Animal Reproduction Science 152 (2015) 39–46

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

The KIT is a putative marker for differentiating spermatogonia in stallions

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ARTICLE INFO

Article history:
Received 10 June 2014
Received in revised form 18 October 2014
Accepted 13 November 2014
Available online 21 November 2014

Keywords:
KIT
Spermatogonial stem cells
Stallion
Germ cells

ABSTRACT

Putative markers have been discovered and are used to identify and separate certain lineage of spermatogonia. The KIT is a marker for differentiating spermatogonial stem cells in several species including mice and goats. The objectives of this study were (1) to investigate reproductive stage-dependent KIT expression patterns in stallions and (2) to identify spermatogonia subpopulations expressing KIT in stallion testes. To achieve these objectives, testicular samples were obtained during routine field castration of stallions. The reproductive stage of the stallions was classified as pre-pubertal (<1 year, $n = 3$), pubertal (1–1.5 year, $n = 4$), post-pubertal (2–3 year, $n = 6$), or adult (4–8 year, $n = 6$). For immunohistochemistry, KIT was used at a dilution of 1:200. In the pre-pubertal and pubertal stage, most germ cells were immunolabeled with KIT. In the post-pubertal and adult stages, immunolabeling of KIT was evident in the germ cells attached to the basement membrane of the seminiferous tubules with exception of some spermatogonia. Co-immunolabeling with KIT and deleted in azoospermia like (DAZL) showed different co-staining patterns, including KIT only, both KIT and DAZL), or DAZL positive germ cell populations alone. The KIT was not immunolabeled in Sertoli or Leydig cells at any reproductive stages. The result of Western blot analysis verified the cross-activity of the KIT antibody with horse testes tissue. In conclusion, KIT appears to be expressed in differentiating spermatogonia, and may be used to identify and isolate differentiating germ cells from stallions.

1. Introduction

Spermatogenesis is the process of germ cell proliferation and differentiation into spermatozoa (Johnson et al., 1997). A malfunction during spermatogenesis will result in stallion infertility or subfertility, which causes financial loss in the horse breeding industry. Little information is available about the biological activity of germ cells in stallions because of the limited laboratory techniques available to identify, isolate, and culture stallion germ cells. In other species, including humans, antibodies against molecules specifically and uniquely expressed in the spermatogonia have been discovered and are used to identify specific spermatogonia subpopulations using immunohistochemistry and immunocytochemistry (Phillips et al., 2010).

The v-KIT Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) also called CD117, C-KIT, mast/stem cell growth factor receptor is a transmembrane

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http://dx.doi.org/10.1016/j.anireprosci.2014.11.004
0378-4320/

tyrosine kinase receptor. It is one of the several molecules expressed in germ cells and has been used as a germ cell marker in several species, including mice (Motro et al., 1991; Rossi et al., 1991; Tajima et al., 1991), rats (Dym et al., 1995), and goats (Heidari et al., 2012). This 145 kDa transmembrane protein has 979 amino acid residues, and as a transmembrane tyrosine kinase receptor, it has three main functional regions, including extracellular, transmembrane, and intracellular domains (Blechman et al., 1995; Zhang et al., 2013). The c-KIT ligand is synthesized in Sertoli cells (Rossi et al., 1991), and it binds to c-KIT, resulting in the transduction of regulatory signals into germ cells. The c-KIT/KIT ligand pathway is important for the proliferation, migration, survival and/or maturation of germ cells (Manova et al., 1990; Godin et al., 1991; Pesce et al., 1993; Albanesi et al., 1996; Ohta et al., 2000). Interestingly, the KIT immunolocalization pattern in male germ cells varies depending on the species. For example in mice, c-KIT expression was found in differentiating type A spermatogonia to pachytene spermatocytes, whereas c-KIT expression was not detected in the undifferentiated spermatogonia (Motro et al., 1991; Rossi et al., 1991; Tajima et al., 1991). In rats, c-KIT is expressed in the cytoplasm of isolated type A spermatogonia (Dym et al., 1995). The c-KIT regulates the survival and/or proliferation of type A1–A4 spermatogonia, but does not affect undifferentiated A single (As or A0) spermatogonia, indicating that c-KIT is expressed in type A1–A4 spermatogonia, but not in the undifferentiated spermatogonia such as As. The results of these studies suggest that c-KIT may be used as a putative marker for differentiating spermatogonia (Yoshinaga et al., 1991). Recently, the presence of c-KIT in spermatogonial stem cells (SSCs) was reported in goats, and a c-KIT antibody was used to identify SSCs in a SSC tissue culture system (Heidari et al., 2012). In contrast to what was observed with rodents, the lineage of germ cells expressing c-KIT in goats is different. These findings in different species suggest that c-KIT plays an important role in spermatogonia, and its expression pattern in germ cells may vary depending on the species.

Expression of KIT in stallion germ cells has not been previously studied. The identification of germ cells expressing KIT should provide an opportunity to understand more about the process of spermatocytogenesis and biological activity of KIT in stallion germ cells. Therefore, the objectives of this study were (1) to investigate reproductive stage-dependent KIT expression patterns and (2) to identify spermatogonia subpopulations expressing KIT in stallion testes. Based on the results of analysis in other species, we hypothesized that KIT is expressed in the membrane of spermatogonia, with different expression patterns during the different reproductive stages and that KIT expression is limited to differentiating spermatogonia.

2. Materials and methods

2.1. Animals

Stallion testes were obtained from a field castration service provided by local veterinarians in the Republic of Korea and through routine castration procedures at the University of California Davis Veterinary Medical Teaching Hospital, with permission from the horse owner. The stallions were all light-horse breeds such as Thoroughbred, quarter, and Jeju horses. Reproductive stages of the horses were categorized based on the age of the horses as follows: pre-pubertal (<1 year, $n = 3$), pubertal (1–1.5 year, $n = 4$), post-pubertal (2–3 year, $n = 6$) and adult (4–8 year, $n = 6$). The reproductive status of stallions prior to castration was not evaluated, but the condition of the testes was evaluated during testicular tissue dissection and histological observation. Only normal testicular samples were used in this study.

2.2. Testicular tissue sample preparation

Testicular tissue samples were prepared as previously described (Yoon et al., 2011), with slight modifications. After castration, testis samples were maintained in an ice box (4 °C) and transported to the laboratory. For testicular tissue fixation, testicular parenchyma samples (1 cm^3) were treated in 4% paraformaldehyde for at least for 24 h. After extensive washing with phosphate buffered saline (PBS) for 24 h, tissue samples were dehydrated using a graded ethanol series and embedded in a paraffin block. For western blotting analyses, a small piece of testicular tissue was and snap-frozen in liquid nitrogen. Frozen tissues were stored at −80 °C until used.

2.3. Immunohistochemistry

Immunofluorescence staining was performed as previously described (Hermann et al., 2007). Briefly, 5-μm sections of testicular tissue were fixed on slides and stored at 4 °C. Tissue slides were treated with xylene to remove paraffin and then rehydrated with a graded series of ethanol. For the antigen retrieval procedure, testicular tissues were treated with citrate buffer at 95 °C for 30 min and blocked with 5% donkey serum (Sigma, St. Louis, MO, USA) diluted in PBS. After a brief wash, the tissues were treated with KIT antibodies (rabbit anti-human, Dako), diluted by 1:200 in blocking serum (PBS containing 5% donkey serum), and incubated for 1.5 h. The KIT primary antibody was detected using donkey anti-goat immunoglobulin (IgG) Alexa Fluor 594 (1:1000, Life Technologies, Grand Island, NY, USA). The tissues were mounted in Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Immunostaining was visualized using a Leica DM 2500 microscope (Wetzlar, Germany), and the images were captured using a Leica DFC 450 C camera. For the double staining procedure, the GATA binding protein 4 (GATA4) antibody (goat anti-human, sigma) was co-incubated with the KIT antibody to identify Sertoli cells. DAZL positive germ cells were treated with KIT antibody to detect differentiated spermatogonia and primary spermatocytes. For counter staining, donkey anti-goat IgG Alexa Fluor 594 (Life Technologies, Grand island, NY, USA) was used at a dilution of 1:1000.