

Analysis of Oct-4 Gene Expression in *in vitro* Cultured Bovine Blastocysts

Kim M. Chau
Southwestern University

Dr. Neal L. First and Dr. Zeki Beyhan
Department of Animal Science
College of Agriculture and Life Sciences

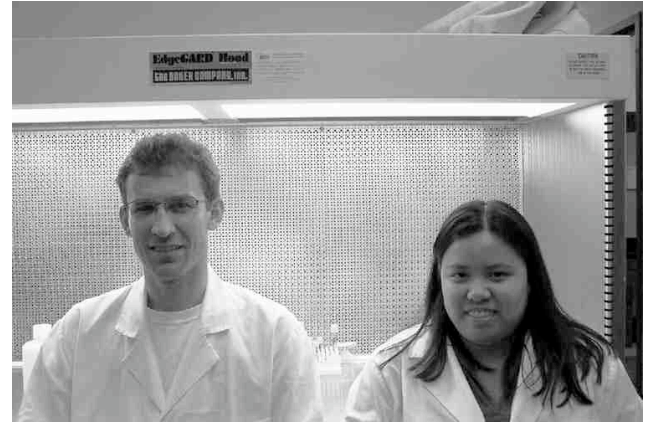
Abstract

The transcription factor Oct-4 is thought to be very important to early embryonic development and differentiation. Most studies on Oct-4 have been performed on murine embryos. These studies show that Oct-4 expression is limited to the inner cell mass (ICM) in blastocysts. The presence of Oct-4 as well as the level of Oct-4 expression has been shown to be important. Oct-4 null embryos fail, as do the majority of cloned embryos, most of which express Oct-4 abnormally. The few studies on Oct-4 in bovine embryos suggest that Oct-4 is expressed in the trophoblast as well as the ICM. However, these studies do not explain why this is the case, nor do they measure the level of Oct-4 expression. In this study, bovine blastocysts were analyzed for Oct-4 gene expression and for localization of Oct-4 protein. We suggest that the level of Oct-4 expression is varied among bovine embryos and that Oct-4 may be localized in the ICM.

Introduction

Oct-4 is a transcription factor that is coded for by the Pou5f1 gene. Transcription factors are proteins that bind to DNA and activate or repress transcription of particular genes. Oct-4 is thought to influence several genes expressed during early embryonic development, and thus, may be very important to the processes of development and cell differentiation.

In mammalian embryonic development, two distinct cell layers are present at the 64-cell stage; the external cells become the trophoblast, which eventually becomes part of the placenta, and the internal cells become the inner cell mass (ICM), which gives rise to the embryo. ICM cells can be isolated and cultured *in vitro* to form embryonic stem (ES) cells. ES cells are pluripotent, which means they are capable of giving rise to all different types of cells found in a particular organism. The trophoblast cells secrete fluid to form an internal cavity called the blastocoel, which lies on one side of the ICM. This resulting structure is called a blastocyst (Fig. 1).



In murine (mouse) blastocysts, Oct-4 expression is limited to the ICM (7). Oct-4 expression is also limited to undifferentiated cells such as ES and embryonic germ (EG) cells. It has been suggested that Oct-4 may be uniquely expressed in pluripotent cells such as ES and EG cells (11).

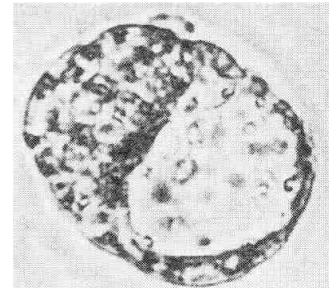


Figure 1. Expanded murine blastocyst *in vitro* (3, courtesy of J. G. Mulnard).

Maintaining Oct-4 expression within a certain range maintains stem cell renewal. However, increased Oct-4 expression triggers differentiation to endoderm or mesoderm and decreased Oct-4 expression triggers dedifferentiation to trophoblast (6) (Fig. 2).

Oct-4 null embryos develop to the blastocyst stage but fail after implantation. Further analysis showed that the blastocysts were composed of trophoblast cells and no ICM cells (5). These data suggest that Oct-4 plays a central role during cell differentiation in developing embryos. The majority of cloned embryos also fail, and analysis showed that most cloned murine embryos expressed Oct-4 in both trophoblast and ICM or expressed Oct-4 irregularly (2) (Fig. 3).

Most Oct-4 studies have been done in murine (mouse) embryos. The very few that have been done on bovine (cow) embryos show a different pattern of Oct-4 expression. Although the murine and bovine Oct-4 gene and Oct-4 protein are highly homologous, it has been shown that bovine Oct-4 expression is not limited to the ICM and is found in trophoblast as well (10, 4) (Fig. 4).

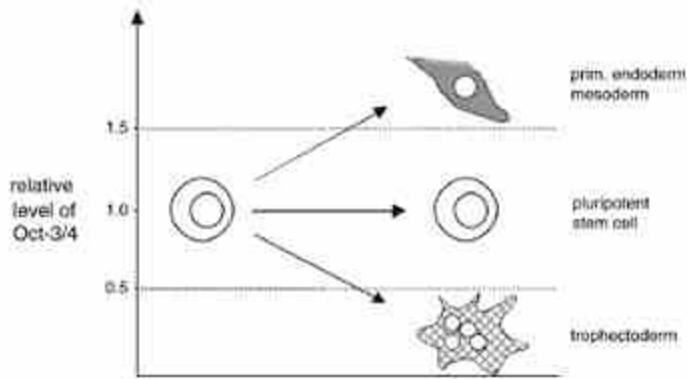


Figure 2. Relationship between Oct4 expression level and stem cell fate. Maintenance of Oct-4 expression within 50% of the normal expression maintains stem cell renewal. Increased Oct-4 expression triggers differentiation into endoderm or mesoderm. Decreased Oct-4 expression results in trophectoderm. (6)

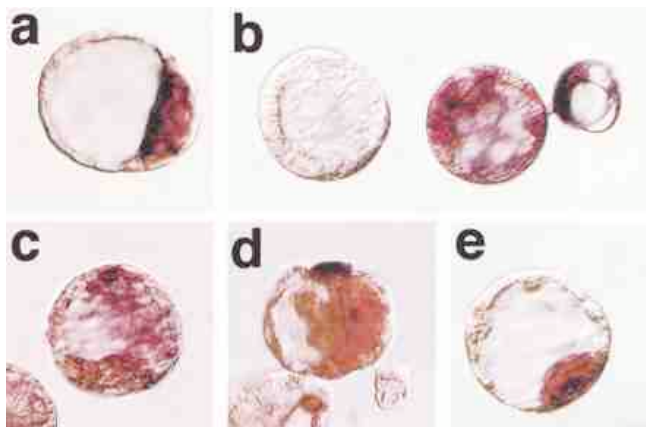


Figure 3. Oct-4 mRNA distribution in murine blastocysts. (a) Fertilized embryo showing Oct-4 localized to the ICM. (b-d) Cloned embryos with abnormal Oct-4 expression. (e) Cloned embryo with Oct-4 expressed in the ICM. (2)

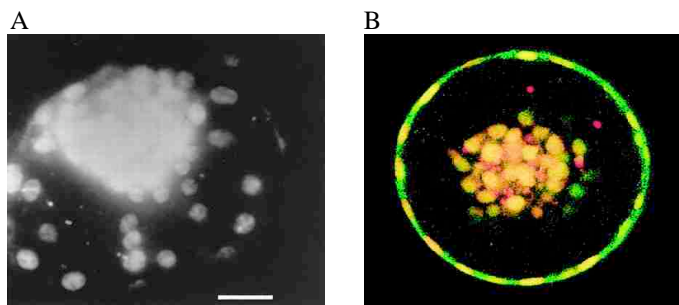


Figure 4. *In vitro* produced bovine blastocysts showing Oct-4 protein in both the ICM and trophectoderm. (a) Hatched bovine blastocyst immunostained for Oct-4. (From van Eijk et al. 1999). (b) Another bovine blastocyst immunostained for Oct-4. (4)

However, these bovine studies do not help explain why this is the case, nor do they measure the levels of Oct-4 expression.

It seems unlikely that early embryonic development would be so varied among mammals, especially between mouse and cow,

since their Oct-4 genes and proteins share high homology. Therefore, we hypothesized that bovine Oct-4 expression would be similar to that of the mouse; i.e., Oct-4 would only be expressed in the ICM and not in the trophectoderm. From these preliminary results, we suggest that bovine Oct-4 expression may be limited to the pluripotent ICM cells.

It is important to study embryonic Oct-4 expression because Oct-4 is the earliest known transcription factor to be developmentally expressed and such studies would further elucidate the mechanisms of embryonic development. Specifically, in the area of bovine cloning and nuclear transfer, since cloned embryos are not very viable, information about the mechanisms of early development would assist in creating more efficient cloning methods or ES cell-based technologies.

In this study, we looked at Oct-4 gene expression at the mRNA level and at the protein level in bovine blastocysts to help evaluate the role of Oct-4 during early bovine development. Oct-4 mRNA was semi-quantitatively assayed in parts of blastocysts, and Oct-4 protein was localized in whole blastocysts. We evaluated distribution of Oct-4 in bovine blastocysts.

Methods

In vitro maturation of oocytes

Bovine ovaries were collected from a local slaughterhouse. Oocytes were obtained by aspirating small antral follicles (2-6 mm in diameter) with an 18-gauge needle. The follicular contents were allowed to settle and the resulting sediment was diluted with wash medium (TL-HEPES, 1) supplemented with 3 mg/ml polyvinylpyrrolidone (PVP, Sigma Chemical Co., St. Louis, MO), 0.2 mM sodium pyruvate, and 25 µg/ml gentamycin (Sigma Chemical Co., St. Louis, MO) and searched for cumulus oocyte complexes (COCs) under a stereo microscope. The COCs were washed 3 times in the wash medium and then matured in 50 µl drops (10 COCs per drop) of maturation medium (TC-199 with Earl's salts, Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-treated fetal calf serum (FCS, Summit, Ft. Collins, CO), 0.2 mM sodium pyruvate, 25 µg/ml gentamycin, 1 µg/ml estradiol-17β, 5 µg/ml luteinizing hormone (LH, NIH oLH, 0.023 units), and 0.5 µg/ml follicle stimulating hormone (FSH, NIH oFSH, 0.02 units) under mineral oil. COCs were matured at 39°C with 5% CO₂ in air and high humidity for 24 hrs.

In vitro fertilization

Matured COCs were transferred to 44 µl drops (10 COCs per drop) of fertilization medium (TL Stock, 8, supplemented with 6 mg/ml bovine serum albumin-fatty acid free (BSA-FAF), 0.2 mM pyruvate, and 25 µg/ml gentamycin) under mineral oil. Sperm was prepared using percoll separation (9) and diluted to 1 x 10⁶ sperm/ml. Sperm, 2 µg/ml heparin, and 1 mM hypo-

taurine/2 mM penicillamine/0.25 mM epinephrine (PHE) were added to the fertilization medium drops. COCs were incubated at 39°C with 5% CO₂ in air and high humidity for 24 hrs.

Embryo culture

Twenty-four hrs after fertilization, *in vitro* fertilized (IVF) embryos were stripped free of cumulus cells by repeated pipetting through a narrow fire-polished pipette, washed once in wash medium and once in phosphate-buffered saline (PBS), and transferred to 50 μ l drops (10 embryos per drop) of culture medium (CR1aa, 1). 10% FCS was added to the CR1aa on the fourth day of culture. Blastocysts were collected on the eighth day of culture.

mRNA isolation from blastocysts

Poly(A) RNA was isolated from blastocysts using the PolyATtract 9600 mRNA Isolation Kit (Promega Corp., Madison, WI). Expanding and hatched blastocysts were collected on the eighth day of culture, put into separate tubes in groups of 3-5, lysed with GTC extraction buffer, and frozen at -80°C until needed. All samples were transferred to a v-bottom 96-well plate containing hybridization buffer with biotinylated oligo(dT) and incubated for 5 min. Cellular debris and precipitated proteins were removed from the mixture by centrifugation in the presence of blocking particles. The samples were then incubated with pre-washed streptavidin-paramagnetic particles (SA-PMPs). mRNA/biotin-oligo(dT)/SA-PMP complexes were collected with a special 96-pin array and a magnet pack. mRNAs were eluted in nuclease-free water and biotin-oligo(dT)/SA-PMP complexes were removed magnetically. The purified poly(A) RNAs were used immediately following for reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR

Poly(A) RNA isolated from individual IVF embryos was reverse transcribed into cDNA in a total volume of 15 μ l using 2.5 μ M poly(T) primers, 50 μ M of each dNTP, and 10 units of AMV-RT in the presence of a ribonuclease inhibitor. Reverse transcription reaction was performed at 37°C for 45 min and stopped by heat-inactivating at 95°C for 5 min. PCR was performed on cDNAs equivalent to half of an embryo using 1 μ M primers for bovine Oct-4 and ATPase (subunit 6) under standard conditions using Taq DNA Polymerase Master Mix (Promega Corp., Madison, WI). To ensure specific amplification, a “hot start” PCR was employed by adding starting materials at 90°C. The PCR program had an initial step of 95°C for 2 min followed by 35 cycles of 30 sec each at 95°C for DNA denaturation, 30 sec at different temperatures for annealing of primers, and 60 sec at 72°C for primer extension. The last cycle was followed by a 7 min extension at 72°C. Tubes prepared without template DNA were used as negative controls. Tubes prepared with bovine genomic DNA were used as positive controls.

Detection and semi-quantification of RT-PCR products

RT-PCR products were separated by agarose gel electrophoresis using a 2% agarose gel stained with ethidium bromide and visualized with UV light. The intensity of each band in the gel was quantitated by densitometry and the relative amount of mRNA was calculated by dividing the sample Oct-4 intensity by the ATPase intensity. Image analysis was performed using NIH Image 1.62 software. ANOVA was used to analyze intensity data.

Immunocytochemical localization of Oct-4

Four embryos per slide were attached to polylysine coated coverslips and fixed in 0.5% formaldehyde in PBS overnight. Embryos were permeabilized in 0.1% Triton X-100 in PBS overnight. Non-specific antibody binding reactions were prevented by incubating Biotechnology, Inc., Santa Cruz, CA) for 40 min coverslips in 1% glycine in PBS and 1% dry milk in PBS solutions before staining. Embryos were incubated with 1:20 anti-human Oct-4 polyclonal antibodies (Santa Cruz at 37°C. The embryos were then rinsed in PBS and incubated for 40 min with 1:100 FITC-labeled anti-goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The embryos were incubated with DAPI (2 μ g/ml) for 10 min. Coverslips were mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA) to retard photobleaching. The slides were examined by Nikon fluorescence microscopy at wavelengths of 565/420 and 485/520-560 for FITC and DAPI respectively. Control embryos were treated similarly, except that Oct-4 antibodies were omitted.

Results

Expression and levels of Oct-4 mRNA in blastocyst-stage embryos

Oct-4 expression analysis was performed in 4 different experiments using a total of 26 bovine blastocysts (Table 1). A sample experiment and corresponding pixel analysis is shown (Fig. 5). Oct-4 relative abundance data was calculated for each sample that expressed Oct-4 by dividing the Oct-4 intensity by the corresponding ATPase intensity. RT-PCR analysis (Fig. 5a, 5c) for Oct-4 mRNA showed that Oct-4 expression is not the same in all embryos. Out of 26 total embryos, 17 (65%) expressed any amount of Oct-4. Among those that did express Oct-4, pixel intensity analysis revealed that the level of expression was highly variable with an average relative abundance of 0.17 ± 0.04 .

Distribution of Oct-4 protein in blastocysts

A total of 2 IVF-produced bovine blastocysts were used for immunocytochemistry—1 for the experiment and 1 as a control

Experiment	Embryos <i>n</i>	Embryos expressing Oct-4 <i>n</i> (%)	Mean level of Oct-4 expression
1	5	3 (60)	0.16 ± 0.08
2	5	3 (60)	0.25 ± 0.10
3	6	5 (83)	0.07 ± 0.04
4	10	6 (60)	0.18 ± 0.05
Overall	26	17 (65)	0.17 ± 0.04

Table 1. Qualitative and quantitative expression of Oct-4 mRNA in bovine IVF blastocysts

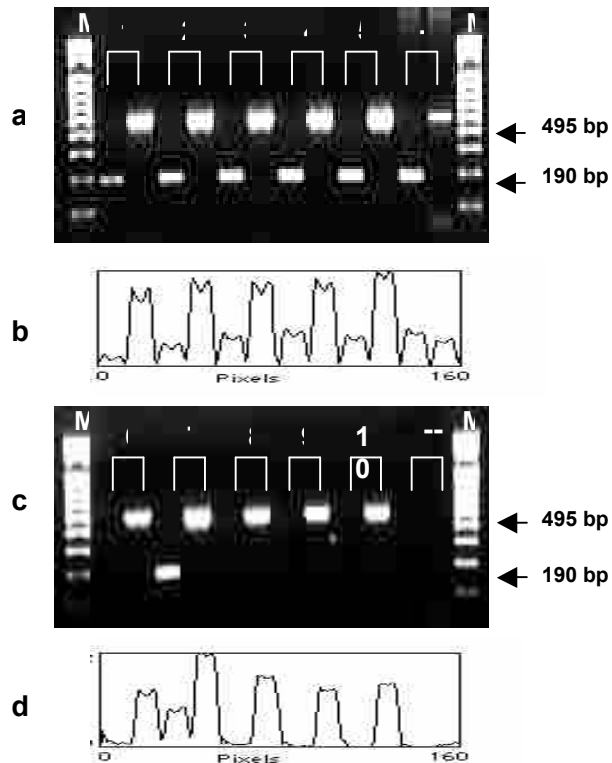


Figure 5. Sample analysis of an experiment. (a, c) RT-PCR analysis of 10 embryo samples (lanes 1-10), a positive control (+), and a negative control (-) separated by agarose gel electrophoresis. Lanes M denote a 100 bp ladder. Arrows point to the Oct-4 bands (190 bp) and the ATPase bands (495 bp). (b) Pixel intensity graph for the bands shown in (a). (d) Pixel intensity graph for the bands shown in (c).

(Fig. 6). In the experimental blastocyst, Oct-4 was detected in the ICM, but not in the rest of the embryo (Fig. 6b). No Oct-4 staining was detected in the control blastocyst in which the primary antibody was omitted (Fig. 6d).

Discussion

In this study, we suggest that bovine IVF blastocysts express Oct-4 variably. This may be due to individual variation among embryos in development. Blastocysts may have been at different developmental stages, and some of them may have

started to die. In this case, a simple apoptosis assay could be used to assess blastocyst quality.

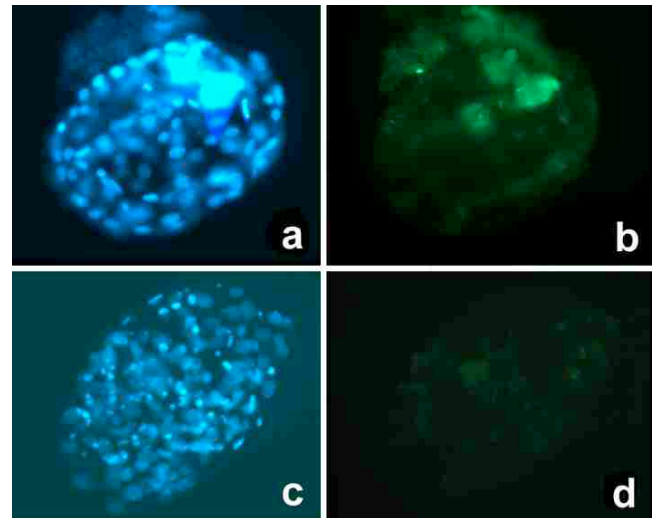


Figure 6. Localization of Oct-4 protein in an expanded bovine blastocyst viewed under fluorescent microscopy at 200X. (a) DAPI staining to visualize the nuclei. (b) Oct-4 staining is limited to the ICM. (c) DAPI staining on a control blastocyst. (d) Oct-4 staining is not visible in the control embryo.

We also suggest that Oct-4 expression in bovine blastocysts may be unlike that of murine blastocysts; i.e., restricted to the ICM. One trial does not give us enough data to support our hypothesis of Oct-4 expression in the ICM, so more replicates will have to be done. However, our preliminary results do support our hypothesis, and we suggest that further study will provide more support. Such experiments should also include *in situ* hybridization to localize Oct-4 mRNA. Such data, along with Oct-4 protein localization from immunocytochemistry, would provide a more complete picture of Oct-4 gene expression.

It has been previously shown that the level of Oct-4 expression is important in mice embryos (6), but such studies have not been performed on bovine embryos. Quantification of Oct-4 expression on whole embryos is not sufficient to correlate expression levels with development. It has been shown in mice that in the blastocyst stage, Oct-4 expression is limited to the ICM (7). Therefore, in future studies, it would be necessary to isolate the ICM from the trophectoderm, and measure the level of Oct-4 expression in each. Even though studies on bovine blastocysts have found Oct-4 to be expressed in the trophectoderm and ICM (10, 4), the level of expression may be the key factor in bovine cell differentiation.

Most cloned murine blastocysts express Oct-4 abnormally, and it is suggested that the frequency of abnormal Oct-4 expression can alone account for the low success rate of cloning (Boiani et al. 2002). It would be interesting to do a similar study on Oct-4 expression in cloned bovine embryos and compare the Oct-4 expression rate with current bovine cloning success rates. Interestingly enough, our experimental rate of Oct-4 expression

in bovine IVF embryos (65%) is not too different from bovine IVF success rates, and might be indicative of embryonic developmental competence.

The processes of development and differentiation are complex, and Oct-4 may be just one of the key regulators. It will be important to learn about these processes to develop more efficient cloning procedures and technologies.

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