UTF1, a Putative Marker for Spermatogonial Stem Cells in Stallions

Heejun Jung1, Janet F. Roser3, Minjung Yoon1,2*

1 Department of Animal and Biotechnology Science, Kyungpook National University, Sangju, Korea, 2 Department of Horse, Companion and Wild Animal Science, Kyungpook National University, Sangju, Korea, 3 Department of Animal Science, University of California Davis, Davis, California, United States of America

Abstract

Spermatogonial stem cells (SSCs) continuously undergo self-renewal and differentiation to sustain spermatogenesis throughout adulthood in males. In stallions, SSCs may be used for the production of progeny from geldings after cryopreservation and therapy for infertile and subfertile stallions. Undifferentiated cell transcription factor 1 (UTF1) is a putative marker for undifferentiated spermatogonia in humans and rats. The main purposes of this study are to determine the following: 1) changes in the expression pattern of UTF1 at various reproductive stages of stallions, 2) subpopulations of spermatogonia that express UTF1. Testicular samples were collected and categorized based on the age of the horses as follows: pre-pubertal (<1 yr), pubertal (1–1.5 yr), post-pubertal (2–3 yr), and adult (4–8 yr). Western blot analysis was utilized to determine the cross-activity of the UTF1 antibody to horse testes tissues. Immunohistochemistry was conducted to investigate the UTF1 expression pattern in germ cells at different reproductive stages. Whole mount staining was applied to determine the subpopulation of UTF1-positive spermatogonia. Immunohistochemical analysis showed that most germ cells in the pre-pubertal and pubertal stages were immunolabeled with UTF1, whereas only a few germ cells in the basal compartment of the seminiferous tubule cross-sections of post-pubertal and adult tissues were UTF1-positive. No staining was observed in the Sertoli or Leydig cells at any reproductive stages. Whole mount staining showed that A0, Apor, and chains of 4, 8, 16 Apor spermatogonia were immunolabeled with UTF1 in the post-pubertal stallion tubule. Isolated single germ cells were also immunolabeled with UTF1. In conclusion, UTF1 is expressed in undifferentiated spermatogonia, and its antibody can be used as a putative marker for SSCs in stallions.

Citation: Jung H, Roser JF, Yoon M (2014) UTF1, a Putative Marker for Spermatogonial Stem Cells in Stallions. PLoS ONE 9(10): e108825. doi:10.1371/journal.pone.0108825

Editor: Jean-Pierre Rouault, Ecole Normale Superieure de Lyon, France

Received May 6, 2014; Accepted August 27, 2014; Published October 1, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: Funding provided by National Research Foundation of Korea (NRF) of the Ministry of Education, Science, and Technology (2012R1A1A039495). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: mjyoon@knu.ac.kr

Introduction

Spermatogonial stem cells (SSCs) undergo self-renewal and differentiation for continuous sperm production throughout adult life. It has become increasingly evident in mice that A single (A0), A paired (Apor), and A aligned (Aal) spermatogonia are undifferentiated spermatogonia [1,2], which later express the c-kit tyrosine receptor as a commitment step to undergo differentiation into spermatozoa [3,4]. In stallions, 8 subtypes of spermatogonia, including A0, Apor, Aal, A1, A2, A3, B1, and B2 spermatogonia were recently introduced [3]. The subpopulation of undifferentiated spermatogonia in stallions has not been established, although A0, Apor, and Aal spermatogonia are considered candidates for SSCs [3,4].

Due to the absence of a reliable marker for SSCs, information on SSCs had been limited until Brinster and coworkers developed a technique for SSC transplantation into the seminiferous tubules of infertile recipient mice in 1994 [3]. Since then, monitoring germ cell colony formation in the seminiferous tubules of recipient mice after transplantation has been used as the key marker for monitoring the presence and activity of SSCs. The application of this SSC transplantation technique to stallions has also been introduced [6]. Transplantation of stallion testicular germ cells in immuno-compromised infertile recipient mice resulted in spermatogonial colony formation; however, spermatogenesis was arrested at the spermatogonial stage due to the incompatibility of donor germ cells to the testicular environment of the recipient [6]. Highly conserved molecules expressed in SSCs are also powerful tools for studying undifferentiated spermatogonia. The putative SSC markers have been used in the localization of SSCs in fixed testicular tissues and SSC colonies of seminiferous tubules using immunohistochemical and whole mount staining techniques, respectively [2,7]. Three molecular markers, GFRα1, PLZF, and CSF1R, have been identified as markers for undifferentiated spermatogonia in stallion, donkeys, and mules [3]. However, expression pattern of these molecules at early reproductive stage, the precise size of spermatogonia colony expressing these molecules by whole mount staining, and possible use of these molecules for immunocytochemistry have remained unclear.

Undifferentied embryonic cell transcription factor 1 (UTF1) is a molecule expressed in embryonic stem cells [8]. UTF1 plays a key role in embryonic carcinoma and ES cell differentiation [8]. UTF1 is also expressed in spermatogonia throughout the human male gonadal development and adult testes [9]. In rats, UTF1 expression is evident in all gonocytes in embryonic and neonatal testes, although restricted to the small subpopulation of the
spermatogonia A during testicular development [10]. Previous studies have suggested that UTF1 is a conserved molecule of undifferentiated spermatogonia and might play critical roles in the self-renewal of SSCs in mammals [9,10]. The main purposes of this study are: 1) to determine the changes in the expression pattern of UTF1 at various reproductive stages in stallions, 2) to identify subpopulations of spermatogonia that express UTF1. Based on previous findings on other species, we hypothesize that UTF1 is expressed in a subpopulation of undifferentiated spermatogonia in post-pubertal and adult stallions, and UTF1 is a putative marker for stallion SSCs. The results of this study and the developed germ cell isolation procedure could be applied to unveil biological activity of SSCs, and it could also be possibly used in infertile and subfertile stallion therapy.

Materials and Methods

1. Animals

Testicular samples were collected through a routine field castration service at private horse farms in the Republic of Korea and through routine castration procedures performed at University of California Davis Veterinary Medical Teaching Hospital. The tests were collected from light-horse breeds including Thoroughbred, Quarter, and Jeju horses. The reproductive stages of the horses were categorized based on the age of horses as follows: pre-pubertal (<1 yr), pubertal (1–1.5 yr), post-pubertal (2–3 yr), and adult (≥3 yr) [11]. Testes were collected during a routine castration service, but not for this study. Because the colts or stallions were not valuable to be a stud, owners of these horses made a decision for the castration to get rid of stallion like behaviors and wanted to use them for a general riding purpose. The colts and stallions were brought in to the UC Davis Veterinary Medical Teaching Hospital by owner. The castration procedure was not modified for this research. Therefore, no approval by an Institutional Animal Care and Use Committee (IACUC) or equivalent animal ethics committee was required for this research. The same testicular samples were also used for several previous studies [11–14].

2. Testicular tissue sample preparation

Testicular tissue samples were prepared as previously described [11], with minor modifications. Briefly, testes were collected and transported to the laboratory in a 4°C icebox. For fixation, testicular parenchyma (1 cm³) was immersed in 4% paraformaldehyde for at least 24 h. Following a dehydration procedure using series of ethanol (Fisherbrand, Hampton, New Hampshire, USA), and tissues were dehydrated in a series of 100%, 90%, and 70% ethanol baths. After antigen retrieval in citrate buffer at 95°C for 30 min, the tissues were treated with an unmasking solution (Vector Laboratories, Burlingame, CA, USA). The slides were immersed in 0.3% hydrogen peroxide in methanol (Fisher Scientific, Pittsburgh, PA, USA) to quench any endogenous hydrogen peroxidase present in the tissues. After blocking with normal goat serum (in Vectastain Elite ABC kit, Vector Laboratories, USA) for 30 min, the tissues were treated with the UTF1 antibody (Millipore) at a dilution of 1:500 overnight. For negative controls, sections were incubated with normal rabbit serum (Sigma, St. Louis, MO, USA) using the same IgG concentration as that of the primary antibody. After secondary antibody incubation using goat anti-rabbit biotinylated antibody (Vectastain Elite ABC kit, Vector Laboratories, USA), tissue samples were then incubated with an avidin–biotin horseradish peroxidase complex (ABC reagent) for 45 min. A Vector AEC peroxidase substrate kit was used as substrate. Counterstaining was accomplished by briefly dipping the slides in hematoxylin (ImmunoMaster Hematoxylin; American Master-Tech Scientific, Inc., Lodi, CA, USA) and tissues were mounted onto glass slides with FaramountTM aqueous mounting medium (Dako, Glostrup, Denmark).

5. Immunofluorescence

Testicular tissues from pre-pubertal (n = 3), pubertal (n = 4), post-pubertal (n = 6), and adult (n = 6) stallions were used for immunofluorescence as previously described [15]. Briefly, 5-μm sections of testicular tissues were treated with xylene to remove the paraffin and then rehydrated in a graded series of ethanol washes. The tissues were incubated in citrate buffer at 95°C for 30 min for antigen retrieval and blocked with 5% donkey serum (Sigma, St. Louis, MO, USA) diluted in phosphate-buffered saline (PBS). The rabbit anti-human UTF1 antibody was diluted at a ratio of 1:500 in blocking buffer and allowed to cross-react with the slides for 1.5 h in a humid chamber. The primary antibody was detected using donkey anti-rabbit IgG Alexa Fluor 488 (1:1,000 dilution, Life Technologies, Grand Island, NY, USA). Tissues were mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI), Vector Laboratories, Burlin-
On Fisherbrand™ Superfrost/Plus microscope slides (Fisher, CA, USA), goat anti-human GATA4 antibody (1:200) and goat anti-human deleted in azoospermia-like (DAZL) antibody (1:200, Sigma, USA) were used as secondary antibody. Donkey anti-goat IgG Alexa Fluor 594 (1:1000, Life Technologies, Grand Island, NY, USA) was used as a secondary antibody for these counterstains. Tissues were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories).

For immunocytochemistry, single germ cells were isolated from testes in pubertal (n = 3) and post-pubertal stage (n = 3) stallions using a two-step enzyme protocol as previously reported [15], with slight modifications. Briefly, a chunk of tissue (10 g) was removed from each testis and sliced (1 cm³). For initial enzymatic digestion, the tissues were incubated with collagenase type IV (1 mg/mL; Sigma) dissolved in Hank’s balanced salt solution (HBSS; Invitrogen) for 10 min with vigorous shaking in a 37°C shaking incubator (Vision Scientific, Yuseong Gu, Daejeon, Korea). Dispersed seminiferous tubules were pelleted by centrifugation at 200 x g and the supernatant that contained Leydig cells was removed. The tubules were then digested with trypsin (2.0 mg/mL trypsin plus 1.04 mM EDTA; Invitrogen) and DNase I (1.4 mg/mL; Sigma) in HBSS for 15 min, and the digestion was quenched using fetal bovine serum (FBS, 10%). The testicular cell solution was filtered through a 70-μm Cell Strainer (Becton Dickinson and Company, Franklin Lakes, NJ, USA). After centrifugation at 600 x g for 10 min, the pellets were resuspended in minimum essential medium (MEM) supplemented with 10% FBS. Approximately 5 x 10⁵ germ cells in MEM supplemented with 10% FBS were loaded onto Fisherbrand™ Superfrost/Plus microscope slides (Fisher Scientific, Fisher Scientific Company, Ottawa, Canada) and subjected to ice-cold methanol as fixation. After overnight air-drying, cells were blocked with donkey serum and followed procedure listed above for immunofluorescence.

6. Immunofluorescent staining of whole-mount tubules

During the germ cell separation process, the dispersed seminiferous tubules were collected and fixed in 4% paraformaldehyde overnight at 4°C. After washing with PBS for 3 times at 60-min intervals, tubules were dehydrated in a series of 25%, 50%, 75%, 95%, and 100% methanol (McOH) for 10 min and permeabilized in 3 mL of MeOH:DMSO:H₂O₂ (4:1:1) for 3 h. The tubules were re-hydrated in 3 mL of 50% and 25% McOH in PBS for 10 min and washed in PBS (2 times, 15 min each). The tubules were blocked with 3 mL of ice-cold PBSMT blocking buffer (2% Bovine milk powder and 0.5% Triton X-100 in PBS) for 2 x 15 min and 1 x 2 h incubated with the UTF1 antibody (1:500, UTF1; Millipore) diluted in blocking buffer at 4°C overnight. After washing with PBSMT (2 x 15 min, 5 x 1 h), the tubules were reacted with donkey anti-rabbit IgG Alexa Fluor 488 (1:1,000, Life Technologies) diluted in blocking buffer at 4°C overnight. After washing with ice-cold PBSMT (2 x 15 min, 5 x 1 h) followed by PBS (2 x 10 min), the tubules were mounted on the Fisherbrand™ Superfrost/Plus microscope slides (Fisher Scientific, Fisher Scientific Company) with Vectashield mounting medium containing DAPI (Vector Laboratories).

7. Imaging

The immunostained tissues were examined using a Leica DM 2500 fluorescence microscope (Wetzlar, Germany) equipped with an EL 6000 external light source (Leica, Wetzlar, Germany), and images were captured using Leica DFC 450 C camera. Green and red fluorescent signals were observed using a dual-emission FITC/TRITC filter. The immunolabeling of single germ cells was observed using a confocal laser scanning microscope (Carl Zeiss, LSM 700). Images were captured using a LSM T-PMT camera (Carl Zeiss, LSM 700). Cell counting was performed manually by a well-trained observer. Cells stained with green or red fluorescent were considered positive cells, whereas cells not stained with any color of fluorescent were considered negative cells.

8. Statistical analysis

The UTF1-positive cell populations in the stallion seminiferous tubule cross-sections at post-pubertal and adult stage were statistically analyzed using a t-test analysis (Excel, Microsoft 2007). Approximately 100 microscopic fields per testicular tissue section were examined. Differences in the ratio of UTF1 positive spermatogonia at various reproductive stages were statistically analyzed by one way analysis of variance [ANOVA, PROC GLM: SAS version 9.0 (SAS Institute, Cary, NC)] followed by Tukey’s post-hoc comparisons. The ratio of UTF1 and/or DAZL positive spermatogonia at each reproductive stage was obtained by counting approximately 2000 spermatogonia in each testis (n = 3 in each reproductive stage). P-values < 0.05 were considered statistically significant.

Results

1. Cross-activity of UTF1 antibody in horse testes

To verify the cross-activity of the UTF1 antibody to horse testes, western blot was performed using the testicular tissue extract from post-pubertal and adult stallions. The protein band showed an approximate molecular weight between 26 and 36 kDa [8]; on the other hand, no band was present on the negative control lane, which was treated with rabbit serum using the same IgG concentration as that of the primary antibody (Fig. 1A). This result confirms that the UTF1 antibody used in the present study cross-reacted with the UTF1 molecules expressed in the spermatogonia of stallion testes.

2. Stage-dependent immunolocalization of UTF1 in stallion germ cells

Immunohistochemistry (colorimetric) was performed to investigate the morphological characteristics and localization of UTF1-positive germ cells within tubules. Various UTF1 positive spermatogonia population was found in the seminiferous tubule cross-sections (Figs. 1 B-I) in adult testes. The UTF1 staining pattern in testicular tissues was investigated at various reproductive stages (pre-pubertal, pubertal, post-pubertal, and adult). At the pre-pubertal stage, spermatogonia were located distal from the basal compartment of the seminiferous tubules, and most of them were immunostained with UTF1 (Figs. 2A and E). Within the spermatogonia, UTF1 expression was exclusively observed in the nuclei of the spermatogonia (Figs. 2A and E). At the pubertal stage, the lumen opening and incomplete spermatogenesis were observed in the seminiferous tubules (Figs. 2B and F). At this stage, immunostaining was observed in the round-shaped nuclei of the spermatogonia (Figs. 2B and F). Interestingly, some UTF1-positive spermatogonia were directly attached to the basement membrane, whereas others were not attached to the membrane (Figs. 2B and F). It appears that as stallions mature from pre-pubertal to pubertal, germ cells are translocated from the luminal compartment to the basal compartment of the tubules. At the post-pubertal and adult stages, the UTF1 protein was also observed in the spermatogonia, and the UTF1-positive cells resided basally within the seminiferous tubules (Figs. 2C, D, G, and H). The nuclear shapes of the UTF1-positive cells ranged from slightly oval to round. The shape of UTF1-positive germ cell translocating from the membrane and moving toward the lumen of the tubules was round, whereas the membrane resting on the basal lamina was

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flattened (Figs. 2C, D, G, and H). UTF1-positive germ cells were rarely observed in the round tubule cross-sections. The mean of UTF1-positive germ cell population on the round cross-section of 100 seminiferous tubules was 1.28 ± 0.41 (n = 3) and 1.59 ± 0.29 (n = 3) at post-pubertal and adult stages, respectively. The UTF1-positive cell population per tubule cross-section was not significantly different between post-pubertal and adult horses (p > 0.05).

UTF1 immunolabeling was not observed in the interstitial space where Leydig cells are localized at various reproductive stages (Figs. 2A–H). At the pre-pubertal and pubertal stages, an intense fluorescent signal was observed in the interstitial space of tissues reacted with the UTF1 antibody (Figs. 2A, B, and F), as well as in the tissues treated with rabbit serum (Figs. 2I and J, negative control). This non-specific staining appears to be due to lipofuscin, a pigment granule composed of lipid-containing residues of lysosomal digestion [16]. This non-specific staining was previously reported in stallion testis of the same reproductive stages [11].

3. Clonal organization of UTF1-positive spermatogonia in the seminiferous tubules

To determine the size of the spermatogonia subpopulations expressing UTF1 in stallions, immunohistochemical analysis was performed on whole mount preparations of seminiferous tubules of pubertal and post-pubertal stallions. In the pubertal tubules, it was demanding to identify the size of UTF1 positive germ cell colony because of massive population of UTF1 germ cells within the tubules. However, most UTF1 positive germ cells in this stage appeared to be A, spermatogonia (Figs. 3A and B). In the post-pubertal stallion tubules, whereas, A, A pr, chains of 4, 8, and 16 A al spermatogonia showed positive signals for UTF1 (Figs. 3C–F). No longer than chains of 16 A al spermatogonia was observed in this study.

4. Absence of UTF1 expression in Sertoli cells of stallion testis

Immunofluorescence was performed to determine the co-immunolabeling pattern of UTF1 and GATA4 (Fig. 4). GATA4 is widely used as a molecular marker for Sertoli cells [2]. In pre-pubertal tissues, GATA4-positive cells resided next to the basement membrane of the seminiferous tubules (Figs. 4A and E). UTF1-positive spermatogonia were not co-immunolabeled with GATA4 (Figs. 4A and E). At the pubertal stage, UTF1-positive cells were located between Sertoli cells in the compartment of basement membrane (Figs. 4B and F). At the post-pubertal and adult stages, a complete generation of spermatogenesis and lumen opening were evident (Figs. 4C and D). At these stages, UTF1 immunolabeling was observed in the nuclei of germ cells adjacent to the basement membrane of the seminiferous tubules, and these cells were juxtaposed to Sertoli cells (Figs. 4C, D, G, and H). No UTF1 immunolabeling was observed in Sertoli cells at any reproductive stage. The rate of UTF1 stained spermatogonia out of randomly selected 2000 spermatogonia
was significantly lower at post-pubertal (2.2 ± 0.25%, n = 3) and adult (1.77 ± 0.32%, n = 3) compared to the ratio at pre-pubertal (64.87 ± 7.36%, n = 3) and pubertal (42.23 ± 10.77%, n = 3) (p < 0.05, Fig. 4 M).

5. Stage-dependent co-localization of UTF1 and DAZL in stallion germ cells

DAZL is a well-known germ cell marker of various species [17–20]. In our laboratory, we reported that most spermatogonia and primary spermatocytes were immunolabeled with DAZL protein at the post-pubertal and adult stages, although some spermatogonia did not show DAZL immunolabeling. To further investigate the subpopulations of DAZL-positive germ cells, co-immunolabeling of UTF1 with DAZL was conducted using testicular tissues of different reproductive stages (A–H). At the pre-pubertal and pubertal stages, most UTF1-positive spermatogonia were co-stained with DAZL (Figs. 5A, B, E, and F) and post-pubertal (42.23 ± 10.77%, n = 3) (p < 0.05, Fig. 4 M).

6. Possible use of UTF1 antibody for immunocytochemistry

We performed immunocytochemistry with UTF1 on germ cells to investigate possible use of the UTF1 antibody as a marker for undifferentiated spermatogonial stem cells in vitro studies. To determine the optimal germ cell fixative for UTF1 immunocytochemistry, four different cell fixatives, including 1) ice-cold 100% MeOH, 2) 100% acetone, 3) 50% MeOH and 50% EtOH, and 4) 50% acetone and 50% EtOH were used to fix single germ cells onto slides. UTF1 immunolabeling of germ cell nuclei was successfully observed in germ cells fixed with all fixatives tested for this study. Co-immunostaining with both UTF1 and DAZL showed that the UTF1 protein was localized in nuclei of spermatogonia at pubertal and post-pubertal stallions, but DAZL staining was detected in the cytoplasm of undifferentiated spermatogonia at pubertal only (Fig. 6A–F).
Discussion

Finding a putative marker for undifferentiated spermatogonia will contribute to the development of a new assistant reproductive technique using SSCs, as well as to better understanding the biological activity of spermatogonia for stallion sperm production. The present study was conducted to identify the subpopulations of spermatogonia that expressed UTF1 and to determine the changes in the pattern of UTF1 expression at different reproductive stages.

Our immunohistological evidence demonstrated that UTF1-positive germ cells were present at every reproductive stage, and the localization of UTF1 was limited to the nuclei of germ cells. The pattern and localization of UTF1 in stallion testes was similar to that observed in humans [9] and rats [10], indicating that the pattern and localization of UTF1 expression are highly conserved in mammals.

Several histological characteristics such as the location of germ cells in the seminiferous tubules and the shape of UTF1-positive cell membrane and nuclei were evaluated to identify UTF1-expressing subpopulations of spermatogonia. In post-pubertal and adult testes, UTF1-positive germ cells reside basally within the tubule. UTF1-positive cells located basally were flattened, whereas the other side of surface facing the other cells ranged from oval to round. These UTF1 immunofluorescence findings indicate that these cells are spermatogonia [21]. A recent study involving equine spermatogonia stem cells demonstrated that the population of As cells at each stage was approximately 200 cells per 1000 Sertoli cell nuclei [3]. In contrast, the population of A1, A2, A3, B1, or B2 spermatogonia was >200 cells per 1,000 Sertoli cell nuclei at each stage [3]. In this study, the number of UTF1-positive germ cell populations per 1,000 Sertoli
cells in post-pubertal and adult stallions was less (78.42±17.83; n=8) than Aund population previously reported in stallions [3].

The number of UTF1-positive germ cells per seminiferous tubule cross-section was 1.48±0.24 (n=8), suggesting that UTF1 is expressed in early subpopulations of undifferentiated spermatogonia. Also immunofluorescent analysis of whole mounted tubules

Figure 4. Stage-dependent co-localization of UTF1 and GATA4 in the seminiferous tubule cross-section of the stallion testes.

Immunolabeling of UTF1 (green fluorescence) and GATA4 (red fluorescence) were observed in pre-pubertal (A and E), pubertal (B and F), post-pubertal (C and G), and adult (D and H) stallion testes. At the pre-pubertal stage, GATA4-positive cells were located close to the basement membrane of the seminiferous tubules. UTF1 was not co-immunolabeled with GATA4 (A and E). At the pubertal stage, UTF1 was localized between the Sertoli cells in the compartment of the basement membrane (B and F). At the post-pubertal and adult stages, completion of spermatogenesis and lumen opening were evident (C and D). UTF1-positive germ cells were observed in the basal compartment of seminiferous tubules. UTF1 immunolabeling was not observed in Leydig, Sertoli, or myoid cells at any reproductive stages. No immunolabeling was observed in the negative control tissues (I, J, K, and L). The area indicated by the broken line-white box (e.g., A, B, C, and D) is enlarged (e.g., E, F, G, and H). The white arrow head indicates spermatogonia immunolabeled with UTF1; the red arrow head indicates Sertoli cells immunolabeled with GATA4. Bar = 200 μm. The graph shows % of UTF1 stained spermatogonia out of randomly selected 2000 spermatogonia with error bars at different reproductive stages (M). Percentage with different superscripts indicate significant different (p<0.05). doi:10.1371/journal.pone.0108825.g004
Figure 5. Stage-dependent co-localization of UTF1 and DAZL in the seminiferous tubule cross-section of stallion testes. Immunolabeling of UTF1 (green fluorescence) and DAZL (red fluorescence) were observed in pre-pubertal (A and E), pubertal (B and F), post-pubertal (C and G), and adult (D and H) stallion testes. At the pre-pubertal stage, most UTF1-positive spermatogonia were co-immunolabeled with DAZL (A and E, red arrow head), whereas germ cells stained with UTF1 only was also observed (A and E, white arrow head). At the pubertal stage, germ cells showed two types of expression patterns, which included germ cells expressing both UTF1 and DAZL (B and F, red arrow) and germ cells expressing DAZL only (yellow arrow). At the post-pubertal and adult stages, UTF1-positive germ cells were not co-immunolabeled with DAZL (C, D, G, H, white arrow). Insets show negative controls treated with normal rabbit serum in place of the primary antibody. No immunolabeling was noticed in the negative control (I, J, K, and L). The area indicated by a broken line-white box (e.g., A, B, C, D) is enlarged in the lower panel (e.g., E, F, G, H). The white arrow head indicates spermatogonia immunolabeled with UTF1, but not co-immunolabeled with DAZL. The yellow arrow head indicates the cytoplasm of single germ cells with DAZL. Bar = 200 μm. The graph shows % of UTF1 and/or DAZL stained spermatogonia out of randomly selected 2000 spermatogonia with error bars at different reproductive stages (n = 3, M).
doi:10.1371/journal.pone.0108825.g005
showed that UTF1-positive germ cells consisted of Λs, Λpr, and 4, 8, 16 chains of Λal spermatogonia. This result led us to conclude that UTF1 can be used as a marker for undifferentiated spermatogonia such as Λs, Λpr, and 4, 8, 16 chains of Λal spermatogonia.

The present study demonstrated that the ratio of UTF1-positive spermatogonia decreases during the transition from pubertal to post-pubertal. This finding was also reported in rats, in which the population of UTF1-positive cells in a cross-section decreased with age [10]. A possible explanation for this change is that UTF1-positive germ cells accumulate in the stunted seminiferous tubule at an early reproductive stage are distributed to the extended seminiferous tubule as the diameter, volume, and length of the single seminiferous tubule increase with active spermatogenesis after puberty [22].

DAZL is expressed in germ cells of mice [18,20], rats [19], pigs [23], rhesus monkeys [15], bulls [24], and humans [17,18], and its expression pattern is species- and developmental stage-dependent. Our research group has previously reported on the stage-dependent DAZL expression in stallion germ cells [12]. In the present study, germ cells co-stained with both UTF1 and DAZL were only observed at the pre-pubertal and pubertal stages, but not at the post-pubertal and adult stages, indicating that co-localization of UTF1 and DAZL in the germ cells is stage-dependent [12]. DAZL is a widely known marker for differentiated spermatogonia [17,19,20]. Schrans-Stassen and coworkers also demonstrated that the differentiation of Λal spermatogonia into Λ1 spermatogonia in mice is blocked by the absence of the RNA-binding protein encoded by the DAZL gene [12,25]. These previous evidences support that UTF1-positive and DAZL negative spermatogonia found in all reproductive stages is undifferentiated spermatogonia. In embryonic stem cells (ESCs), UTF1 expression is rapidly downregulated during ESC differentiation, indicating that UTF1 is a marker for undifferentiated ESCs and possibly plays an important role in maintain this status. Kooistra and coworkers suggested that the function of UTF1 is to maintain undifferentiated status by averting decondensation of chromatin and irregular gene expression of ESCs, which initiate the differentiation process [26]. These findings thus suggest that the UTF1-positive and DAZL-negative germ cells in post-pubertal and adult stallions are possibly undifferentiated SSCs and have the capacity to undergo self-renewal. This fact corresponds with the conclusion of an earlier study involving rats [10]. UTF1 induces rapid cell proliferation and promotes pluripotency of ESCs via coupling the core pluripotency factors with Myc and the PRC2 network [27,28]. Based on the function of UTF1 in embryonic stem cells, we speculate that UTF1 might be involved in promoting self-renewal and maintaining the characteristics of undifferentiated spermatogonial stem cells. However, interestingly some UTF1 positive spermatogonia, but not all, were stained with DAZL in pre-pubertal and pubertal stages. We previously speculated that DAZL positive germ cells are differentiated spermatogonia in pre-pubertal and pubertal stage [12]. However, it is not clear whether these DAZL positive spermatogonia in early reproductive stages are differentiated or undifferentiated because some of DAZL positive spermatogonia are also stained with UTF1. Further study such as co-staining with another undifferentiated spermatogonia marker and DAZL in spermatogonia of pre-pubertal and pubertal stages is warranted to identify the status of the double stained spermatogonia at pre-pubertal and pubertal stage. The reproductive stage-dependent UTF1 and DAZL co-staining pattern also suggests that the status of UTF1 or DAZL positive spermatogonia may change with the reproductive stage of stallions.

The western blot result showed a protein band at 36 and 26 kDa. Despite of the presence of unexpected band at 26 kDa, UTF1 staining pattern in the spermatogonia using the antibody (ab3383) in this present study and another study in rats [10] is
similar with the UTF1 staining pattern in the spermatogonia of other species using different UTF1 antibody [9]. Thus, it appears that the band at 26 kDa is non-specific band.

In summary, the present study has demonstrated that UTF1 is a putative marker for early subpopulations of undifferentiated spermatogonial stem cells in stallion testes, and this marker can be applied to monitoring SSCs for the study of undifferentiated spermatogonial stem cells in vitro.

Acknowledgments

Authors would like to thank Dr. Orwig Kyle (University of Pittsburgh) for providing antibodies and comments on the manuscript, and Dr. Deokjin providing antibodies and comments on the manuscript, and Dr. Deokjin providing antibodies and comments on the manuscript.

Author Contributions

Conceived and designed the experiments: MJY. Performed the experiments: MJY HJJ. Analyzed the data: MJY. Contributed reagents/materials/analysis tools: MJY JFR. Contributed to the writing of the manuscript: MJY HJJ JFR.

References

1. de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. Journal of andrology 21: 776–798.

2. Gassei K, Orwig KE (2013) SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. PloS one 8: e53976.

3. Costa GM, Avellar GF, Rezende-Neto JV, Campoe-Junior PH, Lacerda SM, et al. (2012) Spermatogonial stem cell markers and niche in equids. PloS one 7: e40901.

4. Johnson L (1991) Seasonal differences in equine spermatogenesis. Biology of reproduction 44: 204–291.

5. Brinster RL, Avarbock MR (1994) Germine transmission of donor haplotype following spermatogonial transplantation. Proceedings of the National Academy of Sciences of the United States of America 91: 11303–11307.

6. Dobrinski I, Avarbock MR, Brinster RL (2000) Germ cell transplantation from large domestic animals into mouse testes. Molecular reproduction and development 57: 270–279.

7. Hermann BP, Sukhwani M, Simorangkir DR, Chu T, Plant TM, et al. (2009) Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in chimaeric macaques. Hum Reprod 24: 1704–1716.

8. van den Boom V, Kooststra SM, Boesjes M, Geverts B, Houstmanler AB, et al. (2007) UTF1 is a chromatin-associated protein involved in ES cell differentiation. The Journal of cell biology 178: 913–924.

9. Kristensen DM, Nielsen JE, Skakkebaek NE, Graem N, Jacobsen GK, et al. (2008) Presumed pluripotency markers UTF1 and REX-1 are expressed in human adult testes and germ cell neoplasms. Hum Reprod 23: 773–782.

10. van Bragt MP, Roepers-Gajadien HL, Korver CM, Bonger J, Okula A, et al. (2008) Expression of the pluripotency marker UTF1 is restricted to a subpopulation of early A spermatogonia in rat testes. Reproduction 136: 33–40.

11. Yoon MJ, Berger T, Roser JF (2011) Localization of insulin-like growth factor-I (IGF-I) and IGF-I receptor (IGF-IR) in equine testes. Reproduction in domestic animals = Zuchthygiene 46: 221–228.

12. Jung HJ, Song H, Yoon MJ (2014) Stage-dependent DAZL localization in spermatogonia in rat testes. Reproduction 136: 33–40.

13. Yoon MJ, Berger T, Roser JF (2011) Stage-dependent DAZL localization in spermatogonia in rat testes. Reproduction 136: 33–40.

14. Yoon MJ, Berger T, Roser JF (2011) Stage-dependent DAZL localization in spermatogonia in rat testes. Reproduction 136: 33–40.

15. Hermann BP, Sukhwani M, Lin CC, Sheng Y, Tomko J, et al. (2007) Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult thressus macaques. Stem Cells 25: 2330–2338.

16. Dr. Deo JF, Watiaux R (1966) Functions of lysosomes. Annual review of physiology 28: 435–492.

17. Lin YM, Chen CW, Sun HS, Tai SJ, Hsu CC, et al. (2001) Expression patterns and transcript concentrations of the autosomal DAZL gene in testes of azoospermic men. Molecular human reproduction 7: 1013–1022.

18. Rigo RA, Doelfinan DM, Sleer R, Renshaw AA, Loughlin KR, et al. (2000) DAZ family proteins exist throughout male germ cell development and transit from nucleus to cytoplasm at meiosis in humans and mice. Biology of reproduction 63: 1490–1496.

19. Rocchiarti-March M, Weinbauer GF, Page DC, Nieszlag E, Gromoll J (2000) Dazl protein expression in adult rat testis is up-regulated at meiosis and not hormonally regulated. International journal of andrology 23: 51–56.

20. Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, et al. (1997) The mouse Dazl gene encodes a cytoplasmic protein essential for gametogenesis. Nature 389: 73–77.

21. Russell L, Estlin R, Hikin AS, Clegg E (1990) Histological and Histopathological Evaluation of the Testis. Clearwater, FL: Cache River Press.

22. Johnson L, Neaves WB (1981) Age-related changes in the Leydig cell population, seminiferous tubules, and sperm production in stallions. Biology of reproduction 24: 703–712.

23. Luo J, Rodriguez-Sosa JR, Tang L, Bondareva A, Megre S, et al. (2010) Expression pattern of acetylated alpha-tubulin in porcine spermatogonia. Molecular reproduction and development 77: 348–352.

24. Zhang Q, Li Q, Li J, Li X, Lau Z, et al. (2008) b-DAZL: A novel gene in bovine spermatogonia. Biology of reproduction 65: 771–776.

25. Schrans-Stassen BH, Saunders PT, Cooke HJ, de Rooij DG (2001) Nature of the spermatogonial arrest in Dazl−/− mice. Biology of reproduction 65: 771–776.

26. Kooststra SM, van den Boom V, Thummer RP, Johannes F, Wardenau R, et al. (2012) DAZL: A novel germline-specific gene in testes of human adult rhesus macaques. Stem Cells 25: 2330–2338.

27. Nishimoto M, Miyagi S, Yamagishi T, Sakauchi T, Nica H, et al. (2005) Oct-3/4 maintains the proliferative embryonic stem cell state via specific binding to a variant octamer sequence in the regulatory region of the UTF1 locus. Molecular and cellular biology 25: 5084–5094.

28. Jia J, Zheng X, Hu G, Cui K, Zhang J, et al. (2012) Regulation of pluripotency and self-renewal of ESCs through epigenetic-threshold modulation and mRNA pruning. Cell 151: 576–589.