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Importance of culture conditions during the morula-to-blastocyst period on capacity of inner cell-mass cells of bovine blastocysts for establishment of self-renewing pluripotent cells

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Abstract

The hypothesis was tested that the pluripotency of the inner cell mass (ICM) of the bovine embryo is enhanced by the glycogen synthase kinase-3β inhibitor CHIR99021 and the MAPK1 and MAPK3 inhibitor PD032591. Treatment with the two inhibitors from Days 6 to 8 after insemination increased blastocyst steady state concentrations of mRNA for *NANOG* ($P < 0.05$) and *SOX2* ($P = 0.055$) and tended to decrease ($P = 0.09$) expression of *GATA6*. To evaluate pluripotency, the inner cell mass was isolated by immunosurgery at Day 8, seeded on a feeder layer of bovine embryonic fibroblasts, and cultured in the presence of the inhibitors. Ten of 52 (19%) ICM from control embryos had primary outgrowth formation vs. 23 of 50 (46%) of the ICM from embryos cultured with inhibitors ($P < 0.01$). For ICM outgrowths from embryos cultured without inhibitors, colonies either did not persist through Passage 2 or became differentiated. In contrast, for the inhibitor group, four colonies survived beyond Passage 2, and one line persisted for 19 passages. This cell line possessed alkaline phosphatase activity, expressed several genes characteristically expressed in pluripotent cells, and differentiated into embryoid bodies when cultured in the absence of the signal transduction inhibitors and the feeder layer. Propagation of the cells was difficult due to slow growth and inefficiency in survival through each passage. In conclusion, exposure to inhibitors during the morula-blastocyst transition facilitated formation of self-renewing pluripotent cell lines from bovine blastocysts.

Keywords: Embryonic stem cells; Cattle; Pluripotency; Blastocyst; Morula

1. Introduction

Embryonic stem cells (ESC) are pluripotent, self-renewing cells derived from the inner cell mass (ICM) of preimplantation blastocysts. These cells have important uses for elucidation of developmental mechanisms and for applications in tissue engineering and regenerative medicine [1]. Since the first ESC line was developed in 1981 using the mouse embryo [2], ESC have been established in the human [3], non-human primate [4,5] and rat [6]. In addition, there are reports regarding establishment of "ES-like" cell lines, based on phenotypic characteristics or teratoma formation after transfer

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into immunocompromised recipients, in a variety of species (including cattle) [7–10].

One limitation to establishment of ESC is the difficulty in maintenance of the undifferentiated state. Pluripotency could be maintained by culture with leukemia inhibitory factor (LIF) in the mouse [11], fibroblast growth factor 2 (FGF2) in the human [12], or by culture with mitotically-inactive embryonic fibroblast cells in the mouse [2] and human [4]. In cattle, however, culture of ICM colonies with or without FGF2 on a feeder layer of mouse embryonic fibroblasts did not sustain expression of the pluripotency genes *NANOG* or *SOX2* [12]. Combining feeder cells, LIF and FGF2 caused ICM-derived cells to persist for more than 10 passages [8], but evidence of pluripotency based on expression of differentiation marker genes was not reported. Thus, requirements for maintenance of pluripotency in ICM-derived cells may be species- or developmental stage-specific.

In cattle, it was recently reported that cells derived from parthenogenetic blastocysts remained pluripotent when cultured with a combination of inhibitors against MAPK1 and MAPK3, FGF receptor and glycogen synthase kinase-3β (GSKB) [10]. Indeed, a combination of two inhibitors only, the GSKB inhibitor CHIR99021 and the MAPK1 and MAPK3 inhibitor PD032591, increased efficiency of establishment of ESC lines in difficult strains in mice [13] and rats [6]. Treatment with these two inhibitors is referred to as 2i [6,13]. The adenylate cyclase activator forskolin also promoted ESC formation in the human [14].

In the present study, we tested the hypothesis that the pluripotency of the ICM of the bovine embryo is determined in part by cell signaling mechanisms during the first differentiation event in development, i.e., when the pluripotent morula gives rise to an embryo composed of a combination of differentiated trophectoderm and an ICM that remains pluripotent. This hypothesis was tested by evaluating effectiveness of 2i signal transduction inhibitors beginning at Day 6 of development on competency of the subsequent ICM to give rise to pluripotent daughter cells. A second hypothesis was that addition of forskolin after ICM isolation enhances the efficiency of maintenance of pluripotent cells.

2. Materials and methods

2.1. Blastocyst production

Bovine embryos were produced *in vitro* as described previously [15], unless otherwise mentioned. Fertilization proceeded for 8 h (day of insemination = Day 0). Embryos were cultured in groups of 30 in 50 μL microdrops of SOF-BE1 [16] covered in mineral oil at 38.5°C and in a humidified atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2 with the balance nitrogen. On Day 6, all embryos were transferred to embryonic stem cell medium (ESM) supplemented or not with two signal transduction inhibitors (ESM-2i). The ESM consisted of KnockOut Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 15% (v/v) KnockOut Serum Replacement (KSR, Invitrogen), 1 mM Glutamax (ala–gln, Aldrich-Sigma), 0.1-mM minimal essential medium nonessential amino acids (Aldrich-Sigma), 0.1 mM β-mercaptoethanol (Invitrogen), 100 IU/mL penicillin, 100 μg/mL streptomycin sulfate, and 250 ng/mL amphotericin B (Invitrogen). To prepare ESM-2i, 3 μM CHIR99021 (Stemgent, Cambridge, MA) and 1.2 μM PD032591 (Stemgent) were added to ESM. Embryos were cultured in ESM or ESM-2i at 38.5°C and in a humidified atmosphere of 5% (v/v) O_2, 5% (v/v) CO_2 and the balance N_2 until Day 8 when blastocysts were collected and used for ICM isolation to develop bESC-like cells or determination of expression of embryonic lineage marker genes.

A peri-implantation elongated embryo was recovered by flushing the excised uterus of a cow at Day 21 after AI to serve an as a differentiation control embryo for reverse transcription polymerase chain reaction (RT-PCR). Another cell line used for RT-PCR, the VIVOT trophoblast cell line produced by Neal Talbot, USDA-ARS, was obtained from Dr Alan Ealy, University of Florida.

2.2. Feeder cell establishment

Cell lines of bovine embryonic fibroblast cells (BEF) were developed from fetal skin collected at approximately 2 to 3 mo of gestation and cultured with 10% (v/v) fetal bovine serum in DMEM at 38.5°C and 5% (v/v) CO_2 in humidified air. At the third passage, BEF were treated with mitomycin C (10 μg/mL, Invitrogen) for 3 h to arrest the cell cycle, stored in freezing medium [BEF medium 90% (v/v) and dimethyl sulfoxide, 10% (v/v)] and frozen in liquid nitrogen until use. One day before ICM seeding or ESC-like cell passage, BEF were plated (2×10^4 cells/cm^2) on 0.1% (w/v) gelatin-coated 24-well culture dishes in 0.5 mL culture medium and used as a feeder layer to maintain colonies of ESC-like cells.

2.3. Isolation of ICM and seeding on feeder cell layer

The zona pellucida was removed from blastocysts by exposure to 0.1% (w/v) proteinase from *Streptomyces griseus* (Aldrich-Sigma, St. Louis, MO) in Dulbecco's phosphate buffered saline (DPBS). Zona-free blastocysts were incubated in DMEM (Invitrogen)