cattle (in vitro-produced embryos) and swine (in vivo-produced embryos), Oct-4 protein is present in the embryo throughout early development, and is found in both the trophoblast and ICM of blastocysts. In cattle, Oct-4 mRNA expression is like that of rodents and rhesus monkeys and is only in the ICM of the expanded blastocyst. Finally, in in vitro-produced human embryos the pattern is far more complicated. Human preimplantation embryos express Oct-4 protein in only some of their cells between the 5

cell and compact morula stages, at varying proportions. For example, 5-celled embryos express Oct-4 in 40% of their cells, whereas 9 cell embryos express Oct-4 in only 11.1% and 10 cell embryos in 80% of their cells [21]. At the blastocyst stage of human in vitro-produced embryos, Oct-4 mRNA (based on PCR analysis) and protein are found in both the ICM and the trophoblast. This wide variability, both among species and within single embryos, raises questions as to the true role of Oct-4 in maintaining or signaling pluripotency in these species.

Pluripotency is a trait found in a number of cell types, but the focus for this research is on stem cells derived from embryos. These cells have the potential to generate any cell type, making them a valuable asset to the medical and research communities. The most immediate applications for stem cells today are gene targeting therapy and regenerative medicine (e.g. diabetes treatment) [22-24]. Only twice have ES cells been isolated from equine embryos, and in both cases this was from in vivo-recovered blastocysts [10, 25]. Recovery of ES cells from any in vitro-produced embryo is a rarity, including those resulting from intracytoplasmic sperm injection (ICSI) and in vitro fertlization. In the field of ES cell culture, only 3 studies have successfully recovered ES cells from a nuclear transfer embryo [26-28]. The implications of culturing stem cells from a cloned blastocyst far outstretch those of a natural embryo. To take even the first step towards this—the isolation of ES-like cells from in vitro produced embryos—would be a giant leap for equine science. The most vivid application of this science is in regenerative medicine in athletes. Cartilage and ligament damage in equine athletes is not only a career-limiting condition, but also serves as an

excellent model for such damage in human athletes. Use of stem cells recovered from cloned embryos could theoretically serve to produce cartilage and ligament precursors which when implanted could resolve damage that currently does not respond to treatment. Other possible uses of stem cell production in horses includes gene targeting for research and even re-creation of gametes for the recovery of genetics of valuable animals. Beyond the clinical application of this field, study of the species through this advancing science could clarify our understanding of an unlimited number of physiological process in large animals, using the horse as a model.

ES cells are typically derived in culture by isolating the ICM of a blastocyst-stage embryo and culturing this mass in an environment supportive of stem cell growth. The cells of the ICM are pluripotent, and in appropriate culture conditions they will grow and proliferate continuously. The ultimate proof that a cell is an ES cell involves transferring it to a developing embryo, and demonstrating that the cells are present in a variety of tissues, including the germ line. Cells that appear to be ES cells (e.g. in morphology and protein expression) but that have not been shown definitively to be germ-line transmitted are typically referred to as “ES-like” cells. Successful transfer and germ line transmission has only been applied in the mouse [29], and therefore “ES” cells obtained from any other species are in fact “ES-like” cells. Much is still unknown about why ES cells behave as they do, and what causes these cells to differentiate into a given cell type.

A major focus of modern research is on the differentiation of stem cells into a desired cell type. Left in culture without appropriately-conditioned medium, ES cells

will differentiate into spherical masses of cells known as embryoid bodies (EB), which then produce further outgrowths when attached to a plate surface. Germ cells, osteocytes, cardiomyocytes, and hepatocytes are some of the multitude of cell types that have been derived in vitro from embryoid bodies [30-33]. Consistent advances are being made in finding new agents that induce ES cells to differentiate into cells of interest. Eventually this technology will be put to use to replace damaged organs, or even to fix fertility problems or recover the genetics of a valuable deceased animal.

The first instance of pluripotent cell isolation from an embryo of any species was in 1981, when murine ICM cells cultured in conditioned medium developed into the first ES-like cells in culture [34]. Since then, ES-like cells have been isolated from hamsters [35], pigs and sheep [36], mink [37], cattle [38-40], rabbits [41], rats [42], humans [43], monkeys [44], and most recently horses [10, 25]. Cells were characterized as embryonic stem-like cells by their morphology, undifferentiated culture through serial passages, differentiation into tumors when injected into living creatures or embryoid bodies when growth factors were withheld, induced differentiation into a number of precursor cell types, and in later studies by identified pluripotency markers. Common markers used for identification of stem cells are now sold in kits specific for a given species. For example, 1 commercial mouse and human ES-like cell identification kit contains reagents to identify the presence of Alkaline Phosphatase, cell-surface stage specific antigens SSEA-1 and SSEA-4, and antigens TRA-1-60 and TRA-1-81 (Chemicon International, Temecula, CA). Other common markers used are SSEA-3, ALDH, and of

course, Oct-4. Echoing the expression patterns in embryos, Oct-4 is lost from ES cells when they differentiate [10, 15, 20, 45].

With the exception of the mouse stem-like cells (which used media previously conditioned by other cells), the above cells were all grown on inactivated feeder layers of rat, murine, human or bovine fibroblasts. A common tool in cell culture, feeder layers provide appropriate conditions for difficult-to-grow cells. Occasionally ES cells are grown in specially conditioned medium without a feeder layer, but these occasions are becoming rare since the commercialization of feeder layer products [34, 46-49]. While fibroblasts perform many functions when used as feeder cells, the most important of these are support in cell-to-cell and cell-to-extracellular matrix interactions (attachment of the ES cells to the plate), the production of necessary growth factors and the removal of toxins from the culture medium. Feeder layers allow culture, with minimal effort, of cells that are normally very difficult to grow in vitro. To date, there have been 2 reports (1 full paper and 1 brief report) in which equine ES-like cells have been established in culture, but only 1 of these studies used equine cells to produce the feeder layer, and no information was given on the method of production of those feeder cells [10, 25].

To create a functional and supportive feeder layer, the feeder cells must be prevented from overtaking the slower-growing ES-like cells. Therefore, the feeder cells must be stopped from growing before they can be used in co-culture, but must still be viable to serve their function. The 2 most common methods for inactivation of feeder cells are irradiation and administration of a sub-lethal dose of Mitomycin C (MMC) [50]. Irradiation was used frequently in older studies, but is time consuming and

expensive. Mitomycin C is a more efficient and cost-effective alternative and is readily available and easy to use. A cytotoxin still commonly used in cancer therapy, MMC inserts between the strands of DNA and prevents DNA replication, thus preventing cell division. Once MMC inserts into the genetic material the cell has a finite lifetime, between 1 and 3 weeks. Therefore, the feeder layer for support of ES cells must be replaced periodically during ES cell passaging to provide the best environment to support growth.

The specific aims of this project were:

1. To characterize the expression patterns of Oct-4 protein in *in vitro*-produced equine embryos during early development. In vitro-produced embryos were studied from Day 1 to Day 10 of development, and in vivo-produced embryos (for use as a control for blastocyst development) were studied from Days 7 through 10.

2. To determine the appropriate concentration of MMC for production of a viable inactivated feeder layer from equine fetal fibroblasts. The number of cells present at confluency in 1 well of a 4-well dish, effectiveness of quantification and cell recovery methods, effects of passaging on cell survival, optimal dosage determination of Mitomycin C, and effects of freezing on cell survival were examined to optimize feeder layer production.

3. To evaluate the development of equine ICM cells cultured on feeder layers to determine their capability to produce stem-like cells. These cells were then examined for the expression of Oct-4 protein to further identify ES-like characteristics.

CHAPTER II

EMBRYONIC OCT-4 EXPRESSION

MATERIALS AND METHODS

Approach

Equine embryos were produced by in vitro oocyte maturation, ICSI, and in vitro embryo culture. In vitro-produced embryos at different early stages of development were immuno-stained for Oct-4 expression, and in vivo-recovered blastocysts were stained as well. Oct-4 expression patterns according to day of culture and number of healthy embryonic nuclei were determined. Finally, the results of the immunocytochemistry were validated with a Western blot using murine stem cells to confirm binding ability of the primary antibody.

Embryo origins

Three sources of embryos were used for this phase of study: in vitro-produced embryos, in vivo-recovered embryos, and embryos from a combination of these sources (produced in vitro, transferred to the uterus of a recipient mare, and recovered 2- 3 days later for evaluation).

To produce embryos in vitro, equine ovaries were first recovered from an abattoir and transported at room temperature to the laboratory a maximum of 7 hours post-mortem. Ovaries were trimmed of excess tissue with scissors and scalpel blades were then used to open visible follicles. The granulosa layer of each follicle was scraped using a 0.5 cm bone curette and washed into individual Petri dishes with Hepes-buffered TCM-199 with Hank's salts (Gibco Life Technologies Inc, Grand Island, NY) plus

ticarcillin (0.1 mg/ml; SmithKline Beecham Pharmaceuticals, Philadelphia, PA). After all visible surface follicles were opened the ovaries were cut into 5-mm sections to locate follicles within the ovarian stroma. Petri dish contents were examined at 10-20X magnification under a dissection microscope. Depending on the expansion of cumulus and mural granulosa, cumulus-oocyte complexes located in the dishes were graded as compact or expanded as previously described [51, 52]. Any sign of expansion in the cumulus or mural granulosa caused the related oocyte to be classified as expanded.

Recovered oocytes were matured in TCM-199 with Earle's salts (Gibco) plus 10% fetal bovine serum (FBS, Gibco) and 5 mU/ml follicle stimulating hormone (Sioux Biochemicals, Sioux Center, IA) in droplets at a ratio of 10 µl medium/oocyte under light white mineral oil (Sigma-Aldrich Corp., St. Louis, MO) at 38.2ºC in 5% CO_2 in air. Oocytes were cultured for maturation for 24 hours if recovered with expanded cumulus morphology or 30 hours if recovered with compact cumulus morphology. Oocytes were then denuded of cumulus by pipetting in 0.05% hyaluronidase (Sigma) in CZB-M [53] as previously described [53], and those having a polar body after culture were used for ICSI, performed by Dr. Young-Ho Choi as previously described [53]. Briefly, the denuded, matured oocytes were held in CZB-H [53] prior to ICSI. Sperm were prepared by swim-up in Sp-CZB [53] and injected into the oocyte in CZB-M. Injected oocytes were held in CZB-H containing 10% FBS in 5% CO_2 in air at 38.2 ºC until all manipulations were done. After ICSI, oocytes were cultured in cell culture medium (CCM; Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma) with 10% FBS and 0.5 µg/ml Gentamycin (Sigma)) at 38.2°C in an atmosphere

of 5% CO_2, 5% O_2 and 90% N_2. Medium was completely replaced every 2-3 days. One to 10 oocytes were cultured per drop, and the maximum volume of each droplet was 50 µl.

To evaluate expanded blastocysts, presumptive embryos were cultured for a total of 7-8 days, then the zona pellucida was removed by treatment with acidic Tyrode's solution (Sigma), and the embryos were cultured for additional 2 or 3 days to allow blastocoel expansion.

Embryos were also recovered in vivo. This began with the monitoring of follicular development via palpation and ultrasonography per rectum (performed by Dr. Katrin Hinrichs or Mr. Lance Roasa). When the follicle was >30 mm diameter, mares were artificially inseminated and a dose of Deslorelin (1.5 mg BETpharm, Lexington, KY) given intramuscularly. Mares continued to be monitored to detect the day of ovulation. Seven to 10 days after ovulation, embryos were recovered from the mares by uterine flush as previously described [54].

Finally, embryos resulting from a combination of these procedures were obtained. Embryos were produced in vitro as described above, and follicular growth in recipient mares was monitored and synchronized so that embryos could be transferred to mares that ovulated from the day that ICSI was performed to 3 days afterward. After 7 days of culture in vitro, the in vitro-produced embryos were transferred transcervically to the uteri of synchronized mares then recovered by uterine flush 2-3 days later. Since these embryos developed to the blastocyst stage in vivo, shedding their zonae pellucidae and expanding with formation of an embryonic capsule, they allowed for the study of in

vivo-like embryos while circumventing the difficulty in recovering large numbers of this type of embryo (which is due to the lack of sufficient superovulation technology in the mare).

The numbers of embryos subjected to immunocytochemistry from each of these origins are listed in Table 1.

TABLE 1. Origins of embryos subjected to immunocytochemistry.

Embryo Origin	Number of Embryos Stained
In vitro-produced zygotes[a]	5
Early in vitro-produced ICSI embryos[b]	≥10/culture day
Zona-intact, in vitro-produced ICSI blastocysts[c]	15
Zona-removed, in vitro-produced ICSI blastocysts[d]	5
In vivo-recovered blastocysts[c]	5
In vitro-/in vivo-cultured ICSI blastocysts[e]	11

[a] Day 1
[b] Days 2-6
[c] Days 7-10
[d] Day 9
[e] Transferred at Day 7, recovered after 2 to 3 days in vivo

Immunocytochemistry of early embryos

All embryos described above were subjected to immunocytochemistry. Washing with CCM occurred between each step. Unless otherwise noted, all dilutions were made in phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA). Embryos were first fixed for 20 minutes at room temperature in 4% paraformaldehyde (Sigma) and then permeabilized in 0.2% Triton X-100 (Sigma) and 0.1% Tween-20 (Research Organics, Cleveland, OH) for 20 minutes at room temperature. Non-specific reactions were blocked by 20 minute incubation at room temperature in 10% normal goat serum

(Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Antibodies (1:200) were then administered sequentially at room temperature for 40 minutes each, with extensive washing between antibody treatments. The primary antibody was a mouse monoclonal antibody against amino acids 1-134 of human Oct-4 (Santa Cruz Biotechnology, Santa Cruz, CA), and the secondary antibody was goat anti-mouse antibody conjugated with indocarbocyanine (Cy3; Jackson ImmunoResearch). Ten minute room-temperature incubation with Hoechst 33258 (Sigma) at a concentration of 10 µg/ml was used to counterstain nuclear material. Control reactions for non-specific binding of the secondary antibody were carried out by omitting treatment with the primary antibody. After final washing, embryos were immediately mounted in glycerol (Sigma) under glass coverslips. After examination, samples were stored at -20ºC in the dark.

All embryos were evaluated under fluorescence microscopy. The nucleus number for each embryo as determined by Hoechst staining was recorded. Only normal nuclei were counted; those showing pyknosis or degeneration were disregarded as they do not contribute to the survival of healthy embryos. The nucleus number recorded for embryos at each day of culture was evaluated to establish a normal nucleus number range for each age of embryo (Days 1-6). This was done on the basis that, on average, 30% of embryos develop to the blastocyst stage. During embryo culture, obviously degenerating or retarded embryos are removed on Day 3 and Day 5. Thus, it was estimated that conservatively 50% of equine embryos in culture at a given age are healthy. The top 50 percentile of embryos based on nucleus counts for each age

provided a guideline for a normal nucleus number for each embryo age. Only embryos showing normal development for their age were then evaluated for the number and percentage of nuclei staining for Oct-4 protein. The intensity of Oct-4 staining (on a 0-5 scale, with 0 indicating no fluorescence and 5 the brightest fluorescence seen) and the proportion of total Oct-4 staining nuclei to the total nucleus number were visually determined for embryos of each age.

The proportion of nuclei expressing Oct-4 protein at each embryo stage was found using the equation

[1] $P_x = n_{Oct\text{-}4}/n_{totx}$, x = 1, 2, 3, 4, 5, or 6 days,

where P_x is the proportion of nuclei with any visible Oct-4 staining for age x, $n_{Oct\text{-}4}$ is the total number of nuclei found to be expressing Oct-4 protein in normal embryos, and n_{totx} is the total number of nuclei for age x. The proportion of embryonic nuclei showing Oct-4 staining was compared among embryo ages using analysis of variance, to determine if Oct-4 staining patterns change with embryo age.

Immunocytochemistry of blastocysts

Oct-4 expression patterns in in vitro-produced blastocysts were compared with those for in vivo-recovered blastocysts. Blastocysts were stained for Oct-4 and counterstained with Hoechst 33258, then in vitro-produced and in vivo-recovered blastocysts were immediately mounted in glycerol under coverslips for viewing and in vitro-produced/in vivo-cultured blastocysts were first viewed in 10 µl droplets of glycerol and then mounted. Blastocysts were evaluated under fluorescence microscopy as described above. In addition, the presence of any ICM-like mass based on difference

in fluorescent intensity was noted. Twenty ICSI-produced blastocysts were stained for Oct-4; 1 of these blastocysts was sacrificed as a control for non-specific binding of the secondary antibody. This blastocyst was given the normal staining treatment as described above but the primary antibody step was omitted.

Western blot

Two Western blots were performed, each using 4 in vitro-produced equine embryo equivalents (from a combined stock of 16 equine embryos), to attempt to verify the presence of Oct-4 in equine blastocysts and to validate the specificity of the primary antibody for equine Oct-4 protein. Fetal fibroblasts do not express Oct-4, and both bovine and equine fetal fibroblasts were used as negative controls in the blotting. Positive controls used were 1 µg of a protein fragment corresponding to amino acids 1-134 of the human Oct-4 protein (Santa Cruz) and 10 bovine in vitro-produced embryo equivalents (from a stock of 100 bovine embryos, provided by Dr. Chuck Long). Bovine embryo equivalents also provided a control for Oct-4 content per equine embryo. Embryos or cells were transferred to lysis buffer at a ratio of 1 unit of cell suspension: 2 units of lysis buffer (10mM HEPES, 2 mM $MgCl_2$-$6H_20$, 6 mM β-Mercaptoethanol (Sigma), pH 7.0) and either stored at -20ºC or used immediately. The lysed cell suspension was then diluted with 2 volumes of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 5% β-Mercaptoethanol and boiled for 5 minutes. The solution was centrifuged 30 minutes at 14000 RPM to remove cellular debris and nuclear material. After centrifugation, the supernatant was transferred to a fresh microcentrifuge tube and loaded into the gel immediately or stored at -20ºC. SDS-

PAGE was run at 200V for 35 minutes on 1.0-mm gels with 4% resolving gels and 8% stacking gels under standard conditions using a Mini-PROTEAN 3 Cell (Bio-Rad). All buffers and reagents were supplied by Bio-Rad or Sigma and were prepared according to Bio-Rad protocol. The separated proteins were transferred by electrophoresis in semi-dry transfers to polyvinylidiene difluoride membranes (PVDF, Immobilon-P, Sigma) with a Trans-Blot SD (Bio-Rad) at 15V for 30 minutes in blot transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol), according to manufacturer recommendations. PVDF membranes were washed in Tween-Tris Buffered Saline (TTBS: 0.05% Tween-20, 25 mM Tris, 140 mM NaCl, 3 mM KCl, pH 8.0). Membranes then underwent treatment for 1 hour with 1% normal goat serum and 0.5% skim milk in TTBS to block non-specific reactions. After 3 washes with TTBS, the antibodies were administered at room temperature for 2 hours each at dilutions of 1:1000 and 1:20000 respectively, with 3 30 minute washes of TTBS between antibodies. The primary antibody was that described above, and the secondary antibody was a goat anti-mouse antibody conjugated with Peroxidase (Chemicon, Temecula, CA). After another 3 washes as described above to remove excess secondary antibody, the membranes were incubated with approximately 5 ml of SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) for 3–5 min according to the manufacturer's protocol. The results were photographed on an Alpha-Innotech (IS-500) system. BlueRanger Prestained Molecular Weight Marker (Pierce) provided a reference to determine the weight of the protein to which the antibodies bound; data in other species indicates Oct-4 is a 40-50 kD protein.

Four additional Western blots were performed by Dr. Young-Ho Choi, following the above procedure with the following exceptions: murine stem cells (provided by Dr. Bert Binas) were also used as positive controls and a fresh commercial positive control (same catalog number, but different batch; 20 ng) was used. Instead of bovine or equine fibroblasts, murine fibroblasts (also provided by Dr. Bert Binas) were used as a negative control. These blots were performed with 20 bovine blastocysts and 7 Day 10 equine embryos (subjected to zona removal on Day 7, then 3 additional days of culture). Additional in vitro-produced equine embryos are still being collected by Dr. Choi to continue this work with higher embryo concentrations per blot.

RESULTS

Early embryos

The nucleus number of equine ICSI embryos at Days 1 through 6 of culture and the minimum nucleus number used for selecting embryos for evaluation of Oct-4 staining are presented in Table 2. Embryos meeting the top 50 percentile selection criteria were analyzed for expression of Oct-4. Embryos of the same age showed Oct-4 completely localized to the nucleus, present throughout the cytoplasm of the cells, or distributed at some point in between. In addition, some embryos did not have any Oct-4 present in certain nuclei but stained vividly for Oct-4 in other nuclei. Examples of all expression patterns found can be seen in Figure 1.

Despite the apparent variable diffusion of the protein within the embryo, the presence or absence of Oct-4 in the nuclei of the embryos showed a trend over the early days of development. The percentage of nuclei expressing Oct-4 dipped from 83.3% on

Day 1 to 75.8% on Day 3 and then steadily rose to 100% expression in Day 6 embryos. The proportion of nuclei expressing Oct-4 was significantly higher in Day 6 embryos than in Day 3 embryos (LSD, p=0.032). The number and percentage of nuclei staining for Oct-4 for each embryo age is presented in Table 3.

TABLE 2. Nucleus numbers of early embryos as seen with Hoechst staining and number selected as basis for evaluating Oct-4 staining.

Embryo Age (Days)	Nucleus Numbers of Stained Embryos	Selected Nucleus Number
1	1[a], 2, 2, 3, 4	≥2
2	0, 0, 2, 3, 3, 4, 5, 5, 6, 7	≥4
3	0, 0, 0, 1, 3, 3, 4, 5, 6, 6, 6, 6, 8, 8, 9	≥6
4	0, 1, 5, 5, 7, 7, 8, 10, 11, 11, 13, 17, 18, 38	≥10
5	0, 2, 2, 4, 8, 8, 15, 17, 22, 32, 51	≥15
6	0, 0, 1, 2, 13, 17, 57, 63, 55, 83, 90	≥57

Immunocytochemistry was performed on embryos at Days 1-6 and the number of non-degenerating nuclei was recorded for each embryo. The top 50% of embryos for each age were then selected for further evaluation.

[a] Embryo had 1 intact nucleus and 1 in metaphase, but was considered healthy

FIGURE 1. Oct-4 expression patterns in early equine embryos. Equine embryos produced by ICSI were fixed on Days 1-6 of culture and double labeled with Hoechst 33258 (blue, left) for DNA and Oct-4 (red, right). A and A1) Day 1 embryo with diffuse Oct-4 staining. B and B1) Day 1 embryo with specific nuclei staining. C and C1) Day 2 embryo with diffuse Oct-4 staining and identifiable nuclei. D and D1) Day 2 embryo with specific nuclei staining. E-F1) Day 3 embryos with specific nuclei staining. G and G1) Day 4 embryo with diffuse Oct-4 staining and identifiable nuclei. H and H1) Day 4 embryo with specific nuclei staining. I-J1) Day 4 and Day 5 embryos with specific nuclei staining and non-Oct-4 staining nuclei. K-L1) Day 5 and Day 6 embryos with diffuse Oct-4 staining and identifiable nuclei.

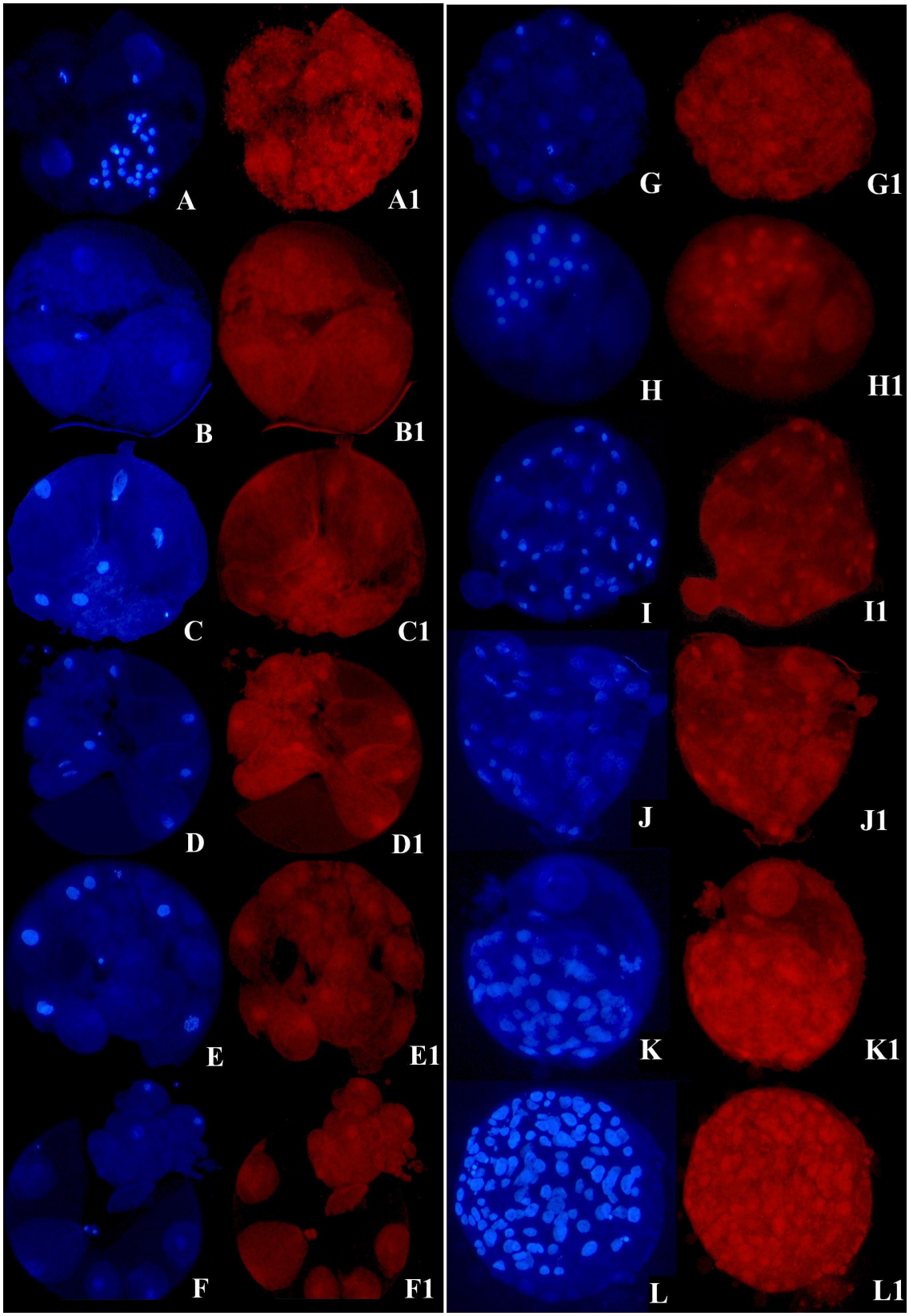
A
A1
B
B1
C
C1
D
D1
E
E1
F
F1
G
G1
H
H1
I
I1
J
J1
K
K1
L
L1

TABLE 3. Oct-4 expression patterns of healthy early embryos.

Embryo Age (Days)	Cell Number	Number of Oct-4 Staining Cells	Percentage of Oct-4 Staining Cells	Intensity of Oct-4 Staining[b]
1	1[a]	0	0	0
	2	2	100	3
	2	2	100	4
	3	2	67	4
	4	4	100	3
Average	**2.4**	**2**	**83.3**	**3.8**
2	4	4	100	1
	5	5	100	3
	5	2	40	4
	6	4	67	3
	7	7	100	3
Average	**5**	**4.4**	**81.5**	**2.8**
3	6	4	67	3
	6	6	100	3
	6	4	67	4
	6	4	67	4
	9	7	78	2
Average	**7[c]**	**5[c]**	**75.8[c,d]**	**3.2[c]**
4	10	10	100	4
	11	11	100	1
	11	11	100	2
	13	10	77	4
	17	17	100	3
	18	18	100	1
	38	22	58	2
Average	**17**	**14.1**	**83.9**	**2**
5	15	15	100	3
	17	17	100	3
	22	22	100	3
	32	21	66	4
	51	51	100	4
Average	**27**	**25.2**	**92.0**	**3.4**
6	57	57	100	2
	63	63	100	3
	66	66	100	2
	83	83	100	1
	90	90	100	4
Average	**72**	**71.8**	**100[d]**	**2.4**

Embryos were stained for Oct-4 and counterstained with Hoechst, then the number of nuclei found under each stain and the intensity of fluorescence of the Oct-4 stain was evaluated.

[a] Embryo had 1 intact nucleus and 1 in metaphase, but was considered healthy

[b] 0 = no visible fluorescence, 5 = brightest visible fluorescence

[c] 2 8 celled non-fluorescing embryos were removed from this group

[d] Within columns, values differ significantly ($p<0.05$)

Two other phenomena of staining were found to apply to all equine embryos studied. Mitotic figures in embryos did not stain for Oct-4 (Fig. 2). In addition, degenerating nuclei (as determined by presence of pyknosis or karyorrhexis on Hoechst staining) did not show any Oct-4 signal (Fig. 2).

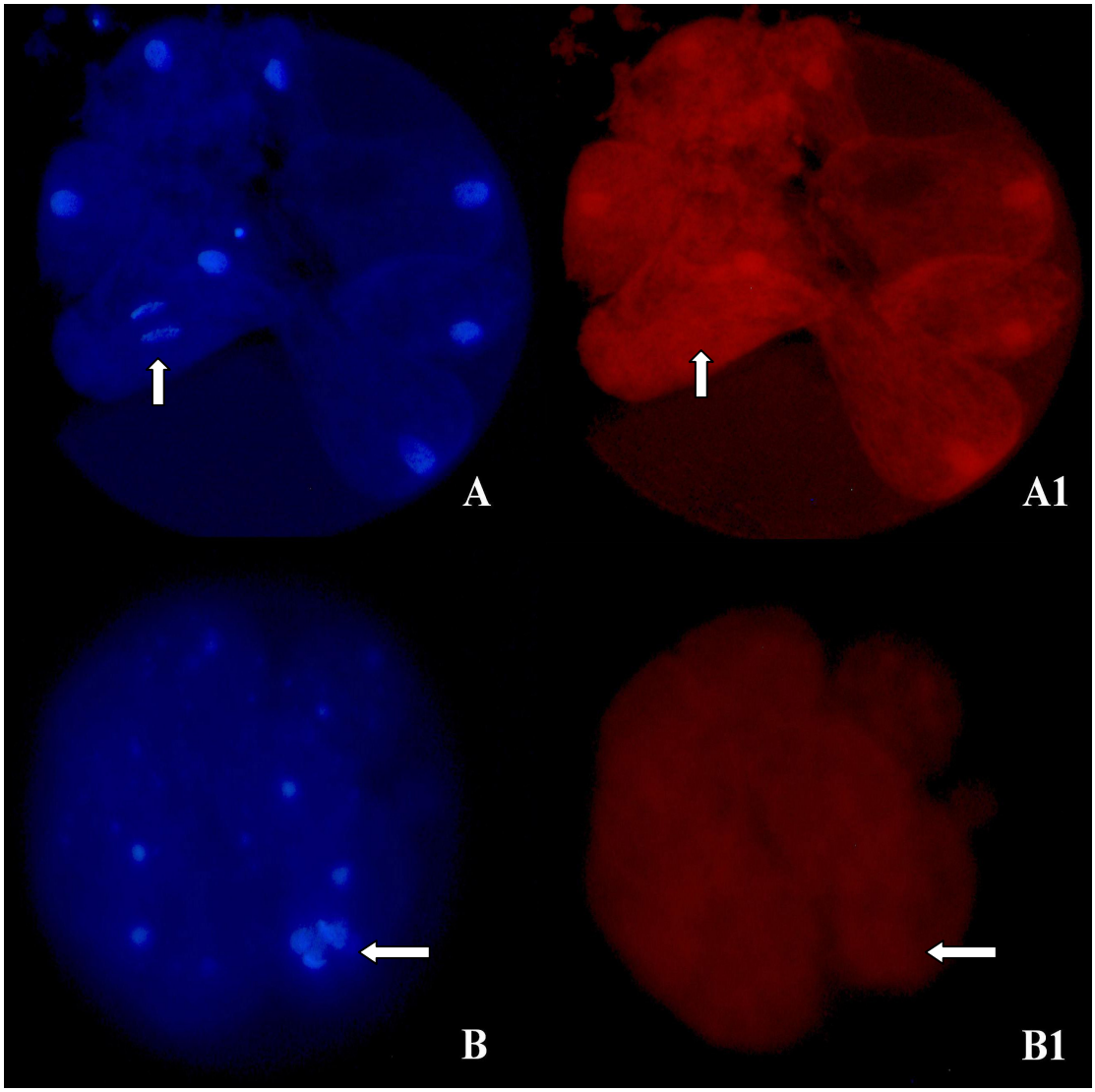

FIGURE 2. Telophase figure and degenerating nucleus staining. Embryos were stained with Hoechst 33258 (blue, left) for DNA and Oct-4 (red, right). A and A1) There is no visible fluorescence in the indicated area of the telophase figure, but the other 7 nuclei in the embryo have visible Oct-4 signal. B and B1) No nuclear material from the degenerating nuclei show any Oct-4 signal.

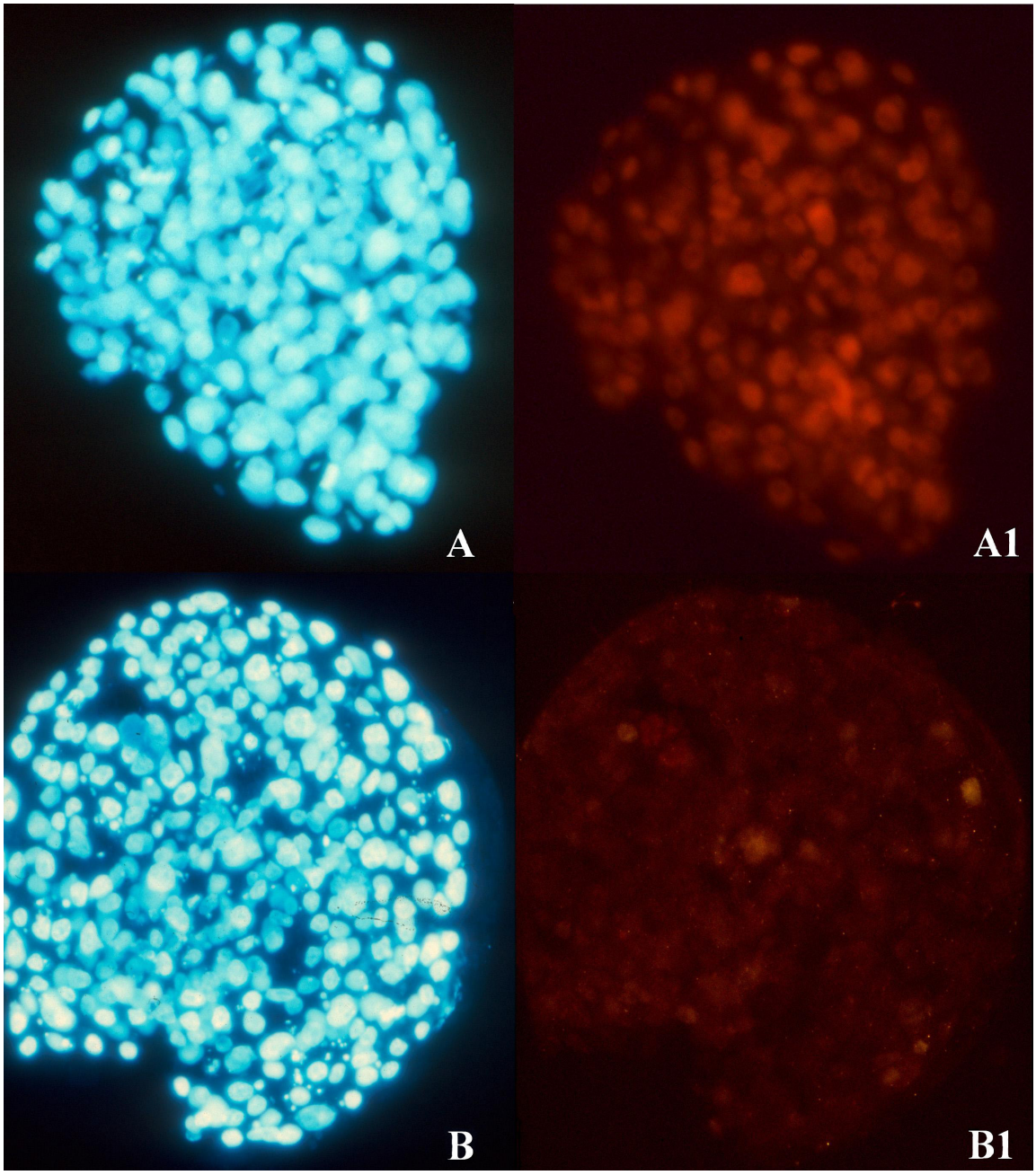

FIGURE 3. In vitro-produced blastocysts with variable Oct-4 expression. Blastocysts were stained with Hoechst 33258 (blue, left) for DNA and Oct-4 (red, right). A and A1) Day 9 ICSI blastocyst with Oct-4 protein localized to nuclei. Zona pellucida was removed at Day 7 to allow expansion. B and B1) Day 8 ICSI blastocyst with diffuse Oct-4 protein throughout.

In vitro-cultured blastocysts

The control blastocyst for secondary antibody nonspecific binding had no visible fluorescence on visualization. Distribution of Oct-4 within the other 19 in vitro-cultured blastocysts was variable: in some blastocysts Oct-4 was localized to the nuclei, and in others it was diffuse throughout the blastocyst (Fig. 3). Once embryos had reached the Day-7 (blastocyst) stage, however, the variable expression of nuclei within a single blastocyst was no longer evident, as all nuclei showed fluorescence. All blastocysts were compressed to the coverslips during mounting, allowing the visualization of a larger proportion of the blastomeres of each blastocyst within a single viewing plane of focus. There was no evidence of differential staining between any of the cells in any in vitro-produced blastocyst.

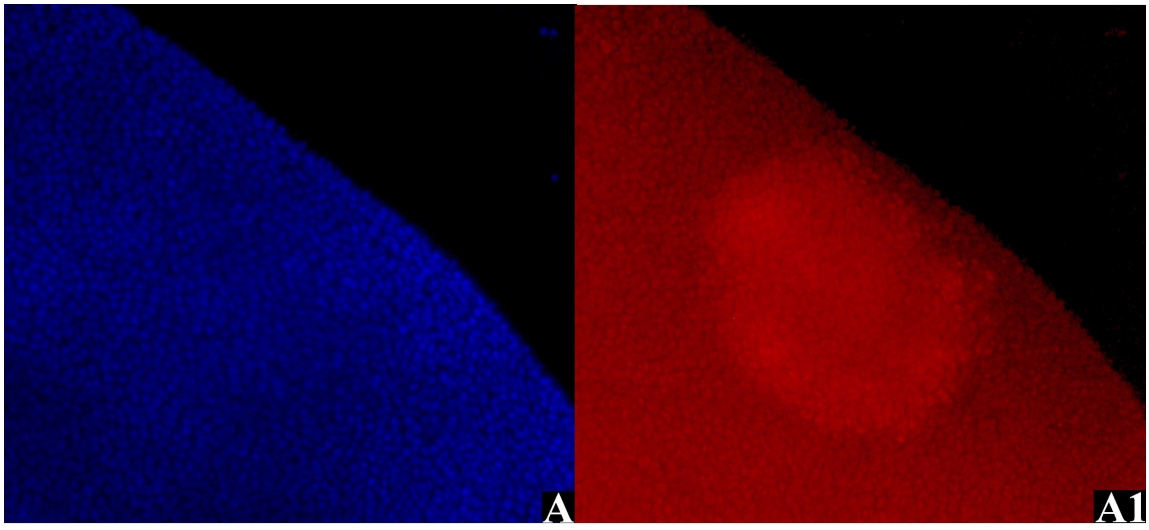

FIGURE 4. Day 8 in vivo-recovered blastocyst with presumptive ICM visible after Oct-4 staining. Blastocyst was stained with Hoechst 33258 (blue, A) for DNA and Oct-4 (red, A1).

In vivo-recovered blastocysts

Of the 5 in vivo blastocysts examined, all had a visible ICM at the time of recovery. All 5 blastocysts were mounted under cover slips before visualization. The presumptive ICM was identified after staining and mounting in 2 blastocysts (aged 8 and 10 days) based on visibly amplified Oct-4 signal in a defined mass of cells in the blastocyst (Fig. 4). In all blastocysts, the Oct-4 signal was localized to the nuclei and was seen in all cells.

In vitro-produced/in vivo-cultured blastocysts

Of the 11 in vitro-produced/in vivo-cultured blastocysts recovered, 7 were evaluated after immunocytochemistry. All of these had identifiable presumptive ICMs as determined by increased intensity of Oct-4 signal in these cells (vs. other cells in the blastocysts) after staining when visualized without compression in a droplet of glycerol. All blastocysts had Oct-4 localized to the nuclei, and the nuclei of cells in the presumptive ICM had a visibly higher intensity signal than those of the rest of the blastocyst (Figs. 5 and 6). When mounted under coverslips, the blastocysts were compressed and some ruptured. In 4 of 7 blastocysts after mounting, the previously visible presumptive ICM was less or no longer identifiable.

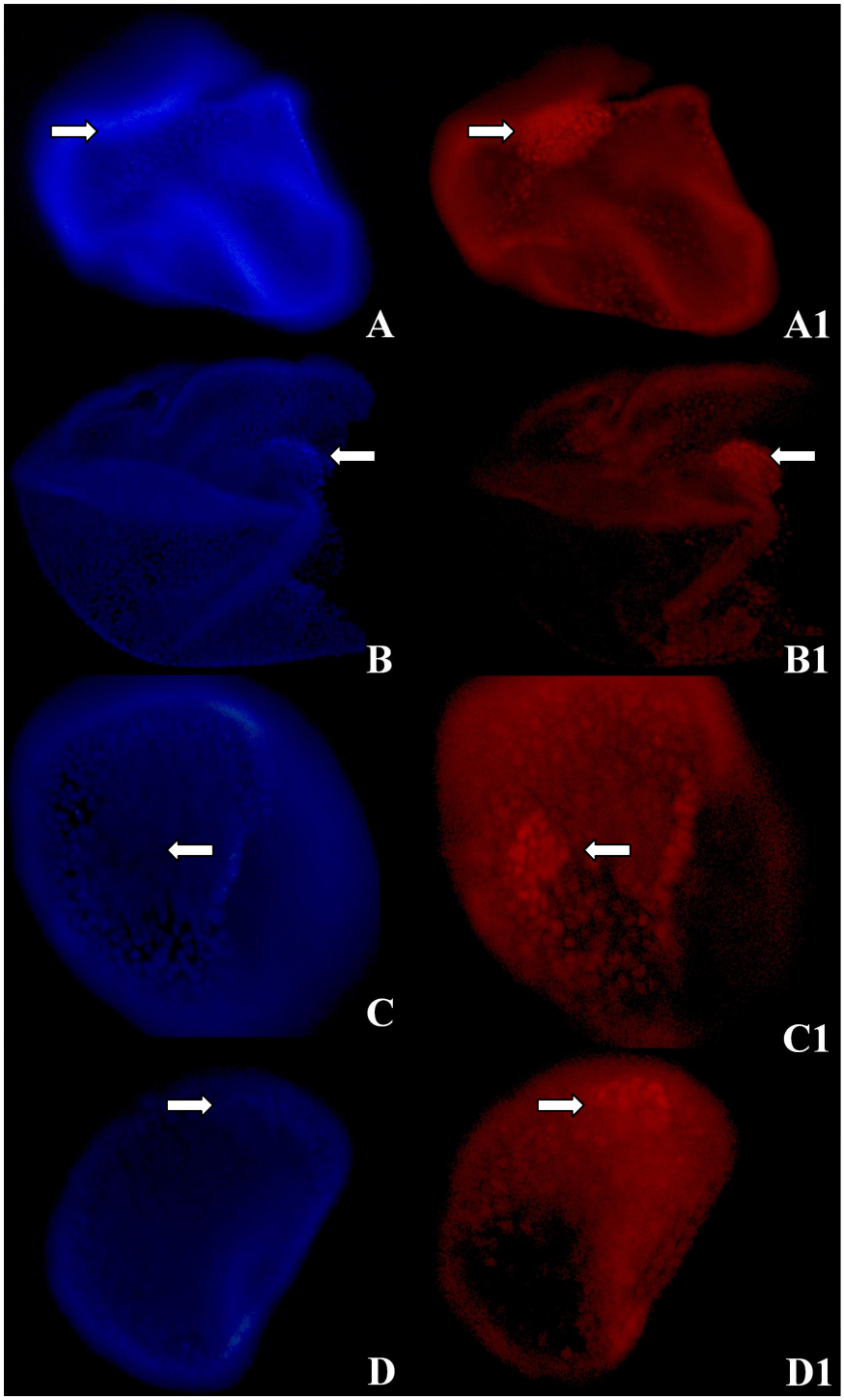

FIGURE 5. Day 9 in vitro-produced/in vivo-cultured blastocysts. Blastocysts were stained with Hoechst 33258 (blue, left) for DNA and Oct-4 (red, right). All blastocysts have presumptive ICMs visible with amplified Oct-4 signal. A-B1) Day 9 ICSI fresh-transferred/recovered blastocysts. Blastocysts collapsed and ruptured during staining. C-D1) Day 9 ICSI vitrified transferred/recovered blastocysts.

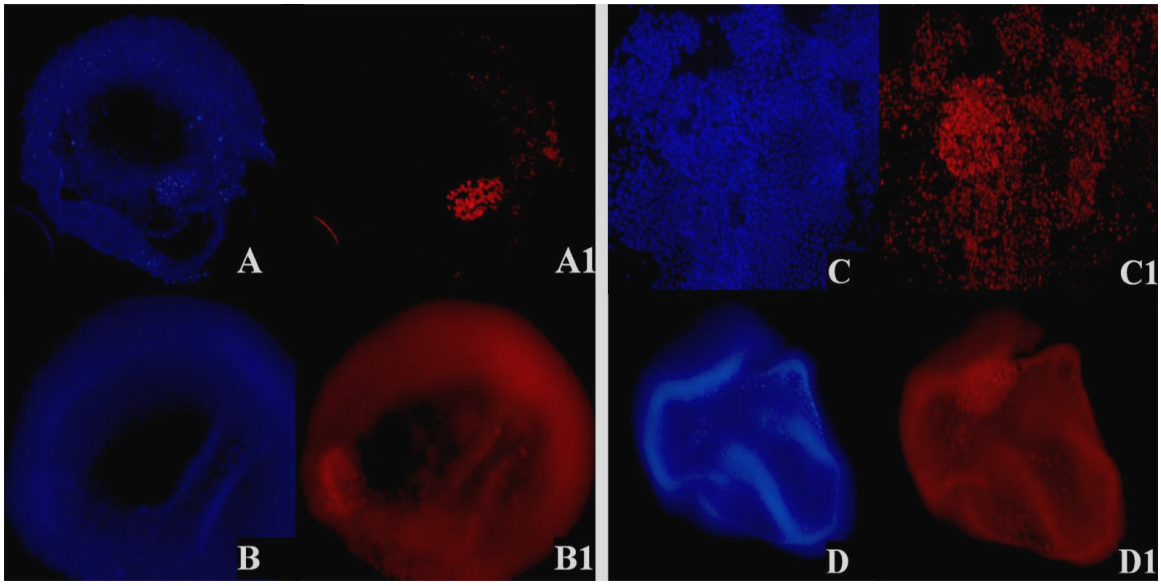

FIGURE 6. Presumptive ICM cells seen before and after mounting. Blastocysts were stained with Hoechst 33258 (blue, left) for DNA and Oct-4 (red, right). In vitro-produced/in vivo-recovered blastocysts mounted under a coverslip (A and A1, C and C1) and free-floating in glycerol (B and B1, D and D1). A-B1) 200x magnification. C-D1) 100x magnification.

Western blot

Western blots using 4 equine embryo equivalents and 10 bovine embryo equivalents did not show any Oct-4 in either lane. In addition, the positive control obtained from Santa Cruz Biotechnology did not perform as expected based on the manufacturer's product information and presented on visualization as a smear with a number of bands rather than a single strong band at 42 kD (Fig. 7).

Further Western blots were performed by Dr. Choi using a fresh positive control. Murine stem cells had a clear band at 42 kD, indicating the presence of Oct-4 in these cells; murine fibroblasts showed no band (Fig. 8). The fresh positive control was visualized as a single thick band at 42 kD, indicating the specificity of the antibody for

Oct-4. The equine and bovine embryos, despite the higher concentrations used, still did not have any visible Oct-4 in the Western blots.

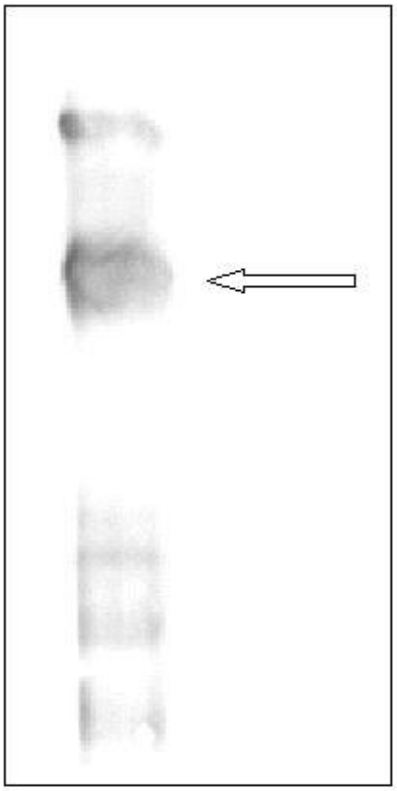

FIGURE 7. Faulty positive control used in initial Western blotting. The thickest band (indicated with the arrow) represents the appropriate molecular weight of 42 kD, but is overshadowed by excessive noise in the lane due to a compromised sample.

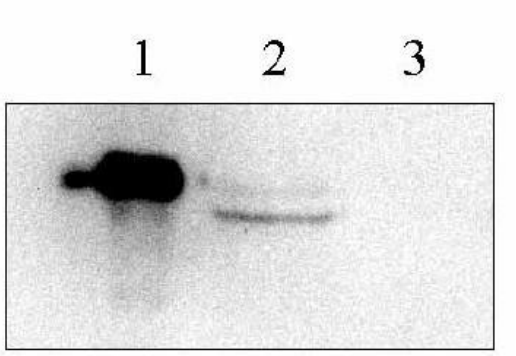

FIGURE 8. Western blot confirming activity of Oct-4 antibody. Blot shows positive Oct-4 control (Lane 1), positive band from murine embryonic stem cells (Lane 2), and negative result with murine fibroblasts (Lane 3).

DISCUSSION

This study reports the expression pattern of Oct-4 in equine embryos from Day 1 after fertilization to the blastocyst stage. Oct-4 was examined in both a temporal and spatial capacity in these embryos. Evaluation was limited to embryos presumed to be growing normally, as defined by the selection criteria.

The results show that Oct-4 is present in early embryos and blastocysts through Day 10 of development. The expression patterns were variable in early embryos but gradually became more regular, with 100% of nuclei in embryos expressing Oct-4 protein at Day 6 onward. This may be related to the maternal-zygotic transition (activation of the embryonic genome), which takes place in equine embryos at the 4-8 cell stage [55]. The steady dip in Oct-4 signal in nuclei over Days 1-3, followed by a rapid recovery, may be related to the exhaustion of maternally generated Oct-4 and then its recovery via expression of zygotic transcripts. It was shown in human in vitro-produced embryos that the presumptive maternal Oct-4 is distributed within the cytoplasm, whereas the embryonic Oct-4 is localized to the nucleus [21]. This supports the finding that nuclear localization was not consistent until 6 days. Data in the rat, mouse, and rhesus monkey indicates that Oct-4 mRNA is produced from the embryonic genome at the time of maternal-zygotic transition [15, 20]. From this point forward the variability within and between embryos decreased significantly in equine embryos, and Oct-4 became more consistently localized to the nuclei of embryos. This may indicate Oct-4 does not play a significant role in very early embryos since it functions as a transcription factor and the genome is not being transcribed; accumulation in the

cytoplasmic space suggests that it is non-functional. With further development, the importance of Oct-4 at these stages is echoed by the specificity of localization to the nucleus. The variability observed in Oct-4 expression of early embryos may be related to many factors. Those nuclei in an embryo not expressing Oct-4 could be preparing to divide; we noted that mitotic figures in embryos did not stain with Oct-4. Additionally, transcription is not occurring when a nucleus is about to degenerate. While we attempted to select only viable embryos for our Oct-4 staining, some of these embryos may have been compromised or may have contained non-viable blastomeres.

Oct-4 was present in all cells of all blastocysts examined, in varying intensities. In vitro-produced equine embryos at 7 days of culture that were considered to be blastocysts (defined as >64 normal nuclei and apparent differentiation of a rim of trophoblast cells) rarely had a visually identifiable ICM. Thus, this section of the study was of particular interest to determine if Oct-4 staining could identify the ICM in embryos of in vitro origin. The ability to identify the ICM of in vitro-produced embryos based on the expression of Oct-4 protein would allow much greater confidence in the developmental capacity of these embryos and their usefulness as a model of in vivo development. In no in vitro-produced blastocysts, however, was there any indication of an ICM based on increased intensity of Oct-4 signal. Mounting under coverslips may obscure the ICMs of equine blastocysts, a phenomenon that went undiscovered until after the in vitro-produced blastocysts had been mounted and studied. However, 40% of in vivo-recovered blastocysts showed stronger Oct-4 signal in a visible, presumptive ICM even when mounted under coverslips. This suggests that there may be in vivo

factors important in promoting viability or maintaining pluripotency of the ICM. In those in vitro-produced/in vivo-cultured blastocysts viewed both before and after mounting under coverslips, all blastocysts recovered had an obvious ICM visible to the eye on initial evaluation before staining, and a mass was also evident after staining. This mass presented as an area of increased Oct-4 signal, with no corresponding increase in Hoechst stain, when viewed in a glycerol droplet. In 4 out of 7 of these blastocysts, the presumptive ICM was more difficult or even impossible to identify once the blastocyst was flattened during mounting. To confirm the benefits of viewing blastocysts in suspension over mounted blastocysts, further studies should be undertaken in which blastocysts are examined under fluorescence microscopy in glycerol droplets before they are mounted for examination on slides.

Obtaining a single equine in vivo-recovered embryo requires many hours of labor and there is only a 50-75% chance of recovery despite this work [56]. A product for mare superovulation has just become commercially available; however it is expensive (~$500 per cycle) and provides an average of only 1.5-2 embryos per flush [57]. Thus, we evaluated both in vivo-recovered blastocysts and in vitro-produced/in vivo-cultured blastocysts. The in vitro-produced/in vivo-cultured blastocysts spent 2 days in utero, and this was associated with expansion of the blastocoel and development of an ICM far exceeding in vitro-cultured counterparts. Blastocysts recovered after this time in utero were up to 1.250 mm in diameter; large when compared with in vitro-cultured blastocysts. These blastocysts were indistinguishable from typical in vivo-recovered blastocysts of the same age. The increased Oct-4 signal in the presumptive ICMs of

these embryos suggests that the uterus supports differentiation between trophoblast and ICM cells.

While no bands indicating the presence of Oct-4 in either bovine or equine embryos were visible in Western analysis to this point, this is most likely due to the low number of cells used for each sample. Week-old embryos are small, averaging 151 cells in a bovine Day 7 embryo [58] and 108 cells in an equine Day 7 embryo [59]. As mentioned above, more embryos are currently being accumulated to allow further exploration of the Oct-4 bands in the Western analysis of these embryos.

Preliminary work has been performed examining the mRNA expression for Oct-4 in equine embryos, in collaboration with the laboratory of Dr. John McLaughlin at the University of Pennsylvania School of Veterinary Medicine. Two in vivo-recovered equine embryos (Days 8 and 10) were evaluated for expression and spatial distribution of Oct-4 mRNA. Using the Day 10 embryo, a reusable equine cDNA library was synthesized as previously described [60, 61]. RT-PCR was performed on this library and on 2 negative controls (equine cumulus cells and fibroblasts) using 2 sets of primers designed based on a published equine expressed sequence tag with sequences corresponding to exons 3-5 of bovine Oct-4. Both sets of primers produced Oct-4 bands (set 1=189bp, set 2=150bp) that were detected only in the blastocyst sample (Fig. 9).

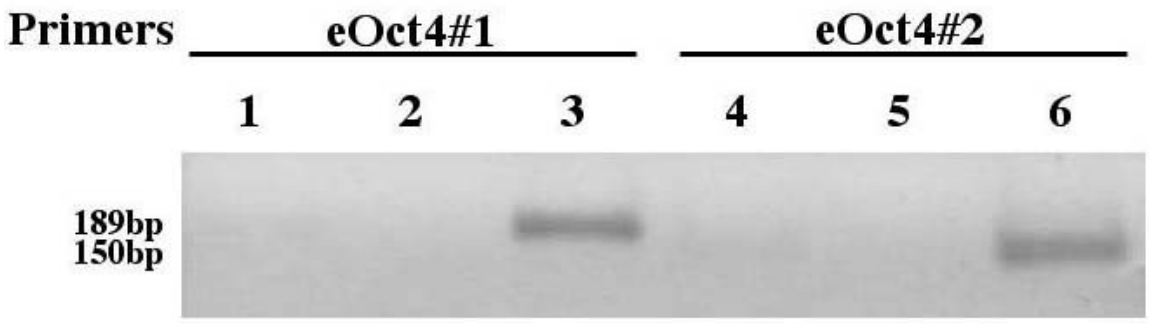

FIGURE 9. Expression of Oct-4 mRNA in equine blastocyst at Day 10. Lanes 1 and 3: fibroblasts, 2 and 4: cumulus cells, 3 and 6: blastocyst. Courtesy of Dr. John McLaughlin, UPenn.

The Day 8 blastocyst was used in whole mount in situ hybridization with an Oct-4-specific antisense mRNA probe that cross-reacts in a number of species with similar specificity to determine spatial distribution of the transcript. The Oct-4 mRNA signal was distributed differently than in murine and bovine samples, which exhibit Oct-4 mRNA in only the ICM (Fig. 10). The Day 8 equine blastocyst had detectable Oct-4 mRNA in the entire inner endodermal layer within the trophectoderm layer (Fig. 11). These results suggest the signal-expressing inner layer either contains pluripotent cells or that the cells of this layer maintain their Oct-4 mRNA expression as they differentiate. Studies are currently underway in Dr. McLaughlin's laboratory to create an equine-specific mRNA probe to ensure species-specific signal.

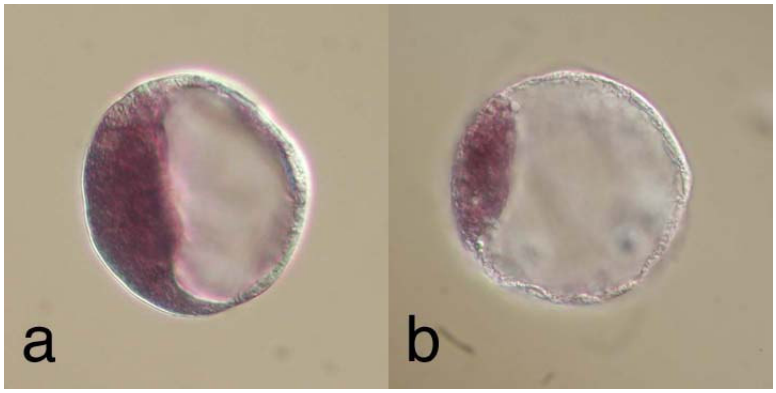

FIGURE 10. Spatial expression of Oct-4 mRNA in mouse and bovine blastocysts. a) Mouse blastocyst at Day 3.5. b) Bovine blastocyst at Day 7. Courtesy of Dr. John McLaughlin, UPenn.

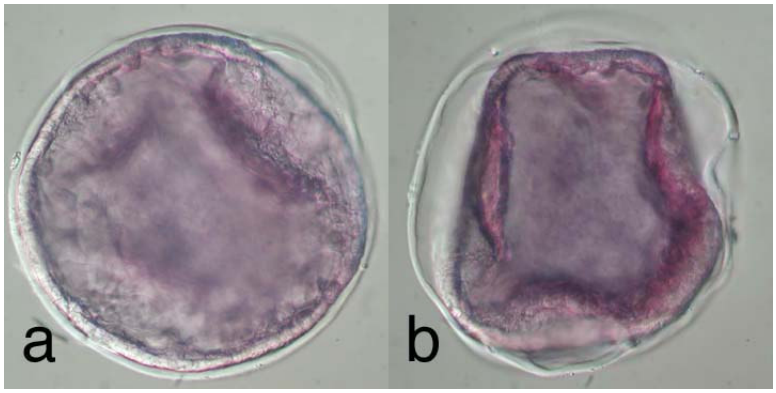

FIGURE 11. Spatial expression of Oct-4 mRNA in an equine blastocyst at Day 8. a and b are the same embryo from a different angle. Courtesy of Dr. John McLaughlin, UPenn.

Forty-three in vitro-produced equine embryos from the 3-cell to blastocyst stages were shipped to the laboratory of Dr. McLaughlin. Of these, 18 were lost during processing (lysed in the hybridization solution) and 12 were subjected to in situ hybridization. Thirteen embryos were fixed for future evaluation with equine Oct-4 mRNA probes. Oct-4 mRNA had low expression in early embryos. Expression in in vitro-produced blastocysts was distributed throughout the embryo (Fig. 12).

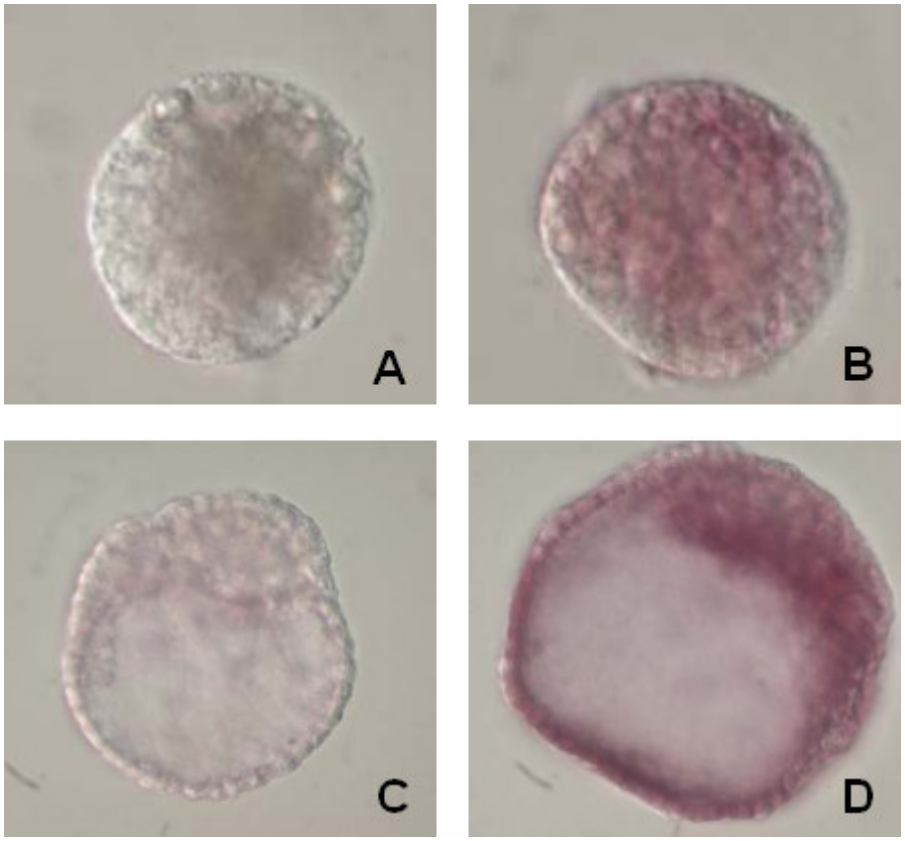

FIGURE 12. Spatial expression of Oct-4 mRNA in in vitro-produced blastocysts. A and B) Day 7 blastocysts; C and D) blastocysts which had the zona removed at Day 7 and were cultured for an additional 3 days. A and C) control; B and D) Oct-4 mRNA. Courtesy of Dr. John McLaughlin, UPenn.

CHAPTER III

FEEDER LAYER DEVELOPMENT

MATERIALS AND METHODS

Approach

To find the appropriate conditions for the immediate (ready within 24 hours of removal from storage) establishment of a feeder layer for stem cell culture, a number of steps had to be taken. It was necessary to establish a line of equine fetal fibroblasts and learn the growth characteristics specific to equine fetal fibroblasts. To determine growth characteristics, increase in numbers of cells over time had to be calculated. Different enumeration protocols were evaluated to determine which most accurately reflected the cell numbers in cell suspensions. Using the technique proven most reliable for counting cells, the number of cells present in a confluent monolayer in 1 well of a 4-well dish (the plate to be used for ES cell culture) was determined in order to provide a baseline for estimating the number of inactivated cells that should be used for plating to immediately obtain a confluent monolayer, and to serve as a basis for further culture calculations. Once this number was determined, the plating density of cells associated with the most rapid growth was determined. This number provided data needed to produce a rapidly growing culture to use in testing different doses of the arresting agent Mitomycin C to inhibit growth in equine fibroblast cells. Using the selected plating density, different concentrations of Mitomycin C were added and the effect on cell growth determined, to establish a concentration of Mitomycin C that was effective in stopping cell growth but that did not cause cell death.

Fibroblast line establishment

Fetal fibroblast cultures were developed from 3 approximately 90-day fetuses recovered from an equine abattoir. The fetuses were transported to the laboratory at room temperature, a maximum of 7 hours from recovery. Skin samples were collected from each fetus and washed extensively with PBS, then finely minced and transferred to 75-cm^3 tissue culture flasks with 5-6 ml of CCM. The tissue was cultured at 38.2°C in 5% CO_2 in air, examined daily, and the medium changed every other day. Approximately 1 week after collection of samples, fibroblasts began attaching to the bottom surface of the culture flask. At this point the intact tissue was removed and transferred to new culture flasks and the cells attached to the flask surface retained in culture an additional 5 days, until they reached confluency. They were then subjected to their first passage.

To passage the fibroblasts, medium from each tissue culture flask was removed and the inside culture surface of the flask was washed 2 times with 8-10 ml of PBS. Each flask was then administered 4 ml of a 0.05% Trypsin-EDTA solution (Trypsin, Sigma) for 2-3 minutes, until cells began to disassociate from one another and detach from the flask surface. Approximately 16 ml of CCM was then added to each flask to dilute the Trypsin and help to suspend any remaining attached cells. This suspension was transferred to 2 15-ml tubes and centrifuged at 3000 RPM for 3 minutes. After centrifugation, the supernatant was removed and the pellets were each resuspended with 1.0 ml of culture medium, and then combined into 1 of the 15-ml tubes before distribution. Each 2.0-ml total cell suspension from 1 culture flask was distributed

between 2-4 fresh culture flasks and returned to culture at 38.2°C in 5% CO_2 in air. These plated cells were considered Passage 1 fibroblasts. Fibroblast cultures were examined daily and the medium changed every other day; most cultures reached confluency after 4 to 5 days and they were subsequently passaged to 2-4 fresh flasks. The fetal fibroblast line with the best growth over the first 3 passages was selected for all further studies and the other lines were discarded. From this point forward a fresh culture of this selected fibroblast line was maintained in the incubator; cells were passaged when they reached confluency (between 5 and 10 days, depending on preliminary cell density) or were needed for further studies.

Counting method and confluency

Cell counts performed with a hemocytometer are accurate in determining the concentration of cells in a suspension. However, to calculate the number of cells representing confluence, and to determine the ability of MMC to arrest growth without cytotoxicity, it was necessary that the number of cells enumerated in the suspension reflected accurately the number of cells present in the culture well. To determine this, 2 cell counting methods, referred to here as “trypsinization-only” and “trypsinization-plus-centrifugation”, were compared over 4 trials. Both counting methods have their applications to particular situations. In more dilute, higher volume solutions centrifugation is necessary, but if the volume can be controlled and the cells can be sacrificed then the trypsinization-only treatment is a valuable option. Centrifugation holds the potential for loss of cells while transferring suspensions between different receptacles and during the centrifugation itself. Cells may remain in the supernatant and

are lost if they are not a part of the pellet. In situations involving dilute cell suspensions, however, centrifugation is the only method available to recover the cells with any efficiency. Trypsinization without subsequent dilution with a serum-containing medium avoids this potential loss of cells through manipulation, but it is not an option if the cells are needed for further studies. If the enzymatic activity of Trypsin continues unquenched, cell membranes are damaged, causing cell death.

Since the ultimate goal of this work is to allow the *immediate* production of a confluent monolayer of previously-inactivated frozen-thawed equine fetal fibroblasts, the baseline number of healthy cells in a confluent monolayer is needed. This will be the number of healthy, surviving cells that will need to be plated into the well, and to this number will be added additional cells to account for any loss due to manipulation and storage damage.

Passage 5 fetal fibroblasts were seeded at an arbitrary low density in the wells of a 4-well dish with CCM and allowed to grow to confluency at 38.2°C in 5% CO_2 in air. Culture medium was changed every other day. Within 2-3 days, fibroblasts formed a 95-100% confluent monolayer on the bottom of the well, and they were then subjected to both counting methods in succession.

First, the medium was removed from the well of interest and the well was washed 2 times with 1.0 ml of PBS. The washed cells were then administered 0.25 ml of Trypsin for 2-3 minutes until cell dissociation was evident, then vigorously pipetted to establish a homogenous suspension and to remove any remaining cells from the well surface. At this point 10 µl of the trypsinized cell solution was removed and diluted

with 90 µl of PBS (1:10 dilution) for counting. This 2-step procedure was necessary because of the limited volume capacity of a single well in a 4-well dish. To eliminate this step, 2.5 ml total volume would be needed in the well, but manufacturer recommendations indicate a maximum volume of 1.9 ml in 1 well of a 4-well dish. Twenty µl of this dilute mixture was then evaluated in a hemocytometer to determine the cell concentration in the suspension. Concentration was determined as the number of cells counted (average of 2 sides of the hemocytometer) x 10^5 cells per ml of fluid. The total cell number recovered from the well using the trypsinization-only method was derived using the equation

[2] $n_{to} = C_1V_1$,

where n_{to} is the total cell number as determined using this technique, C_1 is the concentration of cells as determined with the hemocytometer, and V_1 is the total volume of the cell suspension.

To find the cell number as determined by the trypsinization-plus-centrifugation technique, the remaining trypsinized cell suspension was aspirated from the well and combined with 10-14 ml of CCM in a 15-ml centrifuge tube for washing and removal of Trypsin. The cell suspension was centrifuged at 5000 RPM for 5 minutes. The supernatant was then removed with an electronic pipette and the pellet was resuspended with approximately 0.25 ml of CCM. The actual volume of this solution was measured and recorded and 20 µl of the solution were counted in the hemocytometer as described above, without dilution. To use this concentration to determine the number of cells in the original well, since some medium (and therefore some cells) was removed from the

second measurement technique for use in the first, equation 2 could not completely apply. Therefore, the total cell number was adjusted using the equation

[3] $n_{tpc} = C_2V_2V_1/(V_1-V_r)$

where n_{tpc} is the total cell number as determined using this technique, V_r is the volume of the suspension removed for the previous method, V_2 is the volume after centrifugation and resuspension of the pellet, and C_2 is the concentration of cells as determined with the hemocytometer for this counting method.

On the basis of the results of this trial (see Results), the trypsinization-only technique was selected as the more accurate and simple technique. The approximate number of cells in a 95-100% confluent monolayer in 1 well of a 4-well dish was determined by averaging cell numbers from 4 replicates performed with this technique.

Seeding density

This section of the study was performed to determine the optimum fibroblast seeding number in 1 well of a 4-well dish to achieve the quickest cell growth rate. The original seeding density which grew the most (that which had the highest proportion of final cell density to plated cell density) over a 5-day period was considered the most rapidly growing density. The confluency determination described above found the average cell number in 1 well of a 4-well dish with a just-confluent monolayer of fetal fibroblasts attached to the bottom to be 3.57×10^5 cells. Experimental seeding concentrations for the next step were extrapolated from this data, with an approximation of 4×10^5 cells to allow for some minor cell loss as a result of manipulation. The seeding number with the most rapid growth in 1 well of a 4-well dish over 5 days was

then used in the next phase of study to examine the effect of Mitomycin C on cell growth.

The seeding cell numbers used for study of optimum cell growth were 6.25×10^3, 1.25×10^4, 2.50×10^4, and 5.00×10^4 cells per well. These are based on the assumption that equine fetal fibroblasts divide on average every 24 hours in culture. Assuming 4×10^5 cells are in a confluent well and that the cell number in the well doubles every 24 hours on average, the numbers of cells to plate were based on an approximation of the number of cell doublings needed to reach confluency and the number of days (5) of the study. Cell densities of 6.25×10^3 cells, 1.25×10^4 cells, 2.5×10^4 cells and 5.0×10^4 cells in a well would require 6, 5, 4, and 3 doublings, respectively, to reach 4×10^5 cells in a well for a confluent monolayer. Since the intention of this study is to determine the plating density which gives the fastest growth over 5 days in culture, the assumed number of doublings over 5 days is included in this range. Three replicates were performed using these seeding densities, with each well of a 4-well dish containing a different cell density of passage 6 cells on seeding day (Day 0). The cells were given CCM and cultured for 5 days in 5% CO_2 in air at 38.2°C; medium was changed on Day 3. On Day 5 for each replicate the wells were each subjected to the trypsinization-only counting method. The total number of cells in each well was determined as described in equation 2 above. Growth rate was determined using the equation

[4] $G = (n_f - n_i)/n_i$,

where G is the growth rate over the 5-day period, n_f is the cell number at Day 5, and n_i is the initial cell number in the well. Analysis of variance was then used to examine any

statistically significant differences between the means of the cell numbers recovered between each original cell density.

Cell loss evaluation

During the seeding density study, it was observed that not all cells plated successfully attached to the bottom of the well, and they instead floated in the effluent. These cells were assumed incapable of attachment and were removed with the first medium change. In an attempt to evaluate whether the growth rates determined in the previous study accurately reflected actual growth (number of cells initially plated vs. number of cells present at 5 days) the percent of cell loss associated with passaging was studied in this experiment.

To determine cell loss, passage 7 cells were plated at 1.25×10^4 cells/well in a 4-well dish. The fibroblasts were given 24 hours to attach to the well surface, then the effluent was transferred from 2 wells of the dish into 2 separate 1.5-ml centrifugation tubes; the wells were given fresh medium and returned to the incubator. Cells in all wells were cultured 5 days, and medium for all cells was changed on Day 3. The 2 samples of 24-hour effluent were found to be too dilute to count directly with a hemocytometer, so they were counted using the centrifugation method. The samples of effluent were centrifuged at 5000 RPM for 5 minutes. The supernatant was then removed and the pellet resuspended with 40 µl of CCM, then the cell number recovered from the effluent enumerated with a cell hemocytometer. On Day 5, all wells in the dish were treated with the trypsinization-only cell counting method to examine the growth as calculated based on the number of seeded cells that had attached to the bottom at 24

hours. The potential effect of removing the effluent containing the floating fibroblasts after 24 hours was evaluated by comparing final cell numbers between wells which did and did not have effluent removed at 24 hours.

Mitomycin C concentration

Mitomycin C is an antitumoral antibiotic found to arrest growth in fetal fibroblasts and other cell lines, making the cells useful in cell culture as a feeder layer. This alkylating agent allows the cells to continue their necessary internal processes, yet prevents their division and the reproduction of their DNA. Mitomycin C works by cross-linking complimentary strands of double stranded DNA, a function first studied in 1964 [62]. Under reducing conditions, MMC is converted to a highly reactive compound that reacts in 2 alkylating steps to crosslink the DNA, thus destroying the function of the genome [63]. The fibroblasts continue to metabolize and are useful to support other cells in culture for 10-14 days. The previous 2 studies' parameters were based on the need for this experiment to be a 5-day trial with rapidly growing cells. This experiment was based on technique described previously [50].

To find the dose of Mitomycin C that effectively stops the growth of fetal fibroblasts, it is logical to use the most proliferative cell seeding number to eliminate the variable of cell growth rate. The previous experiment indicated 1.25×10^4 cells in 1 well of a 4-well dish resulted in the most rapid growth of those densities studied over a 5-day period (see Results). This seeding density was used to develop a dose-response curve of a rapidly growing culture to Mitomycin C treatment. This trial was repeated 6 times in 4-well dishes with each well serving as either a control (no Mitomycin C) or 1 of 3

doses: 2 µg/ml, 20 µg/ml, or 200 µg/ml of Mitomycin C in CCM. The doses were chosen based on previous studies of MMC dosing in murine feeder layers [50].

For each replicate, passage 8 confluent fibroblasts were treated with Mitomycin C. To do this, passage 7 fibroblasts in a culture flask were trypsinized, washed, centrifuged, resuspended, enumerated, and then plated at 3.5×10^5 cells/well in all wells of a 4-well dish with CCM and allowed 24 hours to attach to the bottom of the well while incubating at 38.2°C in 5% CO_2 in air. After this trypsinization treatment the cells were considered to be at passage 8. A density of 3.5×10^5 cells/well reflects the number of cells in a confluent monolayer (see Results), and should provide a confluent monolayer immediately without the need for further culture and/or cell division. Mitomycin C treatment of a confluent monolayer allowed a sufficient number of cells to be treated so that the desired starting density of 1.25×10^4 cells/well could be recovered after treatment, washing, centrifugation, and resuspension. The medium was removed from all wells after 24 hours allowed for attachment and replaced with medium containing MMC at the wells' respective treatment concentrations. In each replicate, Well 1 was the control, Well 2 received 2 µg/ml MMC, Well 3 received 20 µg/ml MMC, and Well 4 received 200 µg/ml MMC. The cells were then returned to the incubator for 3 hours.

After the 3-hour treatment was completed, the dishes were removed from the incubator and the medium with Mitomycin C was removed and discarded. The cells were then thoroughly washed 3 times with 1.0 ml of PBS. Each well then received 0.5 ml of Trypsin for 2-3 minutes until cells began to detach from the well surface. When

cells began to disassociate, the Trypsin was quenched with 0.75 ml of CCM. The cell suspension was transferred to 10 ml of CCM in a 15-ml centrifuge tube and centrifuged at 5000 RPM for 5 minutes to wash Trypsin from the cells. The supernatant was removed and the pellet resuspended with 0.5 ml of CCM. The number of cells in each resuspended sample was found by removing 20 µl of the suspension and counting the number of cells in a hemocytometer. The volume of suspension to seed into fresh 4-well dishes to obtain the desired preliminary cell number of 1.25×10^4 was found using the equation

$$[5]\ V = C/1.25 \times 10^4,$$

where V is the volume to be seeded in ml and C is the concentration of cells as found with the hemocytometer. Beginning with the density previously shown to have the most rapid growth allowed examination of the inactivating capabilities of MMC without differing growth rates of the cultures as an additional variable. After the appropriate volume was seeded into 4-well dishes in the same configuration as for treatment with MMC, 1 ml of CCM was added and the passage 9 cells (cells were considered passage 9 after the most recent trypsinization treatment) cultured at 38.2ºC in 5% CO_2 in air. Cells were cultured 5 days, examined daily and medium refreshed on the third day.

On Day 5, dishes were removed from the incubator and each well was subjected to the trypsinization-only method of counting. The number of cells in each well was recorded and compared between each treatment by analysis of variance.

Freezing

Since the Mitomycin C compound, once dissolved in solution, has a short half life (effective period of only 1 week once suspended), preparing Mitomycin C-treated fibroblasts separately for each stem cell passage is inefficient. A method was needed to treat many fibroblasts at once and store them for later use as a feeder layer with ES cell culture. Freezing is the most viable option to store a maximal number of cells at minimal cost.

Freshly trypsinized passage 7 cells (excess cells from the trypsinization performed to obtain cells for the MMC dosing study above) were seeded in the wells of a 4-well dish and allowed to grow to confluency. Confluency was reached after 3-4 days in culture, and the wells were then trypsinized and the cells counted with the trypsinization-plus-centrifugation method. The resuspended cells were combined with an equal volume of 20% DMSO, then divided into 1.0-ml aliquots with a minimum concentration of 1×10^6 cells/ml and transferred to cryotubes, then frozen at a rate of -1°C/minute to -80°C and left overnight. They were then transferred to a liquid nitrogen tank for storage.

To determine the percentage of cells that do not attach within 24 hours after thawing, a cryotube was removed from the storage tank and transferred to 37°C sterile water in a sterile specimen cup for thawing. When thawed, the cryotube was removed from the water and the thawed sample removed and combined with 10 ml of CCM in a 15-ml centrifuge tube, then centrifuged at 5000 RPM for 5 minutes. The supernatant was removed and the pellet resuspended with 0.25 ml of CCM. The cell concentration

was enumerated with a hemocytometer. The passage 8 cells (as a result of the trypsinization treatment) were then plated at a seeding density of 3.5 x 10^5 cells/well, the density determined to give a confluent monolayer with fresh cells, in the wells of a 4-well dish over 6 trials. They were incubated 24 hours in 5% CO_2 in air at 38.2°C, and then the effluent was discarded and the attached cells were subjected to the trypsinization-only counting method to determine the percentage of cells that did not attach within 24 hours of thawing and plating.

Once the effect of freezing was determined through this experiment, all but 1 flask each of passage 8, 9, and 10 cells were sequentially treated with Mitomycin C and frozen for future use as feeder layer cells. The remaining flask from passages 8 and 9 was passaged to 10 fresh culture flasks and allowed to grow to confluency in 5% CO_2 in air at 38.2°C over 7-10 days. The remaining flask from passage 10 was passaged to 4 fresh culture flasks to maintain a fresh stock of untreated cells. Nine of the 10 flasks from each passage were then administered 20 µg/ml Mitomycin C treatment (see Results) as described above, with 6 ml of MMC-containing medium per flask. After treatment, cells were trypsinized and the Trypsin quenched as described for passaging of cells, then the cell suspension was transferred to 15-ml centrifuge tubes and centrifuged to remove the Trypsin. The recovered pellet was resuspended, the resulting volume measured, the concentration of cells determined with a hemocytometer, and the cell suspension combined with an equal volume of 20% DMSO solution. This solution was then aliquoted into 1.0-ml cryotubes and frozen as above, with a final cell concentration of approximately 1.5 x 10^6 cells/ml. The cells were then transferred to liquid nitrogen

for storage in a nitrogen tank until they were needed to rapidly produce a confluent monolayer of inactivated cells for ES cell culture.

RESULTS

Counting method and confluency

Significantly more cells were recovered with the trypsinization-only method than with the trypsinization-plus-centrifugation method (p=0.01). Table 4 illustrates the cell numbers recovered with each method; the trypsinization-plus-centrifugation total cell number has been adjusted to account for the solution removed for direct counting. Not only does it appear that centrifugation causes loss of cells, but this method of cell recovery also results in more variability in calculated cell numbers.

TABLE 4. Comparison of fibroblast recovery methods.

	Trypsinization Only			Trypsinization-plus-centrifugation		
Trial	Cell Conc. (cells/ml)	Total Volume (ml)	Total Cell Number	Cell Conc. (cells/ml)	Total Volume (ml)	Total Cell Number[a]
1	1.20×10^6	0.25	4.03×10^5	5.45×10^5	0.31	2.01×10^5
2	1.60×10^6	0.25	4.00×10^5	8.40×10^5	0.25	2.19×10^5
3	1.55×10^6	0.25	3.88×10^5	5.80×10^5	0.28	1.69×10^5
4	0.95×10^6	0.25	2.38×10^5	1.75×10^5	0.29	5.29×10^4
Avg			**3.57×10^{5b}**			**1.60×10^{5b}**

Passage 5 fibroblasts were allowed to grow just to confluency, then trypsinized and subjected to 2 enumeration methods in succession. Total cell numbers found were averaged over the 4 trials and the averages compared with ANOVA.

[a] Adjusted using cell solution volume and volume removed during trypsinization-only method.

[b] Values differ significantly ($p<0.05$)

Having determined that the use of the trypsinization-only method results in a more accurate representation of the cell number in a given starting sample, the number

of cells in each confluent culture treated with the trypsinization-only method was used to determine the number of cells in a just-confluent monolayer. As seen in Table 4, these cell numbers ranged from 2.38×10^5-4.03×10^5 cells in a well. These numbers indicate that the average number of cells in a confluent monolayer of equine fetal fibroblasts in 1 well of a 4-well dish is 3.57×10^5 cells. Thus, to immediately obtain a confluent monolayer of equine fetal fibroblasts, 3.57×10^5 viable cells should be plated and allowed to attach, and no further culture should be necessary.

TABLE 5. Effect of seeding density on fibroblast growth.

Initial Cell Number	Trial	Final Volume (ml)	Final Cell Concentration (cells/ml)	Final Cell Number	Growth Rate	Average Growth Rate
6.25×10^3	1	0.25	3.90×10^5	9.75×10^4	14.60	18.6
	2	0.25	4.05×10^5	1.01×10^5	15.20	
	3	0.25	6.75×10^5	1.69×10^5	26.00	
1.25×10^4	1	0.25	1.20×10^6	3.00×10^5	23.00	22
	2	0.25	9.00×10^5	2.25×10^5	17.00	
	3	0.25	1.35×10^6	3.38×10^5	26.00	
2.5×10^4	1	0.25	1.75×10^6	4.38×10^5	16.50	14.5
	2	0.25	1.05×10^6	2.63×10^5	9.50	
	3	0.25	1.85×10^6	4.63×10^5	17.50	
5.0×10^4	1	0.25	2.35×10^6	5.88×10^5	10.75	8.75
	2	0.25	1.65×10^6	4.13×10^5	7.25	
	3	0.25	1.85×10^6	4.63×10^5	8.25	

Passage 6 fibroblasts were plated in the wells of a 4-well dish at varying densities and allowed to grow 5 days to determine growth rates.

Seeding density

Table 5 lists growth rates for each seeding density in 3 replicates. Average growth was, in increasing seeding density, 18.6, 22, 14.5, and 8.75 times original seeding density over the 5 days of culture. Though not statistically significant, the

average growth rate from cells originally seeded at a density of 1.25 x 10^4 cells per well was the highest. For the remaining studies in which rapidly growing fetal fibroblasts are needed, cells were seeded at a density of 1.25 x 10^4 cells/well in 1 well of a 4-well dish.

Cell loss evaluation

As Table 6 illustrates, 1.5-2.0 x 10^3 cells were floating in the effluent of the well 24 hours after seeding at 1.25 x 10^4 cells/well (12-16% of seeded cells). From this data it may be assumed that approximately 85% of cells seeded at a density of 1.25 x 10^4 cells in 1 well of a 4-well dish attach within 24 hours; i.e. that the actual original density of cells is 1.06 x 10^4 cells per well. The floating cells did not affect cell growth, as cell numbers found on Day 5 were similar between the 2 groups; no statistically significant differences were present. Therefore the removal of the effluent and unattached cells after 24 hours in culture does not affect cell growth over 5 days. These data showed that while there is some cell death associated with passaging, that it was at a low enough level that it should not alter interpretation of the growth rates obtained in the previous study.

Mitomycin C concentration

Table 7 lists the number of cells recovered after 5 days in culture following plating of 1.25 x 10^4 cells (the most rapidly growing plating density as determined previously) previously treated with varying doses of Mitomycin C. In addition to this quantitative data, cells were examined daily and any noticeable characteristics recorded. Few cells were found floating in the effluent of the wells given no MMC. Cells given any dose of MMC, however, suffered from noticeably higher apparent cell damage (cells

TABLE 6. Equine fetal fibroblast survival post-manipulation.

	Day 1			Day 5		
Well Number & Treatment	Final Volume (ml)	Final Cell Concentration (cells/ml)	Final Cell Number	Final Volume (ml)	Final Cell Concentration (cells/ml)	Final Cell Number
1 Effluent removed	0.04	5.0×10^4	2.0×10^3	1.0	3.00×10^5	3.00×10^5
2 Effluent removed	0.04	3.5×10^4	1.4×10^3	1.0	4.05×10^5	4.05×10^5
3 Effluent undisturbed				1.0	3.20×10^5	3.20×10^5
4 Effluent undisturbed				1.0	3.65×10^5	3.65×10^5

Passage 7 fibroblasts were plated at 1.25 x 104 cells/well and allowed 24 hours to attach. Effluent was then removed from Wells 3 & 4 and the number of cells in the effluent enumerated. Cells were cultured a total of 5 days, then trypsinized and counted.

present in effluent) once the cells were plated and allowed 24 hours to attach. On visual inspection, approximately 50% of the cells in any MMC-treated well failed to attach and remained floating in the effluent. Obvious growth was observed in cells given a dose of 2 µg/ml of MMC after seeding, and excessive cell loss was seen to occur even after cell attachment (e.g. cells detached and were seen in the effluent on Days 2 through 5) in those trials in which 200 µg/ml of MMC was administered. This was determined by the consistent appearance of cells in the effluent of the well over the 5-day culture period and a parallel visible reduction in the number of cells attached to the well surface.

Freezing

Freezing causes significant death in equine fetal cells, and this loss must be accounted for to find an accurate number of cells needed to immediately (within 24 hours of thawing) make a confluent monolayer from a frozen stock. Cells treated with

TABLE 7. Dose-response of equine fetal fibroblasts to Mitomycin C.

Mitomycin C dose (µg/ml)	Day 5 cell number[a]	Average Day 5 cell number
0	8.13×10^4	4.68×10^5
	4.62×10^5	
	4.91×10^5	
	5.04×10^5	
	5.89×10^5	
	6.84×10^5	
2	7.50×10^3	9.79×10^3
	7.50×10^3	
	8.25×10^3	
	1.13×10^4	
	1.13×10^4	
	1.30×10^4	
20	5.25×10^3	7.08×10^3
	5.25×10^3	
	6.00×10^3	
	6.75×10^3	
	6.75×10^3	
	1.25×10^4	
200	7.50×10^2	1.50×10^3
	7.50×10^2	
	7.50×10^2	
	1.50×10^3	
	1.50×10^3	
	3.75×10^3	

Confluent passage 8 fibroblasts were treated with 20 µg/ml MMC for 3 hours, then trypsinized and plated at 1.25×10^4 cells/well. Cells were cultured 5 days and then counted.
[a] Number of cells attached to well surface at Day 5.

MMC, frozen, then thawed and seeded at a density to create confluency immediately if all cells survived (3.5×10^5 cells/well of a 4-well dish) suffered an average of 77.3% cell loss. This data can be seen in Table 8. From these results, it was calculated that to deliver the number of viable, attachment-capable, frozen-thawed MMC-treated cells

needed for immediate establishment of a confluent monolayer, 1.55 x 10^6 total cells would need to be plated in 1 well of a 4-well dish.

TABLE 8. Freezing effects on equine fetal fibroblasts.

Trial	24 hour cell number[a]	Percent loss
1	1.075 x 10^5	69.2857
2	9.625 x 10^4	72.5000
3	6.375 x 10^4	81.7857
4	5.875 x 10^4	83.2143
5	5.875 x 10^4	83.2143
6	9.25 x 10^4	73.5714
Average	**7.96 x 10^4**	**77.3**

Passage 7 fibroblasts were trypsinized and frozen, then thawed, plated at 3.5 x 10^5 cells/well, and allowed 24 hours to attach to the well surface. Cells attached to the well surface at 24 hours were trypsinized and counted.

[a] Number of cells attached to well surface 24 hours after plating

DISCUSSION

Fetal fibroblasts treated with Mitomycin C and frozen for long-term storage provide an efficient and cost-effective culture system for ES cells. The results detailed optimal seeding densities, inactivation treatments, and handling procedures for equine fetal fibroblasts to immediately prepare a confluent feeder layer to support equine ES cell growth. Many of the above procedures required the concentration of a known volume of a cell suspension to be found in order to determine the total number of cells present. It was found that trypsinizing cells without any further treatment such as centrifugation regularly resulted in a higher cell number recovered. This is most likely due to the loss of cells during transfer to and from vessels used for centrifugation and during centrifugation itself. Unlike other cells such as spermatozoa, which have heavy

heads filled primarily with nuclear material, fibroblasts contain a relatively large volume of cytoplasm. This makes them much less dense and they will float and remain in the supernatant of a centrifuged solution. On average, less than half of the cells treated by centrifugation were recovered at the end of the procedure. Therefore, in every situation in which only quantitative data is necessary and the cells are of sufficient concentration to be counted directly on the hemocytometer, the trypsinization-only method is more accurate a tool.

Since the proposed isolation of equine ES cells was to be carried out by culture in 1 well of a 4-well dish, conditions needed to be optimized for that environment. The number of cells that form a 95-100% confluent monolayer in 1 well of a 4-well dish was found to be between 3.5×10^5 and 4.0×10^5 cells. There is some tolerable error in this range that fibroblasts can accommodate; when fewer cells are plated the cells attach and maintain a well spread morphology and when more cells are present the cells attach in a much slimmer compact morphology. The range of cells in a confluent monolayer provided a starting point from which the ideal number of cells to put into a well to immediately achieve a confluent monolayer was determined.

To eliminate as many variables as possible, an additional step was required before MMC dosing could be examined for inactivation of fibroblasts for use as a feeder layer. In a previous study, cells were seeded after MMC treatment at an arbitrary subconfluent density and assessed visually [50]. To increase accuracy, a specific cell density with a predetermined growth rate was used to test the boundaries of the MMC treatment. The most rapidly growing cell density examined in these trials was 1.25×10^4

cells in 1 well of a 4-well dish. The starting density of 1.25x 10^4 cells/well grew to 22 times its original density over our 5-day trial, proving to have the most growth over 5 days out of the densities examined. Presumably, this density allowed enough space for cells to proliferate rapidly and avoid contact inhibition but had a high enough density to allow growth-encouraging cell signaling to occur. In addition, this density corresponds to the general assumption that cells in culture double every 24 hours. Untreated cells plated at this preliminary density and allowed to proliferate just reached confluency, approximately 3.5 x 10^5 cells in 1 well of 4-well dish, at Day 5.

Results indicate between 12% and 16% of equine fetal fibroblasts subjected to passaging or manipulation will not attach once seeded. This loss is minimal and is well accommodated for in the range of cells determined to create a confluent monolayer in 1 well of a 4-well dish.

Methods previously described were used with minor adjustments to allow further quantification of Mitomycin C's effects on equine fetal fibroblasts. Rather than relying purely on visual assessment of growth [50], the actual number of cells present in the well after 5 days was found by counting with a cell hemocytometer. After the different MMC treatments, a known number of cells (1.25 x 10^4) were seeded into each well and the cells allowed to grow, if possible. An unexpectedly large number of cells did not attach post-MMC treatment and remained floating in the effluent of the wells seeded with MMC treated cells. In addition, some cells treated with the highest dosage of MMC attached early on in the trial but later detached and were seen floating in the effluent. During the 5-day culture period, the number of cells attached to the well in this treatment

visually decreased and cells consistently were present in the effluent at media changes. The number of cells in which the inability to attach or remain attached was (presumably) caused by MMC treatment was visually estimated to be 50%. Visual assessment of growth on a daily basis combined with the numbers found as described above indicated 20 µg/ml of MMC for 3 hours is the best treatment to stop cell growth without any excessive loss of attachment ability in equine fetal fibroblasts. This is the concentration of MMC that was used from this point forward to arrest the growth of large colonies of cells for storage and later use as feeder layers.

Results indicate that 77.3% of cells do not attach after the freezing and thawing process and MMC treatment. Many cells are lost in the freezing process due to the formation of ice crystals within cells in the freezing and thawing procedures. Compounded by the recent treatment with a potent poison and the necessary loss while cells are transferred and centrifuged to remove freezing agents, this step is by far the most inefficient in the establishment of a confluent monolayer of equine fetal fibroblasts for use as a feeder layer. Given the estimated cell loss, 1.5×10^6 MMC-treated, frozen-thawed cells should be plated when a confluent feeder layer is desired.

CHAPTER IV

EMBRYONIC STEM CELL ISOLATION

MATERIALS AND METHODS

Approach

Establishing appropriate conditions for ES cell culture is very difficult due to species-specific and even strain-specific requirements. This phase of study began with the equine fetal fibroblast studies described above. Overnight, a feeder layer was established from these pre-treated cells, then blastocysts were co-cultured on the monolayer to encourage attachment and growth of ES-like cells. After 7-10 days in culture with no morphological changes of the embryonic cells, samples underwent immunocytochemistry for detection of Oct-4 protein, as described above.

Culture preparation

Equine fetal fibroblasts were previously inactivated by Mitomycin C and frozen and stored in liquid nitrogen as described in Chapter III. These cells were thawed and washed, then seeded in 4-well dishes at 1.5 x 10^6 cells/well, the number of cells needed for immediate establishment of a confluent monolayer as determined in Chapter III. After the viable cells attached (24 hours) the medium for these cells was changed to one supportive of ES cell growth: CCM plus 0.1 mM β-Mercaptoethanol and 10 ng/ml human Leukemia Inhibitory Factor (LIF, Sigma). LIF is the main additive to ES media believed to encourage the development of ES cells from ICM cells. Once cells were given this medium, the environment was considered prepared for the culture of ES cells.

Embryos used for culture

Five zona-free in vitro-produced blastocysts were cultured intact on the feeder layer. Another 5 embryos of this origin were subjected to zona removal and 2 subsequent days of culture in vitro, then had ICM-like masses mechanically dissected from the trophoblast and placed on the feeder layer. Finally, 5 in vivo-recovered embryos were subjected to trophoblast solubilization with Triton X-100 before the remaining ICM cells were plated on a feeder layer.

Cell culture

Once the culture dish was ready for cell culture, it was returned to the incubator and the ICM was isolated from the blastocyst in those embryos undergoing this treatment. For mechanical dissection, embryos were transferred to 20-µl droplets of CCM in 35-mm petri dishes for handling. The embryo was then rotated and the ICM visually identified. The surrounding trophoblast was sliced away with an 18- to 21-gauge needle, leaving only the ICM behind. Embryos slated for Triton-X digestion treatment were transferred to 20-µl droplets of CCM for holding. They were then transferred to 1% Triton X-100 for 20-30 seconds and moved back to the holding medium for washing. The goal of this treatment was to lyse the outer cell membranes, leaving the ICM as the only remaining viable cells in the blastocyst. The zona pellucida and capsule were then mechanically removed from the digested blastocysts with an 18- to 21-gauge needle. Embryos used for intact culture were removed from embryo culture and held in media as above until transferred onto the feeder layer.

Once the ICM had been isolated (if performed), the whole blastocyst or isolated ICM was placed on the feeder layer and the dish placed back into culture at 38.2°C in 5% CO_2 in air. Over the time the cells were cultured (3-10 days), they were evaluated daily at 200-400X to identify the formation of ES-like colonies. These cells were expected to be in piled colonies and be small and round in appearance if viewed singularly. ES cell-supporting medium was refreshed daily, and after 7-10 days with no change in the morphology of the culture, cultures were subjected to immunocytochemistry to identify Oct-4 expressing cells. For these cultures the failure of attachment (see Results) indicated further culture time would not improve results and all cultures were sacrificed to Oct-4 staining.

Additional trials

After performing preliminary studies on 45 embryos to develop ICM isolation techniques and evaluate response to different culture environments, Dr. Choi attempted to propagate embryonic stem-like cells from 21 additional in vitro-produced embryos. The zonae were removed with acid Tyrode's solution and the embryos were placed back in embryo culture for an additional 2-4 days to allow blastocoel expansion. Following this, the ICM of each embryo was isolated using needle dissection and placed on an inactivated murine feeder cell layer in DMEM knock-out medium (Gibco) with 15% FBS, 10-20 ng/ml LIF, 0.1 mM β-Mercaptoethanol, 1% Non-essential AA (Gibco) and glutamine (Sigma). As above, after 7-10 days with no changes in morphology cells were stained for Oct-4. After this amount of time in culture, cells needed to be passaged to fresh feeder layers, but to do so caused a loss of growth and sacrificed the culture (see

Results). Thus if no identifiable morphological changes had occurred within this amount of time, cultures were stained for Oct-4 rather than passaged.

Immunocytochemistry of cultured cells

Unattached vesicles or embryos were stained for Oct-4 as described above, as were cultures of cells growing on feeder layers. All concentrations and treatment times were identical to those described in Chapter II. Treatments for unattached vesicles were given in the 4-well dish in which cells were cultured, with approximately 250 µl of each solution and 500 µl of medium used for each wash. After staining was complete, unattached cultures were examined in situ on an inverted fluorescent microscope as described above. To stain cultures of cells growing on the feeder layer (designated "putative stem cells"; Fig. 11), randomly-chosen portions of 3 different cultures were dissected away from the plate surface with 21-gauge needles and subjected to immunocytochemistry as described in Chapter II. Cells were then mounted on slides and viewed as described in Chapter II.

Analysis

The number of cultures that attached to the feeder layer and/or formed outgrowths was recorded for each embryo treatment, as was the final number of attempted cultures for each embryo treatment. The proportion of blastocysts establishing a culture was found using the equation

[6] $P_c = A/n$,

where P_c is the proportion of attempts that formed cultures, A is the number of cultures with successful attachment, and n is the total number of attempts. In 9 selected viewing

areas (3 samples of putative stem cells from each of 3 different embryos), an estimation of the number of those cells was made. An estimate was also made based on counterstaining of the total number of nuclei present in these viewing areas under the microscope. Using the equation

[7] $P_{Oct\text{-}4} = n_{Oct\text{-}4}/n_{tot}$,

where $P_{Oct\text{-}4}$ is the proportion of Oct-4 expressing cells in a culture formed from a blastocyst, $n_{Oct\text{-}4}$ is the number of cells in the viewing area with visibly strong Oct-4 signal, and n_{tot} is the total number of cells in the viewing area as viewed via Hoechst staining. These proportions were averaged over each viewing area selected to provide a general proportion of Oct-4 expressing cells in an ICM cell growth.

RESULTS

Cell culture

After overnight culture of inactivated frozen-thawed fibroblasts seeded at 1.5 x 10^6 cells/well, a 95-100% confluent monolayer was observed in all dishes prepared for ES cell culture. Whole embryos cultured on the monolayers failed to attach and remained floating in the effluent, regardless of the length of culture time. These continued to expand in culture, but no outgrowths were observed and no ICM was ever visible. When blastocysts were subjected to trophoblast removal via mechanical dissection or Triton-X 100 treatment and then placed on feeder layers, the pieces of blastocysts formed spherical fluid-filled aggregates that continued to expand in culture but had no obvious ICM and did not attach to the feeder layer.

In the additional trials with murine feeder cells and knock-out base medium, all cell masses attached to the feeder layer and started cell proliferation; however, no growth of morphologically identifiable stem cells was obtained (Fig. 13). Attempts to passage the cells by mechanical dissection or trypsinization resulted in loss of cell growth in the passaged cells.

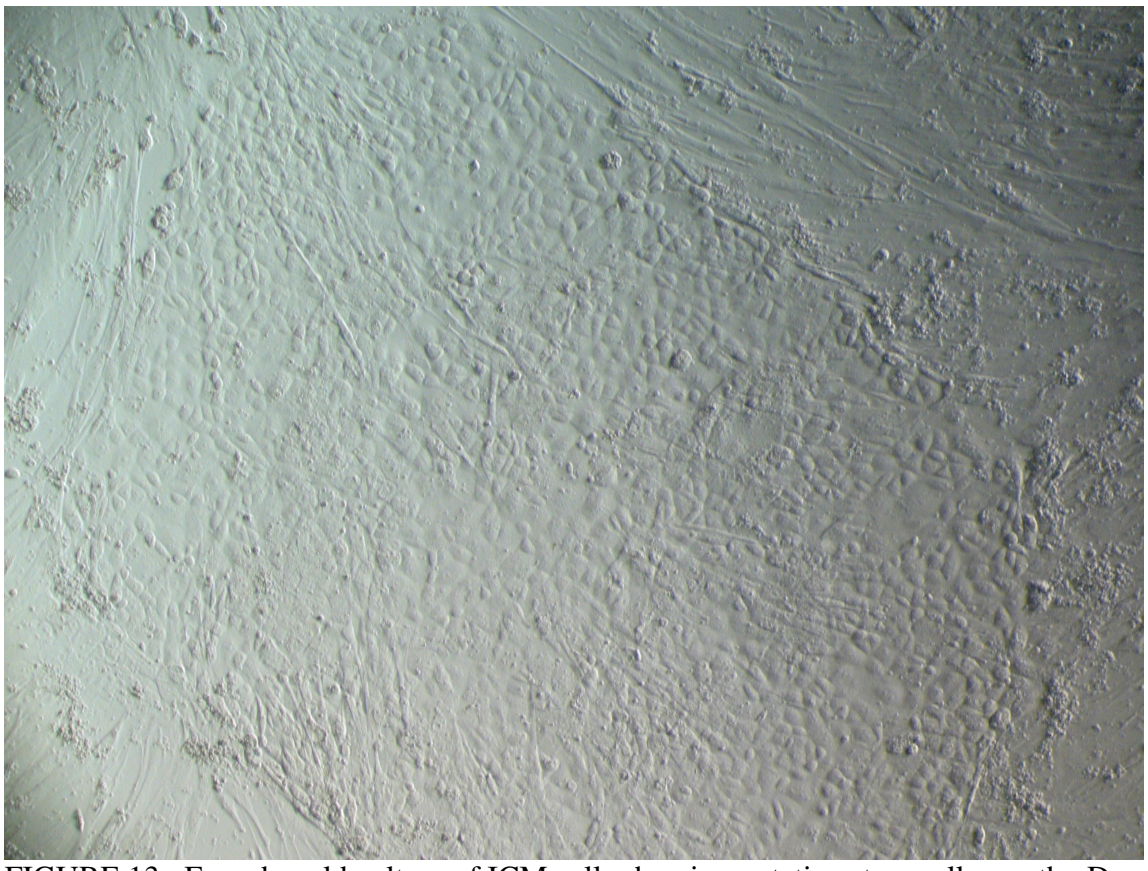

FIGURE 13. Four day old culture of ICM cells showing putative stem cell growth. Day 7 in-vivo recovered blastocyst was mechanically dissected and cultured in ES media on inactivated equine fetal fibroblasts. Passage attempts resulted in loss of growth of these cells.

Immunocytochemistry of cultured cells

All nuclei of the unattached vesicles stained for Oct-4, and the signal was isolated to the nuclei. Notably, there seemed to be some sporadic staining for Oct-4 among the cells of the feeder layer when attached vesicles were stained in situ (Fig. 14). These nuclei had Oct-4 signal equally as bright as the nuclei of the cells with which they were co-cultured. Proximity in culture to the blastocyst or cells appears unrelated to this phenomenon, as cells were evenly distributed throughout the well.

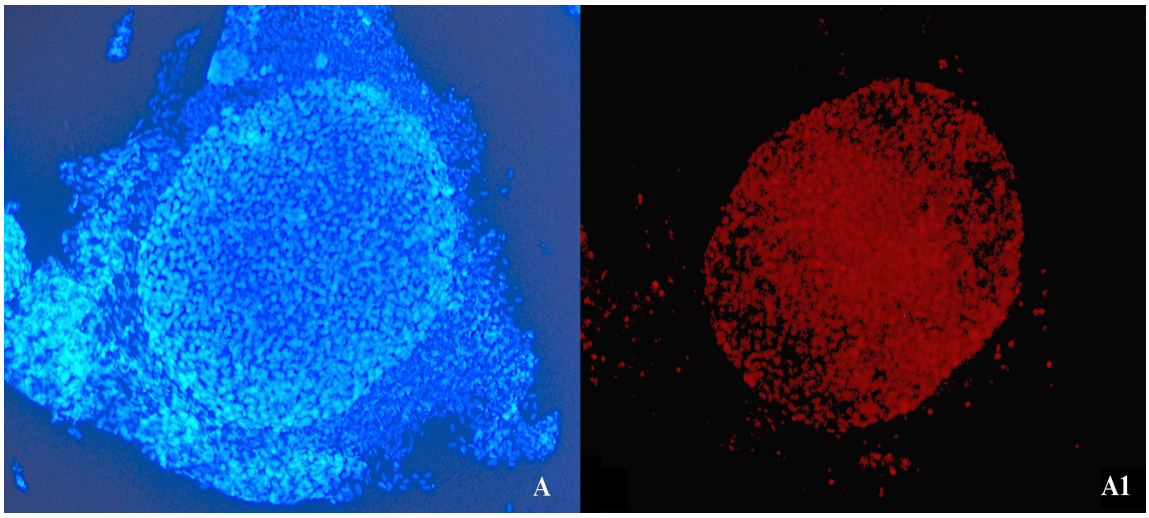

FIGURE 14. In situ Oct-4 staining of unattached vesicle growing in culture on inactivated equine fetal fibroblasts. Image shows Oct-4 staining of the vesicle and sporadic staining of nuclei apparently within the feeder layer. The zona was removed from this Day 7 in vitro-produced blastocyst, and the blastocyst was then placed on confluent, inactivated equine fetal fibroblasts for 3 days before staining. Culture was stained with Hoechst 33258 for DNA (blue, A) and Oct-4 (red, A1).

The embryonic cells which attached and grew on the feeder layer showed sporadic staining Oct-4 protein. In various areas of the same culture, a single cell, a small group of cells (<10), or a tightly packed aggregate of cells showed strong Oct-4 signal on staining, while surrounding and otherwise morphologically indistinguishable cells did not stain for Oct-4 (Fig. 15). As seen in Table 9, of the 9 randomly-selected viewing areas, the percentage of Oct-4 staining cells ranged from 2.04-77.3%, with an average of 19.4% Oct-4 staining cells.

TABLE 9. Expression of Oct-4 in embryonic cell layers.

Layer	Oct-4 nuclei (red)	Hoechst nuclei (blue)	% Oct-4 staining nuclei
A	1	21	4.76
	5	20	25.0
	8	123	6.50
B	2	98	2.04
	13	142	9.15
	221	286	77.3
C	1	10	10.0
	8	42	19.0
	15	73	20.5
AVERAGE			**19.4**

Randomly selected viewing areas of ICM-resultant cultures were evaluated for the proportion of Oct-4 expressing nuclei following immunocytochemistry.

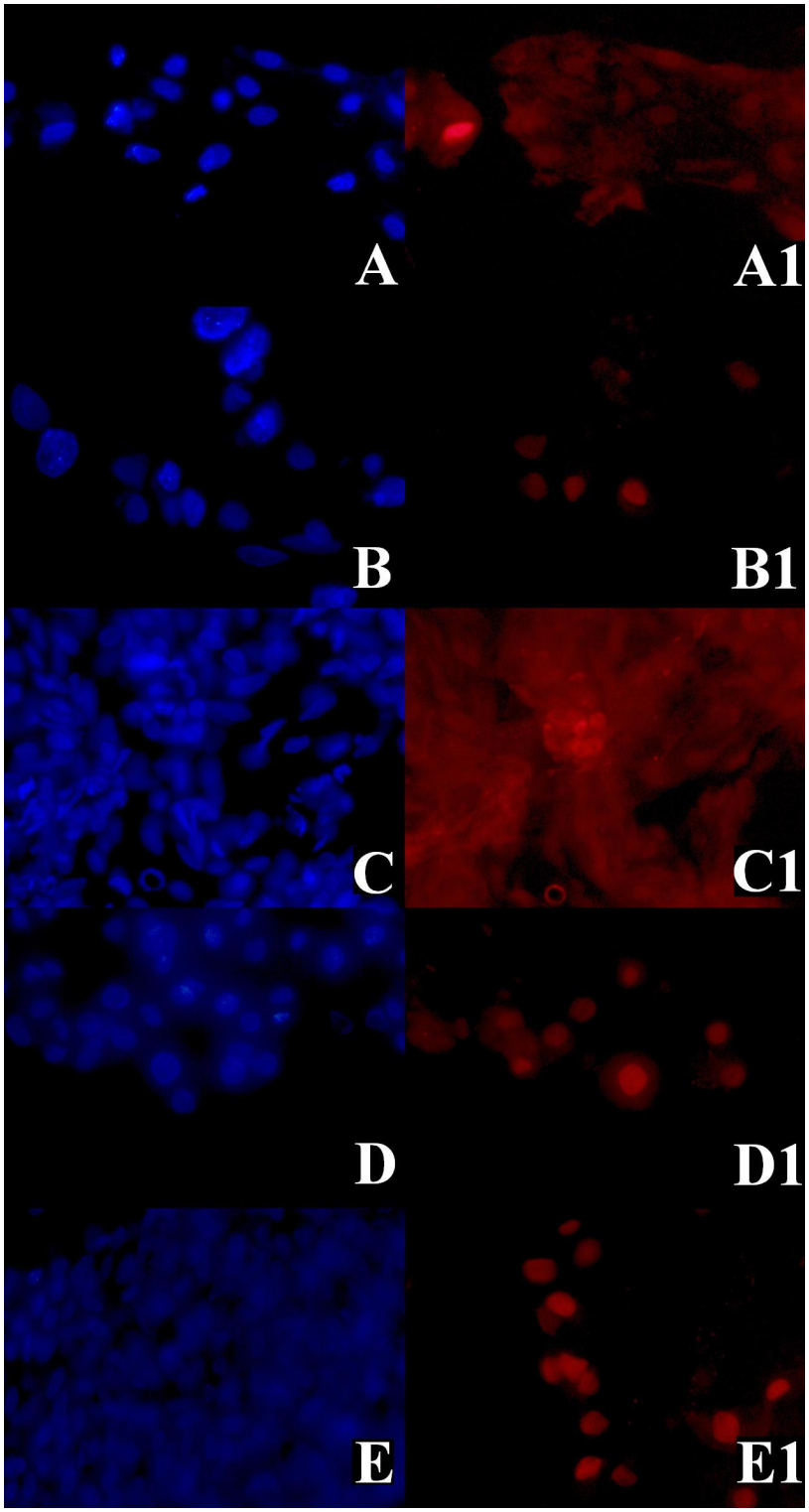

FIGURE 15. Sporadic expression of Oct-4 in ICM cells (7-day culture) growing on feeder layers. Cells were stained with Hoechst 33258 for DNA (blue, left) and Oct-4 (red, right).

DISCUSSION

Despite the many attempts to establish ES cells in culture from in vitro-produced equine embryos, no successful growth of morphologically identifiable stem-like cells was obtained. However, immunocytochemistry revealed pockets of Oct-4 staining (and thus potentially pluripotent) cells within the apparently differentiated cells growing on the feeder layers. If these do represent stem cells (or, because of their sporadic appearance, primordial germ cells) the challenge is to recognize these cells morphologically to select them for passage.

Failure of robust growth of morphologically identifiable stem-like cells may result from a variety of causes, such as the poor development of in vitro-produced embryos, the difficulty in encouraging cells to attach to the feeder layer, and the failure of the support system of the feeder layer or the media. In addition, species differences in morphology and culture requirements of stem cells have been reported [64]. In addition, there are species-specific differences in the expression of the previously discussed pool of well-known marker genes. Since there is only 1 previous report on establishment of embryonic stem cells in the horse [10], little is known about the requirements of this species and its exact expression profile. In this study, ES-like cells were identified by immunocytochemistry for SSEA-1 and STAT-3, RT-PCR for a number of marker genes (including Oct-4), and induced differentiation to 3 precursor cell types.

As evidenced by size and capacity to produce blastocysts, in vitro-produced embryos are developmentally retarded compared to in vivo-produced embryos of the same age. In vitro-produced embryos are much smaller and have a much lower

blastocyst development rate than their in vivo counterparts [65]. Because of this delayed development, in vitro-produced embryos at the stage investigated here (Days 7-10) may not have an ICM capable of forming outgrowths and developing into ES-like cells. The ES-like cells reported by Saito et al. [10] were grown from in vivo-recovered blastocysts, with which 5 attempts were made in this study. The previously described in vivo-recovered blastocyst ICMs were isolated by a different method, however, and bovine feeder cells were used to support growth rather than equine cells. In addition, the ICMs of the 5 in-vivo recovered embryos used here may have been damaged by the Triton-X treatment.

Although ES-like cell isolation in other species has become more efficient as protocols become more refined, there is still a significant amount of failure in this process because the embryonic cells do not attach to the plate surface. Early murine ES cell isolation claimed a success rate of 1/8, with about 30 isolated ICMs plated at a time in a dish [34]. In cattle, 3/14 plated demi-blastocysts formed attachments and created outgrowths [38], and the 1 published equine study reported that 2/3 of their isolated ICMs attached [10]. Another bovine investigation reported plating ICM cells beneath a feeder layer after encountering difficulty getting cells to attach [39]. The attempts to grow ES-like cells depend heavily on the characteristics of the feeder layer and on the medium used; more work is needed to define the optimum conditions for their growth, particularly in equine cells.

Finding an acceptable culture system for equine blastocyst development has been a significant challenge in recent years. Recent use of a complicated medium containing

components shown to be detrimental to embryo development of other species (DMEM/F-12) doubled the equine blastocyst development rate [66]. As mentioned above, however, the ICM is not readily identifiable in in vitro-produced equine blastocysts because of the lack of a well-defined blastocoel. Therefore, this culture medium (which is the base for the ES medium used in this study) could be encouraging the expansion of trophoblast cells while neglecting the ICM. Further, this medium could be beneficial at the blastocyst stage but detrimental once the cells of the ICM have been isolated. In addition, this is the first comprehensive study on the use of equine fetal fibroblasts as a feeder layer; these cells could prove a less hospitable environment than a feeder layer derived from a different species. A direct comparison of the ability of equine and murine feeders to support growth of ICM cells was not performed, and should be in future plans in this area.

Although the goal of establishing ES-like cells in culture was not reached, significant progress was made. Oct-4 staining cells, suggesting pluripotency based on evidence in other species, were found growing in culture. Further work is needed to better define these cells by staining for other stem-cell associated factors, and to refine the culture medium to support these cells.

CHAPTER V

CONCLUSIONS

This work represents the first examination of Oct-4 expression in the equine embryo. Although previously used in RT-PCR with a number of other genes as a marker for pluripotency of stem-like cells [10], no examination of the spatial and temporal expression patterns of Oct-4 protein have been performed in equine embryos of in vivo or in vitro origin. While some questions remain, significant progress has still been made toward understanding the expression patterns of Oct-4 in equine embryos and establishing a method for ES cell isolation from in vitro-produced equine embryos.

Results of Oct-4 staining in these studies indicate equine early development is similar to that reported for human embryos rather than that of murine, bovine, or monkey embryos. As in the human, Oct-4 expression is variable before Day 6 of development. As this timing coincides with the maternal-zygotic transition, it is likely that the expression pattern reflects the depletion of maternal protein and then replenishment after activation of embryonic transcripts. Oct-4 protein and mRNA are present in both ICM and trophoblast up to Day 10, as in human blastocysts. This indicates Oct-4 may play a different or additional role in these species as compared with others in which Oct-4 expression is lost from the trophoblast at the blastocyst stage. In addition, this indicates the equine embryo could be a potential model for the human embryo for embryonic cell pluripotency. In vivo factors appear to influence the ICM (increase expression of Oct-4) when compared with in vitro-cultured blastocysts. We found that the visualization method used to view the embryos after antibody staining (i.e.

in droplets vs. mounted under coverslips) is of importance in identification of an ICM-like mass after Oct-4 staining of equine embryos. Unfortunately, the majority of the work on blastocyst Oct-4 was performed prior to this discovery, but future studies can take full advantage of this and rely on both visualization methods for more accurate results.

Equine fetal fibroblasts have not been previously studied for use as a feeder layer. This study established a number of defined criteria for rapid and efficient production of a feeder layer of MMC-inactivated equine fetal fibroblasts. The equine fetal fibroblasts proved to be hardy and robust, and cell loss due to manipulation (transferring, plating, etc) was minimal (15% loss). However, once cells were treated with Mitomycin C and/or frozen, their resiliency declined dramatically. An improved method to inactivate and store cells while maintaining some of the hardiness of the cells seen before this treatment will greatly improve this process. The results indicate some concentration of MMC between 2 and 20 µg/ml might alleviate some of the damage seen while maintaining deactivation activity. However, these trials provide a guideline for effective establishment of inactivated feeder layer of equine fetal fibroblasts.

Culture on the feeder layers to produce stem cells had equivocal results. Although the morphology of the cells grown in culture did not match those of typical embryonic stem cells as reported in other species (and in the 1 report on work performed with equine embryos), Oct-4 staining cells (potentially indicating pluripotency, based on data in other species) were successfully recovered from equine in vitro-produced embryo ICMs, and these cells grew in culture. These cells cannot be deemed stem-like cells

because they have an atypical morphology and cannot survive passaging, yet individual cells within these growths stained for Oct-4. In addition to finding that Oct-4 staining cells can be derived from in vitro-produced blastocysts in culture, Oct-4 staining cells were also found in the feeder layer following co-culture of an in vitro-produced embryo. The relationship of equine ES-like cells and equine feeder fetal fibroblasts should be further evaluated.

Further work to examine developing equine embryos with improved visualization techniques, test the efficacy of equine feeders, and refine the culture system for equine ES cell isolation are necessary steps to finally grow ES cells in culture and better understand early development of the equine embryo.

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