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# Effects of culture medium and substrate on attachment of *in vitro* produced bovine embryos

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## Abstract

The study of large animal embryonic stem cells (ESCs) *in vitro* has implications for the understanding of lineage differentiation and transgenesis. The first step for ESC derivation is the attachment of the embryo to a substrate on which they can form outgrowths. However, the culture conditions for large animal embryo attachment and ESC derivation have not been studied extensively. Defining culture conditions for embryo attachment such as culture medium and substrate is an important first step for derivation of inner cell mass-derived stem cells. The aim of this study was to compare different types of culture media and substrates for their ability to support attachment of *in vitro* produced bovine embryos in culture. Bovine embryos were produced *in vivo* following established protocols. Blastocysts formed on day 8 after fertilization were transferred to 12-well culture plates containing different types of culture media (Dulbecco's Modified Eagle Medium, DMEM or Medium 199, M199) and substrates [bovine fetal fibroblasts, goat fetal fibroblasts, mouse embryonic fibroblasts (STO) or non-cellular substrates (gelatin, laminin, fibronectin)]. Percentage of attached embryos and number of days since fertilization required for attachment were recorded. Bovine blastocysts preferrably attached to feeder cells rather than non-cellular substrates and there was an interaction of feeder cell type and culture medium used. Therefore, the choice of both feeder cell type and culture medium has to be considered when optimizing conditions to derive cell lines from bovine embryos.

**Keywords**: blastocysts, culture, embryo feeder cells.

## Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of early blastocyst stage embryos. ESCs are pluripotent; they express genes associated with pluripotency (such as Oct-4 and Nanog) and proliferate long term *in vitro* in an undifferentiated state when supplemented with appropriate factors such as leukemia inhibitory factor (LIF; Williams *et al*., 1988; Behboodi *et al*., 2011). In the absence of those factors, ESCs can be induced to differentiate into specific cell types from all the three germ layers (endoderm, mesoderm, and ectoderm).The first studies on ESC derivation were reported from mouse embryos (Evans and Kaufman, 1981; Martin, 1981).

ESCs can be maintained on feeder cells that secrete factors supporting proliferation of ESCs in an undifferentiated state (Reubinoff *et al*., 2000). For example, an immortalized mouse embryonic fibroblast cell line, STO, has been genetically modified to secrete LIF and used as feeder cells (Smith *et al*., 1988). Feeder cells are grown to sub-confluence before they are mitotically deactivated (by irradiation or treatment with mitomycin-c), after which the feeder cells cease to proliferate but still secrete the factors necessary to maintain ESCs in an undifferentiated state. Methods have been established to derive ESCs without the need for feeder cells, as ESCs are to be eventually removed from the feeder cells for further characterization and there is also a need for xeno-free derivation of human ESCs (Xu *et al*., 2001). ESCs derived under feeder-free conditions require constant presence of growth factors such as LIF and/or basic fibroblast growth factor (bFGF) to suppress differentiation and still require an adhesion surface. Various coating layers have been used in mouse and human ESC studies, including gelatin, laminin, fibronectin, poly-d-lysine, and collagen, with varying differentiation tendencies for different ESC lines. For example, one ESC line remained undifferentiated on collagen or poly-d-lysine but differentiated on laminin or fibronectin in a mouse study (Hayashi *et al*., 2007), and a human ESC line remained undifferentiated in collagen, fibronectin, and laminin in a human stem cell medium (Ludwig *et al*., 2006).

ESC derivation has been studied most extensively in mice and humans, and with varying degrees of success in other species such as non-human primates (NHP; Thomson *et al*., 1995), rabbits (Graves and Moreadith, 1993), equine (Saito *et al*., 2002), swine (Chen *et al*., 1999), bovine (Stice *et al*., 1996), and caprine (Behboodi *et al*., 2011). Although NHPs closely resemble humans in their physiology, the use of this model for ESC research is costly, justifying the need for establishing other ESC model organisms that resemble human physiology. Domestic animals (bovine, porcine, caprine) may provide an outbred experimental model in stem cell research and in pre-clinical stem cell therapy

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trials. The use of *in vitro* produced bovine embryos for deriving ESCs may be a suitable approach due to the availability of research materials and past use of bovine embryos in this research area. The study of bovine embryos may also provide a model for studying human embryonic stem cells. For instance, a method was developed for parthenogenetic activation of bovine oocytes or *in vitro* produced embryos as a model to define conditions for producing human embryonic stem cells (Ruggeri *et al*., 2012).

The first step for derivation of ESCs is the attachment of the embryo or ICM cells to a substrate on which they can form an outgrowth in culture; a process which involves interaction between blastocyst and substrate. Mouse and human blastocysts have been systematically studied for their attachment behaviour *in vitro*. For example, the effects of different amino acids on mouse blastocyst attachment and outgrowth were compared (Gwatkin, 1966). Studies of culture conditions for bovine embryo attachment behaviour have also been conducted. Cong *et al*. (2012) used mitomycin-c deactivated STO cells which supported derivation of bovine embryonic stem-like cells that were positive for pluripotency markers such as Oct-4, SSEA-1 and SSEA-4. In another study, mouse embryonic fibroblasts were found to support better attachment of bovine blastocysts and support better growth of ICM-derived ES-like cells than bovine embryonic fibroblasts, although the derived embryonic-like stem cells from both feeder types were positive for Oct-4, SSEA-1 and SSEA-4 (Jin *et al*., 2012). Few studies have specifically investigated attachment rates of bovine embryos on substrate and in different media. Among the first authors to study bovine embryo attachment were Kuzan and Wright (1982b) that compared two types of media and various substrates, and found that bovine embryos attached best on minimal essential medium (MEM) with bovine uterine and testicular fibroblasts. The same authors reported a similar phenomenon for porcine blastocysts (Kuzan and Wright, 1982a). Another study compared the attachment of mouse, human, and bovine blastocysts on uterine epithelial cells cultured in minimum essential medium (MEM), but did not compare the effects of different media or substrates (Lindenberg *et al*., 1989).

We hypothesized that culture media and substrates have differential effects on attachment of *in vitro* produced bovine embryos in culture, and that the interaction between media and substrates can influence embryo attachment. Therefore, the objective of this study was to compare different types of culture media and substrates for their ability to support attachment of *in vitro* produced bovine embryos in culture.

## Materials and Methods

### In vitro *maturation of bovine oocytes*

Bovine embryos were produced using oocytes recovered from slaughterhouse ovaries and cryopreserved sperm, following established protocols (modified from Parrish *et al*., 1986; Krishnakumar *et al*., 2011). All materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. Briefly, two to twenty four hours prior to ovary collection, maturation medium was prepared, containing 200 µM sodium pyruvate, 50 µg/ml gentamycin, 4 µg/ml follicule-stimulating hormone (FSH), 6.4 µg/ml luteinizing hormone (LH), 1 µg/ml estradiol-17β, and 10% fetal calf serum (FSC) in M199 without HEPES (GIBCO, Life Technologies, Burlington ON). Subsequently, 500 µl/well of maturation medium was placed in a four-well plate and covered with embryo culture grade mineral oil at 5% $CO_2$, atmospheric $O_2$, and 39°C. Flushing medium that was used for oocyte washing was also prepared, containing 200 µM sodium pyruvate, 50 µg/ml gentamycin, and 3 mg/ml bovine serum albumin (BSA) in M199 with HEPES (GIBCO, Life Technologies, Burlington ON). The flushing medium was placed at 5% $CO_2$, atmospheric $O_2$, and 39°C.

Upon receiving ovaries carried in a cooler, the ovaries were washed with warm saline solution and antral follicles 3-10 mm in diameter were aspirated for collection of cumulus-oocyte complexes (COCs). COCs were washed in flushing medium and then in maturation medium at 37°C, placed in four-well plates at 20 oocytes/well containing 500 µl/well of maturation medium, and transferred to culture at 5% $CO_2$, atmospheric $O_2$, and 39°C for 22 h.

### *Sperm preparation and in vitro fertilization*

Two to twenty four hours prior to *in vitro* fertilization (IVF), fertilization medium was prepared as previously described (modified from Parrish *et al*., 1986; Krishnakumar *et al*., 2011). Briefly, fertilization medium was supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 18 µM penicillamine, 9µM hypotaurine, 1.8 µM epinephrine, 40 µg/ml heparin, 6 mg/ml BSA, 114 mM NaCl, 3.15 mM KCl, and 25.05 mM $NaHCO_3$. Twenty µl/drop were placed per 35-mm culture dish (BD Biosciences, Mississauga ON), covered with embryo culture grade mineral oil and placed at 5% $CO_2$, atmospheric $O_2$, and 39°C. Briefly, synthetic oviductal fluid (SOF ) medium was prepared containing 0.3 mM sodium pyruvate, 8 mg/ml BSA, 1 mM L-glutamine, 108 mM NaCl, 7.16 mM KCl, 1.19 mM $KH_2PO_4$, 25.07 mM $NaHCO_3$, and 2.5 mM sodium lactate 60% syrup (Krishnakumar *et al*., 2011). Forty µl/drop were placed per 35-mm culture dish (BD Biosciences, Mississauga ON), covered with embryo culture grade mineral oil and placed at 5% $CO_2$, 5% $O_2$, and 38.5°C.

One hour before fertilization matured oocytes were washed with fertilization medium and placed at 10 oocytes/drop in the fertilization medium dishes. Frozen-thawed semen previously obtained from healthy bulls was prepared using the swim-up method (Parrish *et al.*, 1986). Straws containing $50 \times 10^6$ sperm in 0.5 ml

were thawed in a 37°C warm bath for 30s and checked for viability. Sperm were centrifugated at 1600 rpm for 5 min, then the pellet was washed in 200 µl fertilization medium by re-suspension and centrifugation at 1600 rpm for 5 min. The resulting pellet was overlaid with 100 µl fertilization medium and incubated at 5% $CO_2$, atmospheric $O_2$, 38.5°C for 15-20 min. Two-thirds of the swim-up were used for the following steps. Sperm count was determined by using a hemocytometer and $1 \times 10^6$ sperm/ml was prepared. 5-10 µl of the sperm suspension were placed into each drop in the fertilization medium dishes containing the matured oocytes. Day 0 was the day of presumed fertilization. The oocytes and sperm were incubated at 5% $CO_2$, atmospheric $O_2$, and 38.5°C for 18 h.

*Embryo culture: day 1-8*

Eighteen hours after fertilization (day 0), fertilized oocytes were denuded of their cumulus cells by vortexing for less than a minute per 40 oocytes in a small eppendorf tube. The denuded oocytes were then washed in medium consisting of M199 with HEPES and 3 mg/ml BSA, and then washed in SOF medium. The oocytes were placed at 20 oocytes/drop in the SOF medium dishes at 5% $CO_2$, 5% $O_2$, and 38.5°C, cultured until day 8. Medium was changed every 2 days. On day 3 after fertilization unfertilized and degenerated oocytes were removed.

*Embryo culture to test medium and feeder: after day 8*

On day 8 after fertilization, blastocyst-stage embryos (total n = 486) were transferred to 12-well culture plates at one embryo/well containing different types of culture media and substrates (n = number of blastocysts per treatment (medium and substrate)). Each embryo was randomly assigned to DMEM with BFF (n = 47), DMEM with GFF (n = 54), DMEM with a mouse embryonic fibroblast (STO; n = 64), DMEM with gelatin (n = 65), DMEM with fibronectin (n = 50), DMEM with laminin (n = 59), M199 with BFF (n = 46), M199 with GFF (n = 54), M199 with STO (n = 52), M199 with gelatin (n = 50), M199 with fibronectin (n = 58), or M199 with laminin (n = 59).

The different culture media that were tested for their ability to support attachment of bovine embryos were DMEM (commonly used for culturing mammalian embryonic stem cell lines; Martin, 1981) and M199 (commonly used for culturing domestic species blastocysts; Eyestone and First, 1989), each with identical supplements added (15% fetal bovine serum (FBS), 0.1mM MEM non-essential amino acids, 200mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin; GIBCO, Life Technologies, Burlington, ON). The DMEM used in this study contained high concentration of glucose (4.5 g/L), L-glutamine, and no sodium pyruvate, and is widely used as a basal medium for culturing a variety of mammalian cells. The M199 used in this study contained a low concentration of glucose (1 g/L), L-glutamine, and sodium pyruvate, and can be used to culture most types of fibroblasts.

Feeder cells are suggested to facilitate blastocysts to attach and form outgrowths by secreting important growth factors (such as leukemia inhibitory factor, LIF). Homologous (BFF) and heterologous (GFF and STO) feeder cells were used in this study. The STO cells were obtained from the Baylor College of Medicine. The cell line STO-SNL76/7 was modified to express leukemia inhibitory factor (LIF) and was used for culturing the bovine blastocysts. The goat and bovine fetal fibroblast cells were prepared from surgically removed 30-35 day old and 45-60 day old fetuses, respectively. The fetuses were washed with phosphate-buffered saline (PBS) and transferred to a Petri dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin. Forceps were used to remove the viscera, limbs, tail, and head of the fetuses. The trunk of the fetuses were then washed with PBS supplemented with P/S and eviscerated rigorously with razor blades. The resulting tissues were centrifuged in DMEM supplemented with 15% FBS at 100 g for 5 min. The pellet was isolated and cultured in T-175 flasks (Sigma-Aldrich,St. Louis, MO, USA) in DMEM supplemented with 15% FBS for 4 days or until sub-confluence, after which the cells were passaged by trypsinization. Afterwards the cells were digested into single-cell suspension using an appropriate volume of 0.25% trypsin-EDTA for 5 min at 37°C (the trypsinization process was stopped by adding an equal amount of DMEM supplemented with 15% FBS), centrifuged at 500 g for 5 min, and either re-plated onto new T-175 flasks or cryopreserved. To cryopreserve the cells, 1 million cells were placed in 0.5 ml DMEM, 0.3 ml FBS, and 0.2 ml dimethyl sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) in each cryovial, leading to a final concentration of 50% cells in medium, 30% FBS, and 20% DMSO. The cryovials were rapidly transferred to Mr. Frosty freezing containers (Nalgene, Rochester, NY, USA) and placed at -80°C for a minimum of 24 h before transferring to long-term liquid nitrogen storage. Frozen-thawed feeder cells were cultured in DMEM (supplemented with 15% FBS, 0.1 mM MEM non-essential amino acids, 200 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) for two days or until sub-confluence.

These feeder cells were mitotically deactivated by incubation with mitomycin-c (10 µg/ml) for 2-3 hours and washed once with PBS before seeding at $2 \times 10^5$ cells/well in 12-well culture plates in DMEM or M199. After 3-24 hours bovine embryos were randomly distributed to these culture wells at a density of one embryo per well. As embryo-derived cells must eventually be isolated from feeder cells for characterization, non-cellular substrates (gelatin,

fibronectin, and laminin) were also used to eliminate contamination of feeder cells. The substrates were prepared according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA) on 12-well culture plates in DMEM or M199. For gelatin, 0.1% solution was prepared and coated onto culture plates using minimal volume and dried for at least 2 h at 4°C before use. Fibronectin was coated onto culture plates at 2 µg/well using minimal volume and dried for at least 45 min at room temperature before use. Laminin was coated onto culture plates at 1 µg/well using minimal volume and dried for 2 h at 37°C and then washed 3 times with PBS before use.

*Evaluation of embryo culture for attachment*

For each treatment, percentage of attachment (number of attached embryos per 100 embryos used) and number of days since fertilization upon attachment were recorded every 2 days since fertilization for two weeks. Only hatched blastocysts that were expanded and attached were included in the analysis. Embryo outgrowths were cultured in DMEM medium with added supplements (15% FBS, 0.1 mM MEM non-essential amino acids, 200 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin; GIBCO, Life Technologies, Burlington, ON) and 5 ng/ml human recombinant leukemia inhibitory factor (LIF). Growth was observed daily and outgrowths were mechanically passaged when they covered 60-80% of the culture well. Using 20G needles, outgrowths were cut away from feeder cells and plated onto fresh deactivated feeder cells that were seeded at $2 \times 10^5$ cells/well in 12-well plates at a ratio of 1:2 or 1:3. Medium was changed every 1-2 days.

*LIF mRNA expression*

Feeder cells (BFF, STO, and GFF) were analyzed by reverse transcription polymerase chain reaction (RT-PCR) for their expression of LIF. According to the manufacturer's protocol, total RNA was isolated from cells using RNeasy Mini kit (Qiagen, Toronto, ON) followed by treatment with DNase I (Ambion®, Life Technologies, Burlington, ON). RNA quality and quantity were measured using NanoVue Plus (GE Healthcare, Mississauga, ON). A final volume of 25 µl reaction, which consisted of 2 µg total RNA, 0.5 µg Oligo $d(T)_{12-18}$, 1x Reverse Transcriptase (RT) buffer (Invitrogen™, Life Technologies, Burlington, ON), 10mM dithiothreitol (DTT), 0.5mM dNTP, 5U RNase inhibitor, and 10U SuperScript II Reverse Transcriptase (Invitrogen™, Life Technologies, Burlington, ON), was incubated at 42°C for 1 h for reverse transcription. RT-PCR amplification was performed using Thermocycler (Applied Biosystems®, Life Technologies, Burlington, ON). Primer sequences (5'-3') used for amplification of bovine LIF fragments were: Forward (CAG GAG TTG TGC CCC TGC TGC TGG TTC T) and Reverse (CAG GGA GGC GCC CAG GTA CGC GAT GAT G), annealing temperature was 64°C, and length of PCR fragment was 332 bp. Primer sequences (5'-3') used for amplification of murine LIF fragments were: Forward (GAAAACGGCCTGCATCTAAGG) and Reverse (GCCATTGAGCTGTGCCAGTTG), annealing temperature was 61.5°C, and length of PCR fragment was 336 bp. Primer sequences (5'-3') were also used for a housekeeping gene GAPDH: Forward (TCA TGA CCA CAG TCC ATGCCATCACT) and Reverse (GATGTCATCATATTTGGCAGGTTTCTCC), annealing temperature was 60°C, and length of PCR fragment was 253 bp. cDNA was denatured at 94°C for 2 min, followed by 33 cycles of denaturing at 94°C for 30 s, annealing at the specified temperature for 30s, and extension at 72°C for 45 s, and final extension at 72°C for 5 min. PCR products were resolved in 1.1% agarose gel and imaged using ChemiDoc XRS+ Molecular Imager (Bio-Rad, Mississauga ON).

*Statistical analysis*

Statistical analyses were conducted using SPSS 17.0 data analysis software. The distributions of culture conditions and state of attachment were evaluated by $\chi^2$ analysis. One way analysis of variance (ANOVA) was used to evaluate attachment times across culture conditions. A P-value of less than 0.05 was considered statistically significant.

## Results

Blastocysts that were hatched, already expanded, and had an apparent ICM on day 8 since fertilization had a higher chance of attaching to substrate compared to blastocysts that were not yet hatched, not expanded, or had a poorly defined ICM. The blastocysts attached at a higher rate to cellular (n = 140/318) compared to non-cellular (n = 72/341) substrates ($P < 0.05$; Fig. 1). The overall attachment rates between DMEM and M199 were not significantly different, but the mean number of days after fertilization upon attachment was lower in DMEM (13.5 days) than in M199 (14.0 days; $P < 0.05$; Fig. 2).

*Embryo attachment in DMEM medium*

Among cellular substrates in DMEM, blastocysts attached at a higher rate to BFF (n = 30/47) and GFF (n = 33/54) than to STO (n = 27/65; $P < 0.05$); they took longer to attach to GFF (day 13.5) than to BFF (day 12.7, $P < 0.05$) and STO (day 12.7, $P < 0.05$; Fig. 1 and 2). Among non-cellular substrates in DMEM, blastocysts attached to gelatin (n = 14/65), (n = 8/50), and laminin (n = 9/59), although there were no significant differences; they took longer to attach to laminin (day 15.8) than to gelatin (day 14.6, $P < 0.05$) and fibronectin (day 11.9, $P < 0.05$; Fig. 1 and 2).

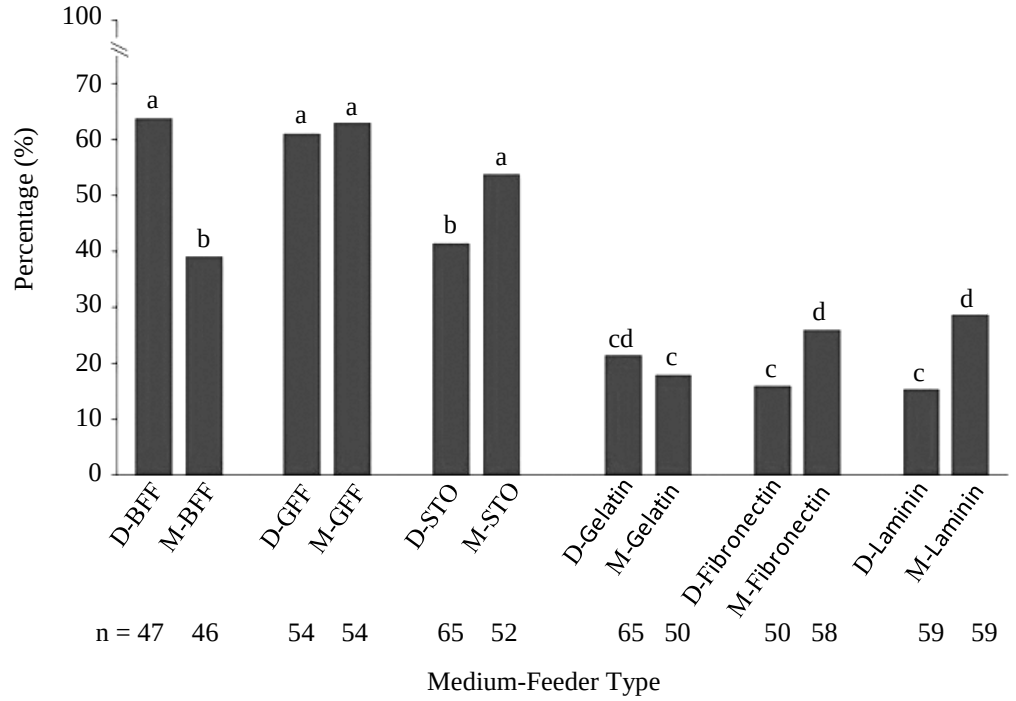


Figure 1. Percentage of bovine blastocyst attachment. D = DMEM, M = M199, n = 340 and 319, respectively. Number of replicates for each treatment is indicated by "n". Letters indicate significant difference ($P < 0.05$).

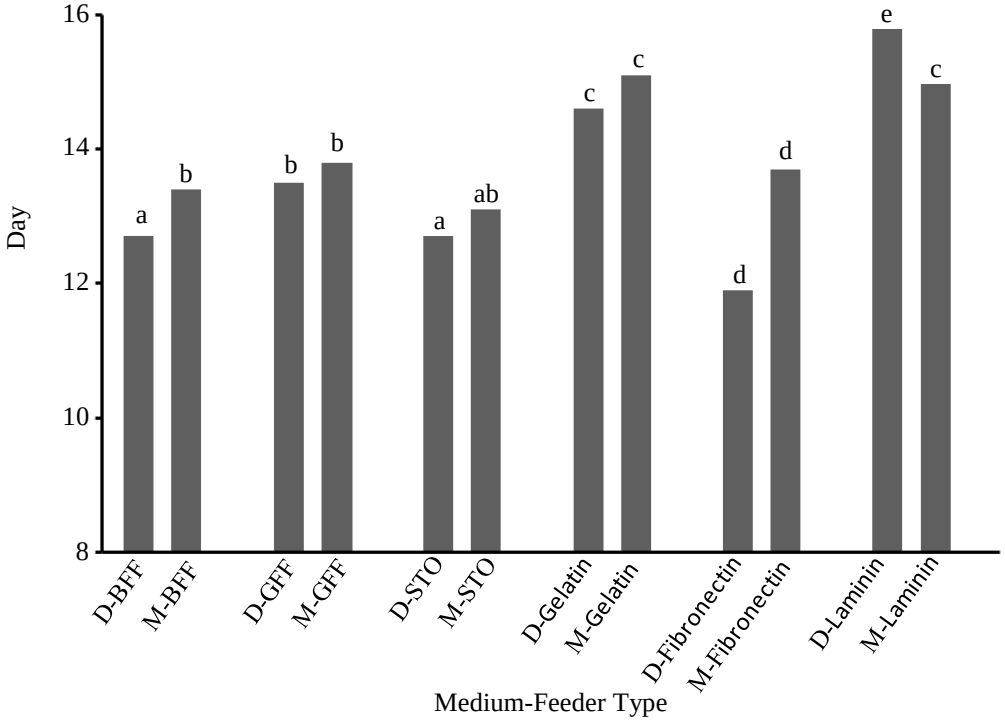


Figure 2. Number of days since fertilization upon attachment. D=DMEM, M=M199, n = 340 and 319, respectively. Letters indicate significant difference ($P < 0.05$).

*Embryo attachment in M199 medium*

Among cellular substrates in M199, blastocysts attached at a higher rate to GFF (n = 34/54) and STO (n = 28/52) than to BFF (n = 18/46, P < 0.05); the number of days to attachment from fertilization were not statistically different among BFF (day 13.4), STO (day 13.1), and GFF (day 13.8; Fig. 1 and 2). Blastocysts attached at a higher rate to laminin (n = 17/59) and fibronectin (n = 15/58) than to gelatin (n = 9/50; P < 0.05); they took longer to attach to gelatin (day 15.1) and laminin (day 15.0) than to fibronectin (day 13.7; P < 0.05; Fig. 1 and 2).

*Bovine embryo outgrowth*

A small number of attached bovine embryo cultured on STO cells in DMEM (n = 6), two embryos cultured on gelatin in DMEM, and one embryo cultured on fibronectin in DMEM also formed outgrowths. They were passaged onto a new feeder and survived a maximum of two passages with addition of LIF to culture medium (see Fig. 3).

*LIF mRNA expression*

Expression of LIF was detected in goat fetal fibroblast cells (GFF), faintly in BFF, and using murine primers, in STO (Fig. 4).

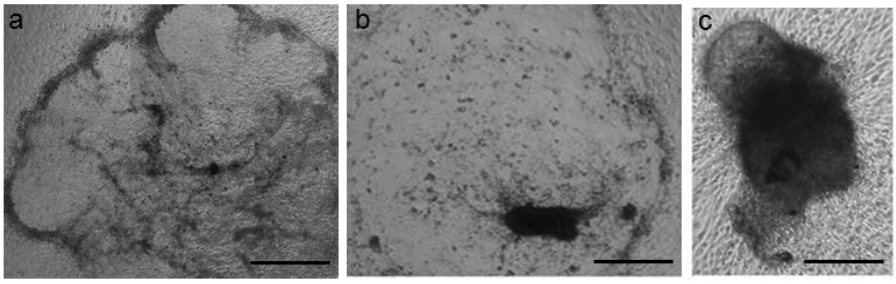


Figure 3. Bovine embryo outgrowths on STO feeder in DMEM. a) Outgrowth on passage 0, scale bar = 5 mm. b) Outgrowth on passage 1, scale bar = 2.5 mm. c) Outgrowth on passage 2, scale bar = 2.5 mm.

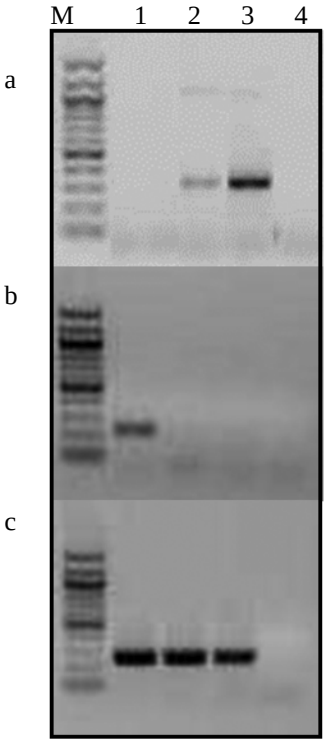


Figure 4. Expression of Leukemia Inhibitory Factor (LIF) in feeder cells detected by RT-PCR. a) Bovine LIF primers, b) Murine LIF primers, c) GAPDH primers. Lanes 1= STO, 2 = bovine fetal fibroblast, 3 = goat fetal fibroblast, 4 = No DNA, ladder (M) = 100 bp markers.

## Discussion

In this study, different feeder cells and culture media were compared for their ability to support the attachment of bovine blastocysts. Although the overall attachment rate was not significantly different between blastocysts cultured in Dulbecco's Modified Eagle Medium (DMEM) and those in Medium 199 (M199), the blastocysts attached at a significantly higher rate to cellular feeders than to non-cellular substrates, which may be expected considering that feeder cells may secrete important growth factors and they provide cell to cell interaction with blastocysts (Smith *et al*., 1988; Stojkovic *et al*., 2005).

The bovine model offers a valuable and convenient source of *in vitro* produced embryos to study culture conditions in embryo development and embryonic stem cell derivation in large animals. These are important in understanding developmental and stem cell biology. Furthermore, these studies have long term implications in transgenesis in large animals, embryonic stem cell lineage differentiation and potential clinical applications in humans and animals. However, not many studies have investigated the medium and feeder requirements for embryos that support embryo attachment and subsequent outgrowth. One study that most closely parallels our study design was done by Piedrahita *et al*. (1990), who investigated the efficiencies of numerous types of feeder layers that produced porcine embryo-derived cells. The main differences in methods were that only one medium type was investigated and time to attachment was not recorded. In that study it was found that STO feeder best supported embryo attachment. To our knowledge, few studies have specifically investigated embryo attachment rates in the bovine model. Kuzan and Wright (1982b) compared development of bovine morulae grown in various types of media (MEM, Ham's F-10 medium, and bovine uterine fibroblast conditioned medium) and substrates (no substrate, collagen substrate, bovine uterine fibroblasts, bovine uterine endometrial tissue, and bovine testicular fibroblasts). Lindenberg *et al*. (1989) compared attachment rates of mouse, human, and bovine blastocysts but did not compare the effects of different media or substrate. The current study extends these findings in identifying the need for cellular substrates and the importance of feeder cell - medium interactions when designing a culture system for derivation of cells from bovine embryos.

### *Cellular feeders may have different energy requirements*

Very little is known about the energy requirements in different cellular feeders and what exactly it is in DMEM and M199 that causes different blastocyst attachment rates. Initially, both high glucose DMEM and low glucose DMEM were considered for this study; however, preliminary studies showed that none of the cell lines proliferated in low glucose DMEM, therefore M199, which has low glucose but contains additional supplements and is widely used for embryo culture, was used in this study. The homologous feeder, BFF, was expected to be beneficial in supporting bovine embryo attachment regardless of medium type, but that was not the case in M199. A recent study also found that bovine ES-like cells grew better on a MEF cell line rather than on homologous bovine embryonic fibroblast (Jin *et al*., 2012). Therefore, it appears that the benefits of using homologous feeder cells depend on the culture medium used.

In DMEM, which has a high glucose content of 4.5g/L, both bovine fetal fibroblast (BFF) and goat fetal fibroblasts (GFF) supported blastocyst attachment at a significantly higher rate than the murine cell line STO. However, in M199 which has lower glucose content of 1g/L, both STO and GFF supported blastocyst attachment at a significantly higher rate than BFF. This shows that the combination of BFF and DMEM is necessary for better blastocyst attachment rates, and the same is true for STO and M199. GFF appeared to be supported in both types of medium so they may be more flexible than BFF or STO in their requirements for energy sources, although it took the longest time for blastocyst attachment compared to other feeders in DMEM. While it is generally understood that mammalian cells require glucose for normal cell functions (Levintow and Eagle, 1961; Rheinwald and Green, 1974), few comparative studies of glucose requirements between different types of feeder cells have been reported. For instance, Rheinwald and Green (1974) compared glucose requirements in human and rodent cell lines. It remains unclear why feeders in DMEM seemed to support blastocyst attachment earlier than that in M199. A positive effect of culture in DMEM may have allowed BFF to support blastocyst attachment in a shorter time period; while on GFF that performed equally in DMEM or M199, attachment time did not differ significantly in either medium. Interestingly, the interaction of culture medium type with STO cells was less evident regarding blastocyst attachment rates than time required for attachment.

Blastocysts also require glucose for hatching, attachment, and outgrowths in mice and humans (Wordinger and Brinster, 1976; Gott *et al*., 1990). According to these studies, the expanded blastocysts used in this study should have high requirements for glucose, and so in theory, should benefit from the higher glucose content in DMEM than from M199. However, higher blastocyst attachment rate in DMEM was only observed in BFF and gelatin. In the current study, *in vitro* produced embryos were used, that differ from embryos developed *in vivo* in developmental rate, lipid content, and energy requirements (Rivera *et al*., 2003). Although it is possible to create ES-like cells from *in vitro* blastocysts, *in vivo*-derived blastocysts produced better quality colonies than those derived *in vitro* as

shown in bovine and ovine studies (Talbot *et al*.,1995; Wells *et al*.,1997). Further study is needed to better define the medium and feeder requirements in *in vivo* produced embryos.

*Non-cellular substrates may share similar adhesion molecules*

Non-cellular substrates may be components of the extracellular matrix, but they do not secrete growth factors and display a limited number of adhesion molecules, which may explain why the blastocyst attachment rates were significantly lower than on feeder cells and that it took longer for blastocysts to attach on the non-cellular substrates. However, non-cellular substrates still provide a valuable tool to study embryo attachment since feeder cells must eventually be eliminated during embryonic stem cell derivation. In DMEM, gelatin supported blastocyst attachment at a higher rate than both fibronectin and laminin, although not significantly; whereas in M199, gelatin supported blastocyst attachment at a significantly lower rate than both fibronectin and laminin. For each substrate type embryos attached at a significantly higher rate in M199 than in DMEM, except for gelatin, where there was no significant difference. Moreover, the differences observed for fibronectin and laminin were more pronounced than that for gelatin. In both media, fibronectin took the shortest time to support blastocyst attachment. Although all three substrates are important in cell-to-cell adhesion processes, very little is known as to whether or not one type is superior over the other types to promote attachment of blastocysts. Numerous cells, including trophoblast cells (Coutifaris *et al*., 2005), have fibronectin receptors and it has been shown that fibronectin-fibronectin binding domains overlap with fibronectin-gelatin binding domains (Chiquet *et al*., 1979), which partially explains the similar attachment rates seen in the current study for embryos cultured on gelatin and fibronectin in DMEM, but does not explain the discrepancies seen when using different media. It has also been suggested that fibronectin, which can also be found in serum, aids cell attachment to gelatin-coated surfaces, so studies combining different non-cellular substrates may provide additional insight (Chiquet *et al*., 1979). Horwitz *et al*. (1985) discovered that cell substrate attachment (CSAT) antigen, one of the many transmembrane proteins that are involved in cell-to-cell adhesion in fibroblasts, binds to both fibronectin and laminin. Fibronectin and laminin may share many other signalling proteins, which may explain their similar attachment rates within DMEM and within M199.

There was a great deal of variability among the non-cellular substrates, as bovine blastocysts attached at the highest rate to gelatin in DMEM but the opposite was true in M199. Substrate stiffness may play a role as it was suggested that collagen, which is stiffer than polystyrene, supported better development of embryos to the blastocyst stage (Rivera and Rinaudo, 2013). However, our study involved embryos that are at a more advanced developmental stage, so further study should investigate if substrate stiffness also affects blastocyst attachment and outgrowth.

*Secretion of growth factors*

Although it may be suggested that blastocysts preferentially attach to a homologous feeder, this was not observed in this study. As suggested earlier, this may be due to medium requirements for feeder cells, as bovine feeders may prefer a medium with high glucose content such as DMEM. Although feeder cells may secrete important growth factors that support embryo outgrowth (Smith *et al*., 1988), it is unclear whether or not one type secretes more than the others or whether different medium types affect this process. In this study the cellular feeders were compared for expression of leukemia inhibitory factor (LIF), a growth factor important in formation of embryo outgrowth. LIF expression was detected in GFF, STO, and BFF. While LIF is often considered key to ESC derivation and maintenance, there are numerous other important growth factors, such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), which are expected to have effects on blastocyst attachment and cell growth and could be investigated further.

*Embryo outgrowths*

LIF was supplied to the medium once blastocysts attached to the feeder and started to form outgrowths. The outgrowths expanded well with LIF addition, which is consistent with the view that LIF is important in the implantation process (Stewart *et al*., 1992). A small number of attached blastocysts that formed large outgrowths were mechanically passaged onto new de-activated feeder cells. Most of the attached blastocysts survived only a maximum of two passages, even in the presence of LIF. It is unclear why LIF addition was useful for the initial expansion of embryo outgrowth but not for the later stages after passage. This may be related to the difficulty of maintaining bovine embryo-derived cells in culture and the derivation of bovine embryonic stem cells, as documented before (e.g. Saito *et al*., 1992). Studies of bovine embryonic stem cell-like (ESC-like) cells obtained from blastocysts have been reported, but with varying degrees of success. For example, Stice *et al*. (1996) derived a cell line solely based on morphology and embryoid body formation, without observation of ESC markers or formation of teratomas or chimeras, whereas Saito *et al*. (1992) derived a cell line that was positive for alkaline phosphatase, Oct-4, and SSEA-1, formed embryoid bodies and formed chimeras. Mouse ESCs are characterized by consistent expression of pluripotency markers, but bovine ESC-like cells are quite variable in

terms of their expression of alkaline phosphatase, SSEA-3&4 across studies (Gjørret and Maddox-Hyttel, 2005; Muñoz *et al*., 2008). Studies such as these vary greatly in terms of growth factors, and so there is great need for more uniform study designs.

## Conclusions and future directions

In this study, bovine embryo attachment was compared in different culture media with various cellular or non-cellular substrates. The data suggest that there is an interaction of feeder cell type and culture medium and therefore these factors need to be carefully matched to optimize embryo attachment. While the current findings provide insight into improving culture conditions for bovine embryo attachment, additional data are needed to define medium and feeder cell combinations that support bovine embryonic stem cell derivation. Future studies should investigate nutrient requirements for various feeder cell types and the effect of nutrient availability on embryo-feeder interaction.

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