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Characterization of goat inner cell mass derived cells in double kinase inhibition condition

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ABSTRACT

The identification of small molecular inhibitors, which were reported to promote the derivation of mouse and human embryonic stem cells (ESCs), provides a potential strategy for the derivation of domesticated ungulate ESCs. In present study, goat inner cell mass (ICM) derived cells in the double inhibition (2i) condition, in which, mitogen-activated protein kinase kinase (MAP2K) and glycogen synthase kinase 3 (GSK3) were inhibited by PD0325901 and BIO respectively, were characterized. The results showed that goat ICM derived cells in 2i medium adding leukaemia inhibitor factor (LIF) possessed a mouse ES-like morphology. But these cells had much compromised proliferation capacity, resulting in difficulty in expansion. In 2i alone medium, goat ICM derived cells possessed primate ES-like morphology. These cells expressed pluripotent markers and could differentiate into derivatives of three germ layers *in vitro*. However, these cells could not be proliferated in long-term (persisted for 15 passages) because of spontaneously neural differentiation. Additionally, goat ICM derived cells could be inducing differentiated into neural lineage *in vitro*. Although goat ESCs could not be established in PD0325901 and BIO alone medium, this derivation condition provides a useful research system to find signaling molecular those regulate early embryonic development and pluripotency in goat.

1. Introduction

Embryonic stem cells (ESCs) provide a promising tool for gene editing and improving production traits in goat. Although establishment of ESCs has been reported in some domesticated ungulates [1–5], it is difficult to derive bona fide ESCs from goat [6]. Lack of appropriate derivation system is one of obstacles to establishment of goat ESCs. Because of difference in developmental biology, conventional ESCs derivation system for mouse or human is inadequate to maintain self-renewal and pluripotency of goat ESCs [7].

Recently, some small molecular inhibitors, which silence differentiation cue signaling pathways and activate pluripotency maintaining signaling pathways, were reported to facilitate the derivation of mouse and human ESCs [8–10]. By double inhibition (2i) of mitogen-activated protein kinase kinase (MAP2K) and glycogen synthase kinase-3 (GSK3), bona fide ESCs have been derived from refractory mouse strains [11] and rat [12,13], from which ESCs were previously difficult to be derived. These results suggest a potential utilization of small molecular inhibitors in derivation of domesticated ungulates ESCs. However, whether both inhibitions of MAP2K and GSK-3 by small molecular inhibitors could facilitate the derivation of goat ESCs is unclear. In present study, goat inner cell mass (ICM) derived cells in the double inhibition (2i) condition, in which, MAP2K and GSK3 were inhibited by PD0325901 and BIO respectively, were characterized.

2. Materials and methods

2.1. Embryo culture and inner cell mass isolation

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Goat morula stage embryos were recovered through uterine flushing. The study was approved by Animal Care and Use Committee of College of Veterinary Medicine, Northwest A&F University (Yangling, China). Morula stage embryos were cultured in 2i medium or control medium. 2i medium consisted of DMEM/F12 (Life Technologies, Carlsbad, CA), containing 10% FBS and 10% KO-SR (Life Technologies), 1% nonessential amino acids (Life Technologies), 0.1 mM β-

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mercaptoethanol, 2.5 μM BIO and 1 μM PD0325901 (Stemgent, Cambridge, MA). Control medium was similar with 2i medium except replacing BIO and PD0325901 by their solvent dimethyl sulfoxide (DMSO). Embryos were cultured under mineral oil in the condition of 38.5 °C, 5% CO_2, and saturated humidity. After 2 days, inner cell mass (ICM) were isolated from blastocysts by micromanipulation.

2.2. Isolation and culture of goat inner cell mass derived cells

Isolated ICM were seeded on inactivated mouse embryonic fibroblast (MEF) in 2i medium or 2i+LIF medium respectively. 2i+LIF medium is consisted of 2i medium and 1000 IU/ml leukaemia inhibitor factor (LIF, Life Technologies). After 48 h, ICM attached to the bottom of dishes and the first medium change was carried out. About 8~10 days later, the ICM outgrowth were manually dissociated into small cell clumps with a microscalpel and transferred onto new feeder cells. After the first passage, colonies with ESC-like morphology were selected for further propagation and characterization.

2.3. Differentiation in vitro

For embryoid body (EB) formation, the colonies with ESC-like morphology were transferred into agar-coated dishes and cultured in suspension. After 7~10 days, EBs were transferred into gelatin-coated dishes allowing to attachment for further differentiation.

2.4. Differentiation in vivo

The colonies with ESC-like morphology were harvested and injected into the hind leg muscle of nude mice using an 18 G needle. Six to seven weeks after injection, mice were sacrificed, and teratomas were dissected and histologically characterized.

2.5. Induced differentiation of neural lineages

The neural differentiation was performed according to established methods [14]. The EBs cultured in suspension for 5 days plated onto gelatin-coated culture dishes in medium consisted of DMEM/F12 containing 10% FBS, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol. When rosette structures appeared in EB outgrowths, the rosette areas were mechanically selected and transferred onto poly-ornithine coated culture dishes in N2 medium. N2 medium consisted of DMEM/F12, 10 ng/ml FGF-2 (Merck Millipore, Darmstadt, Germany) and 1% N2 supplement (Life Technologies). After cells migrated from attached rosette and reached about 80% confluency, the cells were sub-cultured and characterization. For neural differentiation, FGF-2 was withdrawn from N2 medium. Cells were cultured in medium without FGF-2 for 10 days until cells were fixed for immunofluorescent assays.

2.6. Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized in 0.1% Triton X-100 in PBS for 10 min. After blocking with 3% BSA, the cells were incubated with primary antibodies overnight at 4 °C. The cells were then rinsed three times with PBS and incubated for 60 min with fluorescein isothiocyanate (FITC)-conjugated second antibody (Santa Cruz Biotechnology). DNA was counterstained with 1 μg/ml Hoechst 33342. Samples were mounted and observed under laser scanning confocal microscopy (Nikon).

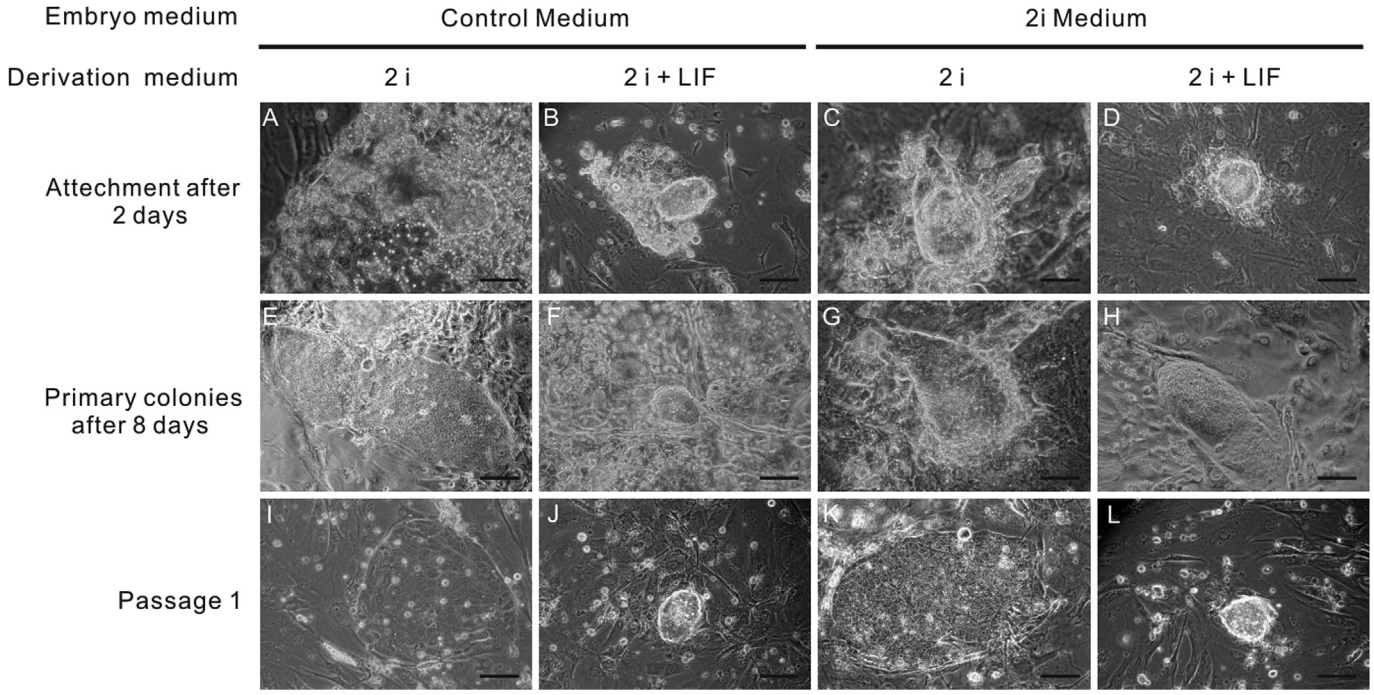

Fig. 1. Isolation of ICM derived cells in 2i or 2i+LIF medium. Isolated ICM from blastocysts cultured in control (A, B, E, F, I, J) or 2i medium (C, D, G, H, K, L) were seeded on inactivated feeder cells in 2i (A, C, E, G, I, K) or 2i+LIF medium (B, D, F, H, J, L) respectively. After 2 and 8 days, ICM attached to feeder cells (A–D) and formed primary ES-like outgrowth (E–H). The outgrowth were manually dissociated and replanted onto fresh feeder cells. For ICM isolated from blastocysts cultured in control medium, most of colonies evidently differentiated at passage 1 in 2i medium (I). In 2i+LIF medium, although colonies maintained mouse ES-like morphology at passage 1 (J), these cells had very limited proliferation capacity. For ICM isolated from blastocysts cultured in 2i medium, colonies maintained primate ES-like morphology in 2i medium at passage 1 (K). In 2i+LIF medium, colonies maintained mouse ES-like morphology at passage 1 (L). ICM, inner cell mass. LIF, leukaemia inhibitor factor. Bars = 100 μm.