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Stage-specific expression of Sal-like protein 4 in boar testicular germ cells

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ABSTRACT

Spermatogenesis, the complex process of sperm cell development including mitotic cell division and meiosis, relies on spermatogonial stem cells (SSCs). While markers for developing germ cells have been well investigated in mice, developmental stage-specific markers of germ cells in domestic animals have not been identified. Sal-like protein 4 (SALL4) is known as a putative marker for undifferentiated spermatogonia in rodents; however, its expression in domestic animals has not been investigated. The objective of this study was to characterize the expression of SALL4 in the developmental stages of boar testes and SSCs. Interestingly, all SALL4-expressing cells responded positively to PGP9.5, which is known as a spermatogonia marker in boar testes, while some PGP9.5-positive cells did not express SALL4 in pre-pubertal boar testes. At this stage, the expression of SALL4 was observed in GFRα1-positive cells, and its expression was maintained in cultured pSSCs *in vitro*, suggesting that SALL4 is a marker of early-stage boar spermatogonia that express GFRα1 in pre-pubertal testes. Additionally, SALL4 expression was observed in c-Kit-positive but not in PGP9.5- or SCP3-positive cells in post-pubertal testes. In conclusion, SALL4 is expressed in early undifferentiated spermatogonia in pre-pubertal boar testes and in primary spermatocytes in post-pubertal boar testes. Therefore, SALL4 can be used as a stage-specific marker of developing germ cells in boar testes.

1. Introduction

Spermatogonial stem cells (SSCs) are present within seminiferous tubules, which are composed of a basal membrane surrounded by a layer of peritubular cells in the testis [1]. Spermatogonial stem cells maintain a balance between self-renewal and differentiation capacities in order to perform spermatogenesis in the adult testis. Such cells have been identified in many species, including rodents, pigs, and cows, and they can be used as a tool to recover fertility [2–6].

Mouse SSCs are well characterized and are easily identified by specific cell-surface molecules such as integrin α6 and β1, THY-1 (CD90), and CD9 [7–10]. Recent research on mouse SSCs has also identified intracellular molecules as SSC markers, such as the transcription factors PLZF, OCT4, and neurogenin 3 [11–13]. Additionally, a number of protein markers have been identified that are expressed in differentiated spermatogonia, including STAT8, KIT, SOHLH1, and SOHLH2 [14].

Kanatsu-Shinohara et al. first reported the derivation of embryonic stem (ES)-like cells from neonatal mouse testes [7]. These multipotent germ line stem cells exhibited similar morphology and gene expression patterns to mouse ES cells and developed three germ layers, both *in vitro* and *in vivo*, contributing to chimera formation [15].

Furthermore, mouse SSCs express several pluripotency marker genes, including *Oct4*, *Sox2*, *Klf*, and *c-Myc,* that are required to reprogram fibroblasts into a pluripotent state [16]. Extensive gene expression profiling of mouse germline stem (mGS) cells, mouse ES (mES) cells, induced pluripotent stem (iPS) cells, and SSCs revealed that the gene expression patterns of mGS cells are closer to those of mES cells than to those of iPS cells [17].

Sal-like protein 4 (SALL4) is a member of the *SALL* gene family of zinc finger transcription factors that was first discovered in *Drosophila* and later found to be conserved in *Caenorhabditis*

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elegans, *Xenopus*, mice, and humans [18]. Mouse SALL4 is essential for inner cell mass proliferation in early embryonic development and interacts with other transcription factors, such as TBX family proteins, to control the development of various organs including the brain and heart [18–20]. Several studies have demonstrated that SALL4 interacts with important pluripotency genes such as *Oct4* and *Nanog*. Functional studies using SALL4-deficient and SALL4-overexpressing ES cells showed that a loss of SALL4 is associated with accelerated differentiation, while overexpression of SALL4 reduces ES cell differentiation [19,21,22].

In mice, SALL4 is expressed in the adult testis and is undetectable by northern blotting in all other organs except the ovary. However, its expression is detectable in mouse gonads by reverse transcription-polymerase chain reaction (RT-PCR) [21,23].

Conditional SALL4 knock-out mice failed to exhibit a robust ES cell pool, demonstrating that SALL4 can interact directly with PLZF, a transcription factor required for SSC function [24]. Zhang et al. report that only one isoform of SALL4B has been cloned and sequenced in pig tissues and embryos, revealing that SALL4 is expressed most strongly in the ovary, spleen, lung, heart, and testis [25]. Based on these previous studies, the objective of this study was to determine the expression patterns of SALL4 during the different developmental stages of boar testes, testicular germ cells, and cultured pSSCs. The potential of SALL4 for use as a putative intracellular marker of boar germ cells is also discussed.

2. Materials and methods

2.1. Derivation of pSSC and in vitro culture

Culturing and derivation of pSSC were carried out according to the methods described by Lee et al. [26]. Briefly, pSSCs were derived during the castration of 5-day-old crossbred piglet testes at the Sam-Woo Breeding Farm, South Korea. Each testis from five piglets was encapsulated and dissociated with Collagenase IV and DNase I for 15 min at 37 °C before being filtered through a 40-μm nylon mesh. Red blood cells (RBCs) were eliminated using RBC Lysis Buffer (Sigma-Aldrich, St. Louis, MO, USA). Isolated testis cells were seeded onto 0.2% (w/v) gelatin-coated 12-well plates (2×10^5 cells per well) and incubated with Stempro-34 medium (Gibco; 10640-019) at 31 °C in 5% CO_2. To maintain the pSSC population *in vitro*, every 7 or 8 days, upon confluence, pSSCs from testicular fibroblast cells were trypsinized using 0.005% Trypsin-EDTA (Gibco; 25300-054). Following trypsinization, 0.5×10^5 pSSCs and newly prepared testicular fibroblasts were seeded into 0.2% (w/v) gelatin-coated 12-well plates.

2.2. Histology, immunohistochemistry, and immunocytochemistry

First, testes taken from 5-, 30-, 60-, 90-, 120 and 150-day old boars were obtained from the National Institute of Animal Science (NIAS), according to guidelines approved by the Institutional Animal Care and Use Committee at the NIAS (approval no. NIAS2015-120), and from the Sam-Woo Breeding Farm in Korea. Each stage of two pairs of testes samples were rinsed in phosphate-buffered saline (PBS), fixed in Bouin's solution (Sigma-Aldrich; HT10132) overnight at 4 °C, and dehydrated through an alcohol gradient for 120 min. Dehydrated tissues were cleaned in xylene, infiltrated with melted paraffin for 2 h at 65 °C, and embedded in paraffin blocks. Testes were sectioned at a thickness of 5 μm using a microtome (Leica, Nussloch, Germany), and sections were placed on glass slides. Hematoxylin and eosin staining were carried out using Gill's hematoxylin and eosin Y solution (Sigma-Aldrich).

For immunohistochemistry, tissue sections were rehydrated using xylene and 50–100% ethanol. Antigen unmasking was carried out by boiling sections in 10 mM sodium citrate buffer for 10 min. Non-specific binding was blocked using 1% bovine serum albumin (BSA) in PBS for 30 min at 22 °C. Samples were incubated overnight at 4 °C with primary antibodies including SALL4 (1:100 dilution; Abcam), PGP9.5 (1:500 dilution; Serotec), SCP3 and C-Kit (1:100 dilution for each; Santa Cruz Biotechnology), and GFRα1 (1:50 dilution; Santa Cruz Biotechnology; Table 1). After washing each tissue section five times in PBS for 10 min, tissue sections were incubated with fluorescent-conjugated secondary antibodies diluted 1:300 in 1% BSA in PBS or with horseradish peroxidase (HRP)-conjugated secondary antibody (1:500 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at 25 °C, followed by incubation in 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA). For immunofluorescence staining, secondary antibodies included Alexa Fluor® 568 Donkey Anti-Rabbit IgG, Alexa Fluor® 488 Goat anti-Mouse IgG, Alexa Fluor® 568 Donkey anti-Mouse IgG, and Alexa Fluor® 488 Goat anti-Rabbit IgG from Life Technologies, and Goat anti-Mouse IgG-HRP and Donkey anti-Goat IgG-HRP from Santa Cruz Biotechnology. Tissue sections were incubated with or without 1 μg/mL 6-diamidino-2-pheylindole (DAPI) in PBS for 10 min, and coverslips were applied with mounting solution (DAKO; Carpinteria, CA, USA; S3025).

Total boar testis cells and *in vitro*-cultured pSSC colonies were rinsed in PBS and fixed in 4% paraformaldehyde for 10 min followed by membrane permeabilization with PBS containing 0.05% Triton X-100 for 10 min. Non-specific protein binding was blocked with 1% BSA in PBS for 30 min at room temperature (RT), and cells were incubated overnight at 4 °C with the following primary antibodies: SALL4 (1:100 dilution) and PGP9.5 (1:500 dilution). Cells were then washed with PBS and incubated for 1 h at RT with Alexa Fluor® 568 Donkey anti-Rabbit IgG to detect PGP9.5 and Alexa Fluor® 488 Goat anti-Mouse IgG to detect SALL4. For nuclear staining, DAPI was added at a concentration of 1 μg/mL for 10 min. Finally, mounting solution was used to fix pSSC colonies. Immunostained tissue and pSSC colonies were observed using a fluorescence microscope (Nikon, Tokyo, Japan).

2.3. Isolation of RNA and RT-PCR

Total RNA was extracted from pSSC colonies (passage 2) and testis fibroblasts (pFeeder) using an RNeasy Mini Kit (Qiagen) with on-column DNase treatment (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen, California, USA) with the Oligo(dT)30 primer, according to instructions specified by the manufacturer. Target gene PCR amplification was carried out using 30 cycles of 30 s at 95 °C, 10 s at 57 °C, and 20 s at 72 °C. Primers were designed using Primer3 (http://frodo.wi.mit.edu). The following primers were used to detect boar transcripts: SALL4-sense 5′-GACTCACTGCCCTTGTCCTG-3′, SALL4-antisense 5′-CTTCGGCTCTGGTACTCCTG-3′; PGP9.5-sense 5′- GAGATGCTGAACAAAGTGCTG-3′, PGP9.5-antisense 5′-CATGGTTCACCGGAAAAGG-3′; and B2M-sense 5′-TTCACACCGCTCCAGTAG-3′, B2M-antisense 5′-CCAGATACATAGCAGTTCAG-3′, with B2M functioning as a control gene.

Table 1
List of antibodies for immunostaining.

Antibody	Company	Catalogue number	Diluted
SALL4	Abcam	ab57577	1:100
PGP9.5	Serotec	7863–1004	1:500
SCP3	Santa Cruz Biotech	SC-33195	1:100
C-Kit	Santa Cruz Biotech	SC-1494	1:100
GFRα1	Santa Cruz Biotech	SC-6157	1:50