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Identification and IVC of spermatogonial stem cells in prepubertal buffaloes

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ABSTRACT

Development of suitable selective marker for buffalo spermatogonial stem cells (SSCs), optimization of long-term IVC conditions, and their pluripotent retention capacity in buffaloes can be of prime importance in selective genetic modifications of this species. In the present study, we identified CDH1 as a specific marker for buffalo SSCs and revealed that it existed in two protein isoforms (large [135 kDa] and small [90 kDa] subunits) in the buffalo testis; furthermore, immunohistochemical analysis revealed that CDH1 expression was present in spermatogonia but absent in the somatic cells of 4-month-old buffalo testis. After 7 days of enrichment, expression of CDH1 was also detectable in IVC colonies (~53% enrichment efficiency by Fluorescence-activated cell sorting (FACS)). For long-term culture of SSCs, proliferation studies with different factors showed that combination of 20 ng/mL GDNF, 10 ng/mL FGF2, and 1000 U/mL LIF could significantly promote number of colonies (~two folds) and proliferation of buffalo SSCs (~three folds) compared with those of control or single-treatment groups; furthermore, addition of these combination growth factors significantly upregulated the messenger RNA level of spermatogonial-specific and pluripotency-related markers (*BCL6B*, *GFRA1*, and *POU5F1*), whereas downregulated receptor tyrosine kinase (*KIT*). For confirmation of their stem cell potential, Dolichos biflorus agglutinin–stained cells were identified in the basal membrane of seminiferous tubules of xenotransplanted mice testis. These findings indicate the identification of a new buffalo SSCs marker; furthermore, it may help in establishing long-term culture that would assist in genetic modification of these buffaloes.

1. Introduction

Spermatogonial stem cells (SSCs) have unique capacity for self-renewal in the seminiferous tubules and continuously support spermatogenesis throughout the life of a male animal. Genetically modified SSCs can give rise to a vast number of spermatozoa that produce offspring following transplantation into a recipient testis, which makes it a potential novel technology with feasible application to various animal species like mouse, rat, and goat [1–3]. Recently, successful germ-cell transplantation into bull testis has resulted in the production of donor-derived sperm cells [4]. Chinese crossbred buffalo (Murrah × Swamp) has been originated by crossing local swamp and river buffaloes [5]. The selective breeding has improved the overall production level in buffaloes; however, these animals are poor breeders as indicated by poor fertility and low reproductive efficiencies. IVC of buffalo SSCs can provide genetic modification of this species; however, no information is available about buffalo SSCs.

Over the years, culture conditions for mouse SSCs have been established, facilitating the characterization of these

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cells and factors involved in self-renewal and differentiation [6]. However, no male germ stem cell line has yet been established for livestock species. The reason for this may be because of the lack of sufficient understanding regarding spermatogonial-specific markers. Recently, Dolichos biflorus agglutinin (DBA) [7–9], UCHL1 (ubiquitin carboxyl-terminal esterase L1, previously known as PGP 9.5) [10–12], and POU5F1 [13] have been reported as specific markers for spermatogonia in prepubertal buffalo testis, and are further utilized for SSCs characterization in short-term culture. However, stem cell potential of buffalo spermatogonia has not been determined yet. In the present study, for the first time, we reported spermatogonial-specific markers UCHL1 and CDH1 in prepubertal crossbred buffalo testes and used them to identify the efficiency of spermatogonial enrichment and characterization during IVC.

Several approaches have been proved to be critical for IVC and expansion of mouse and bovine SSCs. Using a combination of growth factors and a special stem cell medium, Kanatsu-Shinohara, et al. [14], have achieved expansion of mouse SSCs by a factor of 1012-fold; furthermore, combination of GDNF, GFRA1, and FGF2 has been shown to induce self-renewal [15]. In domestic animals, Kuijkwe, et al. [16] investigated the effect of LIF, GDNF, EGF, and FGF on primary cell cultures of pig neonate testis and demonstrated that EGF and FGF had a large influence on the formation of SSC-like colonies and on gene expression patterns. Similarly, various growth factors and their combinations were tested for their *in-vitro* effects on qualitative and quantitative aspects of bovine SSC behavior [17].

Because the growth factors that regulate the balance of self-renewal and differentiation of SSCs are of great interest, we sought to provide a suitable system based on morphology and transcriptional and translational evaluation of SSC-specific factors. Here, we used a factorial design to investigate the effects of FGF2, GDNF, and LIF on primary cell culture of buffalo spermatogonia.

Furthermore, stem cell potential of buffalo spermatogonia after IVC was determined by xenotransplantation assay. Although germ cell transplantation from nonrodent species into mouse testis did not result in complete spermatogenesis, till date it is the only available bioassay for detecting the stem cell potential of germ cells in a given population of donor testis cells from any species [18,19].

2. Materials and methods

2.1. Animals and ethical statement

The BALB/c-nu mice were obtained from Hubei Disease Control Center (Wuhan, China), and the protocol for animal use in the present investigation was approved by the University's Institutional Animal Ethics and Care and Use Committee. The mice were maintained in a germ-free isolation facility at 22 ± 1 °C with 70% relative humidity. The food and water were autoclaved and were generally offered *ad libitum*.

Testes were collected from 3- to 6-month-old male Chinese crossbred buffalo (Murrah × Swamp) calves (Hubei Jinniu Animal Husbandry Ltd., Jing Men, China) after aseptic surgical castration.

2.2. Immunohistochemistry

Fixed tissues from prepubertal and adult buffalo testes were dehydrated, embedded in paraffin, and sectioned (6 mm thick). Dilutions of primary and secondary antibodies were done in PBS with 1% BSA (Sigma-Aldrich Co.). Briefly, sections were dewaxed, rehydrated, and blocked with 10% fetal bovine serum (Gibco) in PBS for 30 minutes and incubated with mouse antihuman UCHL1 (1:1000, cat. no. sc-1183; Santa Cruz Biotechnology) and rabbit antihuman CDH1 antibodies (1:500, cat. no. sc-7870; Santa Cruz Biotechnology) overnight at 4 °C. After washing three times with PBS, testicular tissues were placed in 3% H_2O_2 (Dingguo Co., Beijing, China) for 10 minutes, washed three times with PBS, and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies, i.e., goat antirabbit immunoglubulin G (IgG) and goat antimouse IgG (Boster Co., Beijing, China; 1:100) for 30 minutes at 37 °C. After this, the tissues were rinsed three times with PBS, incubated for 3 to 5 minutes in DAB substrate kit (Vector Laboratories) according to the manufacturer's instructions. After rinsing thoroughly in distilled water, tissues were counterstained with hematoxylin, dehydrated, and photographed by inverted fluorescent microscopy (Eclipse TE2000-U; Nikon, Tokyo, Japan). For negative controls, primary antibody was omitted, and instead, the section was incubated with 1% BSA in PBS.

2.3. Western blot assay

Testes were washed with PBS twice and lysed by ultrasonic cell cracker with the radioimmunoprecipitation assay buffer (Dingguo) already mixed with protease inhibitor. After placing on ice for 5 minutes, the cell protein buffer was separated from cell debris by centrifugation at 13,300 × *g* for 5 minutes. The total protein concentration was measured by bicinchoninic acid assay (Dingguo), and a total of 80 μg of total protein was prepared for each sample before loading into 12% PAGE gel.

After electrophoresis, the proteins were transferred to the PVDF membranes (Millipore). After blocking nonspecific sites by 5% nonfat milk diluted in Tris-buffer saline supplemented with 1% Tween-20 buffer (TBST) at room temperature for 1 hour, the membranes were incubated at 4 °C overnight with primary antibodies (UCHL1, 1:1000; CDH1, 1:800; ACTB, 1:500; Santa Cruz Biotechnology). The membranes were washed three times in TBST on low-speed orbital shaker to remove residual primary antibodies. Later, the membranes were incubated with secondary antibodies (1:3000, HRP-labeled goat antirabbit or rabbit antimouse IgG; Santa Cruz Biotechnology) at 37 °C for 1 hour. After rinsing with TBST three times, the HRP immunoblots were detected by Clarity western ECL substrate (Bio-Rad) and exposed to X-ray films for visualization of the protein bands.

2.4. Isolation of spermatogonia

Testes were collected from buffalo calves (3- to 6-month old, 10 heads) by standard castration procedure. A two-step