Journal of Integrative Agriculture 2017, 16(11): 2547–2557

Available online at www.sciencedirect.com

ScienceDirect

RESEARCH ARTICLE

Establishment and characterization of immortalized bovine male germline stem cell line

LEI Qi-jing[1], PAN Qin[1], MA Ju-hong[1], ZHOU Zhe[1], LI Guang-peng[2], CHEN Shu-lin[1], HUA Jin-lian[1]

[1] *College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering and Technology, Northwest A&F University, Yangling 712100, P.R.China*

[2] *Key Laboratory for Mammalian Reproductive Biology and Biotechnology, Ministry of Education/Inner Mongolia University, Hohhot 010021, P.R.China*

Abstract

Male germline stem cells (mGSCs) are unique adult germ cells with self-renewal potential and spermatogenesis function in the testis. However, further studies are needed to establish a long-term cultural system of mGSCs *in vitro*, especially for large animals such as bovine mGSCs. In this study, we first established a stable immortalized bovine male germline stem cell line by transducing Simian virus 40 (SV40) large T antigen. The proliferation of these cells was improved significantly. These cells could express spermatogonial stem cell (SSC)-specific markers, such as PLZF, PGP9.5, VASA, LIN28A, and CD49F, both in the mRNA and protein levels. Additionally, these cells could be differentiated into three germ layer cells to enter meiosis, form colonies, and proliferate in the seminiferous tubules of busulfan-induced infertile mice. The immortalized bovine mGSCs maintain the criteria of mGSCs.

Keywords: male germline stem cells, immortalization, bovine, proliferation, cell transplantation, testis

1. Introduction

Male germline stem cells (mGSCs), also named spermatogonial stem cells (SSCs), are the only stem cells in organisms that transmit genetic information into the next generation (Kubota *et al.* 2003; Oatley and Brinster 2003). Production of functional germ cells is essential for the continuation of the germline of mammalian species. In mammalian testis, 1 g of tissue can produce more than 20 million sperms everyday (Amann 1986). Moreover, SSCs can transdifferentiate directly to other crucial type of cells *in vitro* (Zhang *et al.* 2013; Chen *et al.* 2016). Spermatogonia have traditionally been subdivided into A and B (Rooij *et al.* 1998; Nayernia *et al.* 2003).

Bovine, as an important economic animal, provides premium beef and hides. Expanding the bovine mGSCs *in vitro* is difficult, and a long-term culture system of bovine mGSCs is still lacking (Sahare *et al.* 2015; Cai *et al.* 2016). Therefore, establishing the immortalization of bovine germline stem cells is necessary. Feng *et al.* (2002) and Hofmann *et al.* (2005) have reported that mGSCs could be immortalized by outer cause, such as telomerase reverse transcriptase (TERT) and Simian virus 40 (SV40) T antigen. However, the high expression level of telomerase is a fun-

Received 22 February, 2017 Accepted 14 April, 2017
LEI Qi-jing, E-mail: qijinglei@nwafu.edu.cn; Correspondence HUA Jin-lian, E-mail: jinlianhua@nwsuaf.edu.cn; CHEN Shu-lin, E-mail: csl_1359@163.com; LI Guang-peng, Tel/Fax: +86-471-4992495, E-mail: gpengli@immu.edu.cn

doi: 10.1016/S2095-3119(16)61625-8

damental characteristic of germline stem cells (Pech *et al.* 2015). In contrast, SV40 large T antigen was an efficient factor for animal cell immortalization (Hofmann *et al.* 2005; Hou *et al.* 2015). In this study, bovine mGSCs were immortalized by SV40 large T antigen, and the cells fulfilled the criteria of mGSCs and maintained the stemness markers and self-renewal and meiosis potential.

2. Materials and methods

2.1. Isolation and enrichment of male germline stem cells (mGSCs) from bovine testis

The 4–5 month old Qinchuan bull calves were killed and their testes were harvested. All the procedures were carried on under the supervision of Chinese Association for Laboratory Animal Science, and approved by the Ethics Committee of Northwest A&F University, China. Bovine mGSCs were isolated and purified as previously reported (Li B *et al.* 2016). Bovine mGSCs were purified by magnetic activated cell sorting (MACS) with THY1 antibody, the detailed procedure as previously reported (Reding *et al.* 2010; Zheng *et al.* 2016).

2.2. Culture of bovine mGSCs

The culture medium consisted of DMEM/F12 supplemented with 10% KSR (Gibco, Massachusetts, USA), 0.1 mmol L^{-1} β-mercaptoethanol (Sigma, Missouri, USA), 2 mmol L^{-1} L-glutamine (Invitrogen, Carlsbad, CA, USA), 1% non-essential amino acids (Invitrogen), 1% FBS (Gibco), 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (Invitrogen). The growth factors used in this study were 20 ng mL^{-1} GDNF (Peprotech, Rocky Hill, USA), 20 ng mL^{-1} bFGF (Millipore, Massachusetts, USA) and 10 ng mL^{-1} LIF (Millipore).

2.3. Lentivirus preparation and the immortalization of bovine mGSCs

Lentivirus production was referred as Auvergne *et al*. (2013) described. Briefly, HEK293T cells were seeded in culture plates, the plasmids pLOX-Ttag-iresTK (Addgene, Cambridge, USA) containing SV40 large T antigen along with the plasmids pVSVG and pPAX2 were added into culture medium together 24 h after the seeding and incubated for 20–30 min. The virus-containing supernatant was harvested 48 h after transfection and used for transduction after the cell debris were removed by filter. For cells immortalization, 1×10^5 of bovine mGSCs were plated in a 35-mm dish. 12 h later, the cells were transduced with virus containing supernatant and 10 μg mL^{-1} polybrene (Sigma), incubated overnight at 37°C and 5% CO_2. The medium was replaced with fresh DMEM/F12 medium supplemented with 10% FBS (Gibco), 0.1 mmol L^{-1} β-mercaptoethanol (Sigma) and 2 mmol L^{-1} glutamine (Invitrogen). The culture medium would be changed every 2–3 days, and then cultured for more than 3 months until the cells were immortalized.

2.4. Population doubling time (PDT) determination

The population doubling time (PDT) of bovine mGSCs were measured following the protocol described previously (Zhang *et al.* 2011). In brief, cells were serially subcultured. We calculated the initial seeding cell number and the total cell number after culture for 48 h according to the formula below, $PDT=[lg2/lg(Nt/No)]\times t$, where, Nt is the number of cells after *t* h of culturing, No is the number of cells seeded initially.

2.5. RT-PCR

Total RNA for reverse transcription-PCR (RT-PCR) analysis was extracted from the 15th passage bovine mGSCs under normal conditions using RNAiso (TaKaRa, Biotech. Co., Ltd., Dalian, China). The cDNA was synthesized based on 500 ng RNA with a TransScript II First-Strand cDNA Synthesis Kit (TaKaRa, Biotech. Co., Ltd., Dalian, China) according to the producer's instruction. The primers were designed based on the sequences of the open reading frame from the NCBI GenBank and synthesized by Sangong Biotech (Sangong Biotech, Shanghai, China). Specific PCR primers and the length of the amplified products are listed in Table 1. The PCR products were evaluated by agarose (Invitrogen) gel electrophoresis, visualized with UV illumination after stained with ethidium bromide (Invitrogen).

2.6. Immunofluorescence staining

The 15th passage bovine mGSCs cultured in normal conditions were fixed with 4% PFA for 15 min at room temperature. After washed with cold PBS, cells were blocked with 1% BSA for 30–60 min and incubated in primary antibodies including CD49F (1:500, Bioss, Beijing, China), OCT4 (1:300, Abcam, MA, Cambridge, USA), STRA8 (1:500, Abcam, MA, Cambridge, USA), and VASA (1:500, Abcam, MA, Cambridge, USA). After washed with PBS, cells were incubated with the corresponding FITC conjugated secondary antibodies according to the manufacturer's manual (1:500, Invitrogen). The nuclei were stained by 1 μg mL^{-1} Hoechst 33342 (Sigma) for 5 min at room temperature. Meanwhile, the negative controls were stained with the appropriate fluorescent-conjugated secondary antibodies.

2.7. CDy1 staining

CDy1 staining was performed following to the manufacturer's