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Full Length Research Paper

Growth inhibition of mouse embryonic stem (ES) cells on the feeders from domestic animals

Hongguo Cao*, Zhifei Xie, Yong Pu, Pan Yang, Ya Liu, Yunsheng Li, Fugui Fang, Zijun Zhang, Yunhai Zhang, Yong Tao and Xiaorong Zhang

Anhui Local Livestock Genetic Resources Conservation and Breeding Laboratory, College of Animal Science and Technology, Anhui Agricultural University, Hefei, Anhui 230036, China.

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Mouse embryonic stem cells (mESCs) can be propagated *in vitro* on the feeders of mouse embryonic fibroblasts. In this study, we found growth inhibition of mESCs cultured on embryonic fibroblast feeders derived from different livestock animals. Under the same condition, mESCs derived from mouse embryonic fibroblast feeders were seen on the mass-like colonies and round or oval images, and more significant growth in the total number of colonies ($p<0.05$) and viable cells in the colonies ($p<0.01$) than that from goat embryonic fibroblast feeders, and viable cells in the colonies ($p<0.05$) than that from porcine embryonic fibroblast feeders. The feeders from bovine embryonic fibroblasts also reduced viable cells in the colonies, but were not significantly different in the total number of colonies and viable cells in the colonies with mouse embryonic fibroblast feeders. mESCs on the different embryonic fibroblast feeders were expressed as stem cell-specific markers Oct 4 and stage-specific embryonic antigen 1 (SSEA 1). Here, our results indicate that the feeders from goat, porcine and bovine embryonic fibroblasts inhibit the proliferation of mESCs.

Key words: Domestic animals, feeders, mouse embryonic stem cells (mESCs), growth.

INTRODUCTION

Embryonic stem cells (ESCs) derived from inner cell masses of blastocysts have strong self-renewal capacity and plasticity, and are very suitable for exogenous gene knock-in or knock-out genetic modifications. In addition, ESCs as donors for nuclear transfer have a high cloning efficiency (Hochedlinger and Jaenisch, 2002; Jaenisch et al., 2005), and can solve the problems of low efficiency and instability on the expression of foreign genes in transgenic animals by related diploid chimeric embryos, tetraploid complementary embryos or nuclear transfer technologies. Meanwhile, ESCs can be induced and differentiated into all types of cells in the body (Tonti-Filippini and McCullagh, 2000), and even female (Hübner et al., 2003) and male germ cell (Geijsen et al., 2004; Kerkis et al., 2007) under certain conditions of *in vitro* culture, thus ESCs provide an ideal cell model for the study of reproduction, development and disease treatment.

The current system for mESC culture uses mouse embryonic fibroblasts as a feeder supplemented with leukemia inhibitory factor (LIF). Among them, the type of feeder is an important element. Therefore, in order to explore the growth characteristic of mESCs on the feeders with several different feeders, feeders from porcine, bovine, goat and mouse embryonic fibroblasts were used for the culture of mESCs in this study. The study shows that the feeders from goat, porcine and bovine embryonic fibroblasts inhibit the proliferation of mESCs, so it provides a model for insight into the functional difference in the support of ESC culture.

MATERIALS AND METHODS

Cell culture

The primary embryonic fibroblasts from 75-day-old and 6 cm long goat fetuses, four-month-old and 20 cm long bovine fetuses, 30-

*Corresponding author. E-mail: caohongguo1@yahoo.com.cn. Tel: +86 (0)551 5786357. Fax: +86 (0)551 5786357.

day-old and 8 cm long porcine fetuses, and 12.5-day-old and 5 mm long mouse fetuses were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone, China) with 10% fetal bovine serum (FBS) (Hyclone, Australia), respectively. After covering the dishes, the embryonic fibroblasts were treated for 2.5 h with 10 µg/ml mitomycin C (Sigma, America), digested with trypsin solution (Gibico, Australia), seeded at 1.2×10^5 cells/cm^2 in dishes treated with 0.1% gelatin, and cultured at 37.5°C (Kim et al., 2012; Ma et al., 2012). 29th generation mESCs from male R1 cell line were seeded at 1×10^5 cells/dish on the feeders from goat, bovine, porcine and mouse embryonic fibroblasts with stem cell medium consisting of 1000 U/ml LIF (Chemicon, America), 15% FBS and high glucose DMEM respectively, and cultured at 37.5°C. mESCs were subcultured at 1:4 dilutions two days after culture, and analyzed over three times after continuous culture and passage under the same condition.

Proliferation analysis

For the proliferation of mESCs on the different feeders, the cells mediated above were fixed with 4% paraformaldehyde for 15 min, permeablized with 0.5% Triton X -100 in phosphate buffered saline (PBS) for 10 min. After staining with 1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Roche, Switzerland), the cells were examined with a fluorescence microscope.

Gene expression

For immunocytochemistry, mESCs on the different feeders were fixed with 4% paraformaldehyde for 15 min, permeablized with 0.5% Triton X -100 in PBS for 10 min, blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. The primary antibodies anti-Oct 4 in rabbit (Abcam, United Kingdom) and anti- stage-specific embryonic antigen 1 (SSEA 1) 1 in mouse (Chemicon, America) were diluted 1:200, respectively, added onto cells and incubated at 4°C overnight. Cells were washed three times with PBS, and incubated with CY3 labeled secondary antibody (1:200 dilution) at room temperature for 1 h, washed three times with PBS. The cells were finally stained with DAPI and examined.

Data analysis

All data were collected from three independently replicated experiments, and values were analyzed by student's t-test to determine the significance of differences. P value < 0.05 was considered statistically significant.

RESULTS

Growth characteristics in the different feeders

mESCs derived from mouse embryonic fibroblast feeders were in good condition, which mainly had higher colony density, larger colony size in the unit feeder than others. Under the same condition, mESCs derived from bovine embryonic fibroblast feeders had a large colony number, but smaller colony size and less cell number in the colony than that from mouse embryonic fibroblast feeders, especially, part of the colonies which showed flat and no stem cell-specific nest-like colony morphology. In addition, part of the cells stained with nucleus-specific dye DAPI in the colonies had larger nuclei under a fluorescence microscope. mESCs derived from goat embryonic fibroblast feeders also had smaller colony, less cell number in the colonies under a fluorescence microscope than that from mouse embryonic fibroblast feeders, and most colonies consist of two to three cells. mESCs derived from porcine embryonic fibroblast feeders were poor, mainly minor colony number and less obvious colony image, and had larger nuclei and less cell number in the colonies than that from mouse embryonic fibroblast feeders (Figures 1 and 2).

Proliferation difference in the different feeders

Under the culture condition of different feeders, mESCs derived from mouse embryonic fibroblast feeders had higher colony density than that derived from goat embryonic fibroblast feeders ($p<0.05$), also higher colony density in the unit feeder than that derived from porcine embryonic fibroblast feeders, but no difference between mouse and porcine. mESCs derived from bovine embryonic fibroblast feeders had higher colony density in the unit feeder than that derived from goat or porcine embryonic fibroblast feeders ($p<0.05$), but total cell number in the colonies derived from mouse embryonic fibroblast feeders was higher than that derived from porcine ($p<0.05$) or goat ($p<0.01$) or bovine (Figure 3 and Table 1).

Expression of stem cell-specific markers on the different feeders

Under the culture condition of different feeders with ESC medium, mESCs derived from mouse embryonic fibroblast feeders were in good condition, expressed stem cell-specific markers nucleoprotein Oct4 and membrane protein SSEA1. In addition, mouse ES cells derived from bovine, porcine and goat embryonic fibroblast feeders also expressed stem cell-specific markers nucleoprotein Oct 4 and membrane protein SSEA 1 (Figure 4).

DISCUSSION

Under the condition of no suitable feeder and differentiation inhibitory factor *in vitro*, ESCs are easy to differentiate into other cell types. So, culture system of feeder and differentiation inhibitory factor plays an important role in the process of maintaining the undifferentiated state of ESCs. In general, feeder, as an important factor for the isolation and culture of ESCs, can secrete cytokines promoting the proliferation of ESCs. Conventionally, mouse embryonic fibroblasts as feeders are suitable for culturing mouse (Evans and Kaufman, 1981), human (Thomson et al., 1998; Shetty and

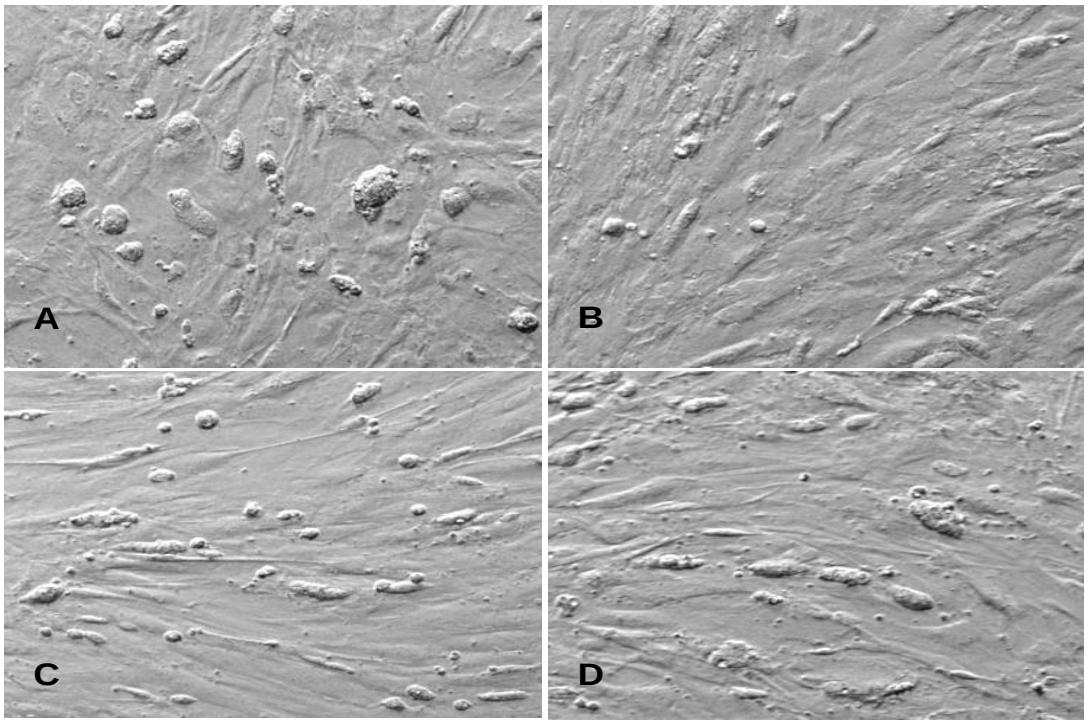

Figure 1. The images of mESCs on different feeders 24 h after 3rd passage. Feeders from: A, mouse; B, porcine; C, goat; D, bovine.

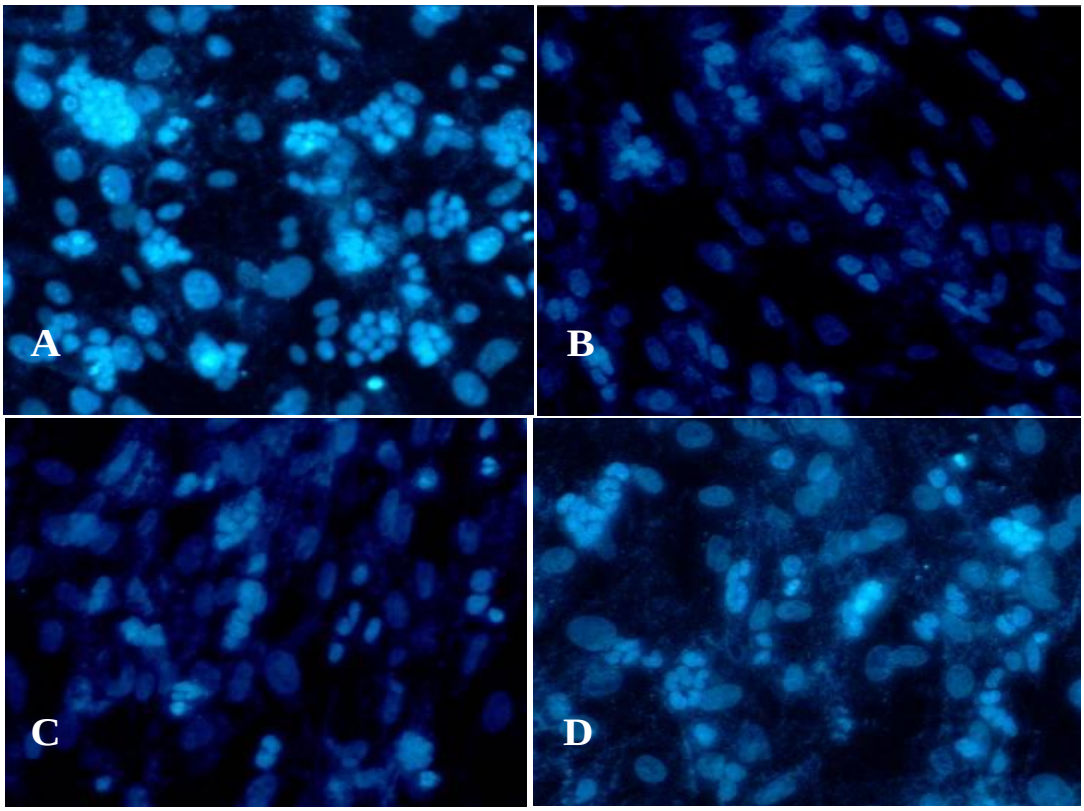

Figure 2. Staining images of mESCs with DAPI dye on different feeders 24 h after 3rd passage. Feeders from: A, mouse; B, porcine; C, goat; D, bovine.

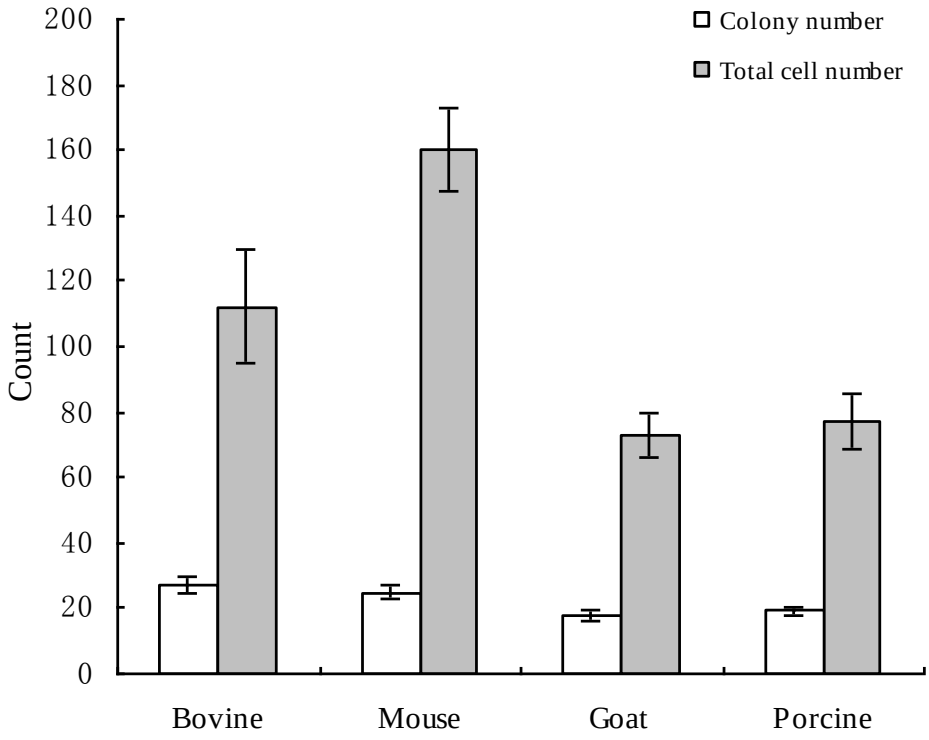

Figure 3. The difference between mESC colony and total cell number in the colonies of different feeders.

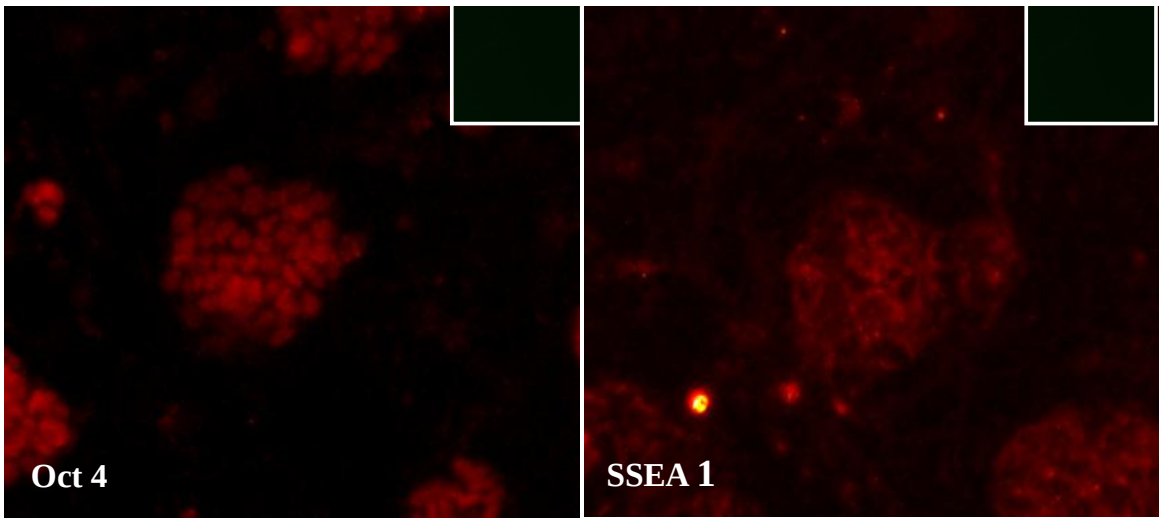

Figure 4. Immunocytochemistry of mESCs with Oct 4 and SSEA 1 antibody. Negative control on top right corner.

Inamdar, 2012), rat (Buehr et al., 2008) and monkey ESCs (Thomson et al., 1995; Chen et al., 2012), but feeder cells, which can be used to support mESCs self-renewal capacity and plasticity in other species, derive from human being, for example, human foreskin fibroblasts, human foreskin fibroblast can produce interleukin-6 to support derivation and self-renewal of mESCs. The current system for mESCs culture uses mouse embryonic fibroblasts as a feeder supplemented with LIF, because LIF is an interleukin 6 class cytokine that affects cell growth by inhibiting differentiation (Ma et al., 2012). In addition, mESCs can be propagated *in vitro*

Table 1. The effect of the proliferation of mESCs on different feeders.

Species	Colony number	Total cell number
Bovine	27.25±5.6199	112.25±34.5386
Mouse	24.75±3.9476[a]	160.25±25.5522[a]
Goat	17.50±3.3166[b]	73.25±13.5247[c]
Pig	19.25±2.3629	77±17.3013[b]

Means in the same columns with similar superscripts are not significantly different (P>0.05).

on feeders of mouse STO cells, because the STO cells secrete several cytokines that are essential for mESCs to maintain their undifferentiated state, but after STO cells were infected with adenovirus containing a mutant form and overexpressed, the cytokine-bone morphogenetic protein 4 (BMP 4), overexpression of BMP 4 in STO feeder cells repressed the proliferation of mESCs *in vitro* (Kim et al., 2012). In this experiment, the feeders from goat, porcine and bovine embryonic fibroblasts inhibited the proliferation of mESCs, therefore, the results suggest that it probably provides a model for insight into BMP 4-related cytokine signal pathway in goat, porcine and bovine embryonic fibroblasts.

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