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Optimization of the conditions of isolation and culture of dairy goat male germline stem cells (mGSC)

Haijing Zhu, Chao Liu, Mingzhao Li, Junwei Sun, Wencong Song, Jinlian Hua*

College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Key Lab for Animal Biotechnology of Agriculture Ministry of China, Northwest A&F University, Yangling, Shaanxi 712100, PR China

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ABSTRACT

Male germline stem cells (mGSC) reside in the basement of seminiferous tubules of the testis and have the capacity of self-renewal and differentiation into sperm throughout the life of animals. Reports on mice and human mGSC have demonstrated that mGSC are an unlimited resource of pluripotent stem cells for sperm production. The conditions of isolation and culture of mouse and human mGSC are well developed; however, the systematic culture conditions of dairy goat mGSC are still deficient although there have been several reports of successful cultures. With the present research, several key elements of isolation and culture of dairy goat mGSC have been determined. Details for the conditions of isolation of dairy testicular spermatogonium cells were optimized, and effects of several extracellular matrix types, ages of dairy goat, and cytokines on enrichment and culture of mGSC were compared. Biological characteristics of the cells were also evaluated by RT-PCR and immunofluorescent staining. The results indicated there is one kind of enzyme cocktail (CTHD (1 mg/ml collagenase, 10 μg/ml DNase, 1 mg/ml hyaluronidase and 1 mg/ml trypsin) combined TD (0.25% trypsin and 10 mg/ml DNaseI)) that can be used to successfully isolate dairy goat testicular spermatogonium cells efficiently; and fibronectin as well as laminin were efficient extracellular matrix to enrich mGSC among the extracellular matrix types evaluated. Age of dairy goat clearly influenced the cultures of dairy goat mGSC with the efficiency of establishment of an mGSC line being greater if the age of the dairy goat is younger. Some cytokines *e.g.* BIO (A GSK3 inhibitor, 6-bromoindirubin-3′-oxime) and basic fibroblast growth factor (bFGF) acted positively on the maintenance of proliferation and pluripotency of mGSC. Leukemia inhibitory factor (LIF) might, however, inhibit the proliferation of dairy goat mGSC. These cultured mGSC maintained similar characteristics as mouse and human mGSC. These results provide an efficient system to isolate and culture of dairy goat mGSC.

1. Introduction

Male germline stem cells (mGSC) are the only kind of adult stem cells that exist in seminiferous tubules of adult testis which can transmit genetic material to the next generation (Kubota et al., 2004a). The capacities of these cells for self-renewal and differentiation into sperm support spermatogenesis throughout the life of the animal. Studies on mouse, primate and human mGSC have been conducted for many years, and pluripotent cell lineages derived from mGSC have been obtained from mouse, primate, and human testes (Guan et al., 2006; Conrad et al., 2008; Hermann et al., 2012).

Male GSCs retain the characteristics of stem cells and male germline cells and these features help in isolating and identifying the mGSC in testis (Dym et al., 2009). Because no specific marker is available for mGSC, scientists used

* Corresponding author at: Tel.: +86 29 87080068; fax: +86 29 87080068.
E-mail address: jinlianhua@nwsuaf.edu.cn (J. Hua).

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several markers in combination to identify and purify the mGSC in testis. Genes expressed in mGSC vary among species. CD49f, CD9, GFRa1, and CD90 are surface markers for mouse and human mGSC (Conrad et al., 2008; Hofmann et al., 2005; Kubota et al., 2003; Kanatsu-Shinohara et al., 2004; Shinohara et al., 1999). Whether one or several markers are expressed in dairy goat mGSCs, and it may also be validity to identify and purify mGSCs from dairy goat testis were investigated in this study.

Dairy goats are one type of livestock of great importance for milk and meat production as a human food source. Male GSCs derived from dairy goat testis are a valuable resource for genetic material conservation and optimization. The studies on dairy goat mGSC will be beneficial for developing the systems of isolation, purification, culture, and exploration of the mechanisms of self-renewal and differentiation of mGSC.

In vitro culture provides an efficient method to study the molecular mechanism and cellular pathways that sustain the functions of mGSC. Mouse and human pluripotent mGSC lines have previously been established (Guan et al., 2006; Conrad et al., 2008). Hua et al. (2011) reported that fetal dairy goat mGSC can be successfully cultured *in vitro*. However, the studies on isolation and culture systems of dairy goat mGSC have not been defined.

In the present study, comparisons were made on enzyme cocktails in digesting dairy goat testis at ages from newborns to adults and the effects of different extracellular matrix types on purifying mGSC by counting the percentage of CD49f positive cells were compared. Also, the roles of cytokines and small molecules in promoting mGSC colony formation and proliferation were determined. Furthermore, attempts were made to establish an efficient system to isolate and culture dairy goat mGSC.

2. Materials and methods

2.1. Isolation of male germline stem cells (mGSC) from goat testis

The goats were killed to conduce the experiment, and testes from newborn to adult dairy goats were collected. All the procedures were conducted under the supervision of Chinese Association for Laboratory Animal Science. Goat testes were washed 5–10 times with phosphate buffered saline (PBS) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. The seminiferous tubules were stripped from each testis and then dissected into small pieces using forceps. Seminiferous epithelial cells were dissociated using three different enzyme cocktails: (1) CDH, 2 mg/ml collagenase (Invitrogen), 20 μg/ml DNase (Sigma) and 2 mg/ml hyaluronidase (Sigma); (2) CDD, 2 mg/ml collagenase (Invitrogen), 20 μg/ml DNase (Sigma) and 2 mg/ml dispase (Invitrogen); and (3) CTHD, 1 mg/ml collagenase (Invitrogen), 10 μg/ml DNase (Sigma), 1 mg/ml hyaluronidase (Sigma) and 1 mg/ml trypsin (Invitrogen). All the enzyme cocktails were dissolved in Dulbecco's phosphate buffered saline. The digestion was conducted at 37 °C for 15 min with pipetting every 5 min for the first step. After centrifugation at 100 × *g* for 5 min, the fragments of seminiferous tubules were collected and digested with TD (0.25% trypsin and 10 mg/ml DNaseI) for the second step for 10 min at 37 °C. The effects of different enzyme combinations were identified by the total number of cells per gram testicular tissue and cell viability was determined by Trypan blue staining.

Table 1
The cytokines and small molecule used in culture mGSC.

Group	Control	LIF	bFGF	BIO	LIF + bFGF	LIF + BIO	BIO + bFGF
LIF	–	+	–	–	+	+	–
bFGF	–	–	+	–	+	–	+
BIO	–	–	–	+	–	+	+

2.2. Purification of dairy goat mGSC

The dairy goat testicular cells were cultured on extracellular matrix of 1 μg/ml Fibronectin (Sigma), 5 μg/ml Laminin (Sigma), 2 μg/ml Gelatin (Sigma) or Matrigel (BD) coated plates at 37 °C, 5% CO_2 and at a saturated humidity for 2 h, and the cells were seeded at the concentration of 10^6 cells/ml, respectively. Subsequently, the attached cells were fixed with methanol/acetone (*V*:*V* = 1:1) for 15 min and identified by immunofluorescence staining using CD49f (1/500, Chemicon) antibody, which is believed to be a marker for mGSC (Shinohara et al., 1999). The efficiency of each extracellular matrix in dairy goat mGSC purification was measured by the percentage of CD49f positive cells of the total number of cells that attached.

2.3. Optimization of the culture conditions for dairy goat mGSC

The purified cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% Knockout serum replacement (KSR, Invitrogen), 4 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen) which were comprised using different combinations of the cytokines and small molecular BIO, such as 1000 units/ml human recombinant leukemia inhibitory factor (rhLIF, Millipore), 5 ng/ml recombinant human basic fibroblast growth factor (bFGF, Millipore) and 2.5 μM 6-bromoindirubin-3′-oxime (BIO, Merck). The effects of the cytokines and small molecular BIO (Table 1) were determined by the number of colonies and total cells after being cultured for 1 week.

2.4. Influence of age on the culture of dairy goat mGSC

To test whether the age of dairy goat influence the passage of mGSC *in vitro*, mGSC derived from goats of different ages were cultured and passaged under normal conditions (with the medium of DMEM supplemented with 20% KSR, 4 mM L-glutamine, 1% non-essential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 5 ng/ml bFGF and 2.5 μM BIO). The effects were measured by assessing the greatest passage and average passage rates of cells at different ages.