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Conservation of spermatogonial stem cell marker expression in undifferentiated felid
spermatogonia

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Abstract

Spermatogonial stem cells (SSCs) are distinct in their ability to self-renew, transmit genetic information, and persist throughout the life of an individual. These characteristics make SSCs a useful tool for addressing diverse challenges such as efficient transgenic production in non-rodent, biomedical animal models or preservation of the male genome for species in which survival of frozen-thawed sperm is low. A requisite first step to accessing this technology in felids is the establishment of molecular markers. This study was designed to evaluate, in the domestic cat (*Felis catus*), expression both *in situ* and following enrichment *in vitro* of six genes (*GFRA1*, *GPR125*, *ZBTB16*, *POU5F1*, *THY1*, *UCHL1*) that had been previously identified as SSC markers in other species. Antibodies for surface markers GFRA1, GPR125, and THY1 could not be validated, while western blot analysis of prepubertal, peripubertal, and adult cat testis confirmed protein expression for the intracellular markers UCHL1, ZBTB16, and POU5F1. Co-localization of the markers by immunohistochemistry revealed that several cells within the subpopulation adjacent to the basement membrane of the seminiferous tubules and identified morphologically as spermatogonia, expressed all three intracellular markers. Studies performed on cheetah (*Acinonyx jubatus*) and Amur leopard (*Panthera pardus orientalis*) testis exhibited a conserved expression pattern in protein molecular weights, relative abundance, and localization of positive cells within the testis. The expression of the three intracellular SSC marker proteins in domestic and wild cat testes confirms conservation of these markers in felids. Enrichment of marker transcripts following differential plating was also observed. These markers will facilitate further studies in cell enrichment and *in vitro* culture of felid SSCs enabling both production of transgenic domestic cats and preservation of the male genome from rare and endangered felids.

Keywords

Spermatogonial stem cell, domestic cat (*Felis catus*), cheetah (*Acinonyx jubatus*), Amur leopard (*Panthera pardus orientalis*), testis

1. Introduction

Spermatogenesis is one of the most proliferative mechanisms in adult animals, as cells are continually being produced and lost. The constant rate of sperm production is heavily reliant on regulation of the adult stem cell population of the testis, the spermatogonial stem cell (SSC). SSCs are unique among adult stem cells in that they possess the ability to transmit genetic information to the next generation. This makes them an attractive target for genetic manipulation, especially in species such as the cat in which we lack germline competent embryonic stem cells (ESCs). In contrast to ESCs that often develop genetic and epigenetic changes over time, a study involving two years of continuous culture demonstrated that mouse SSCs could maintain euploid karyotypes, normal methylation patterns, and germline competence during long-term culture [1].

Additionally, the ability of this stem cell population to self-renew makes it a promising alternative for genetic preservation of rare and endangered animals [2]. Cryopreservation of spermatozoa has served as the primary means for preservation of the male genome (reviewed by [3]). However, spermatozoa are fully mature cells which cannot undergo further replication. Furthermore, sperm collection is restricted to reproductively mature individuals, preventing the recovery of valuable genotypes from immature animals. This is especially pertinent in felids because many species exhibit a high rate of juvenile mortality [4]. SSCs appear early during the postnatal period [5]. Therefore, in contrast to spermatozoa, SSCs can be collected not only from adults, but also from reproductively immature animals. Moreover, SSCs can be cryopreserved

using simple cryopreservation techniques as first demonstrated in the mouse [6], and subsequently used in a diverse group of species. This is in sharp contrast to mature spermatozoa, in which cryopreservation protocols must be developed on a species by species basis and, despite decades of research, yield low post-thaw survival (reviewed by [7]). Such is the case with felids; the 38 members of this Family exhibit striking variability in the types and severity of sperm cryosensitivities. Many of these species are notorious for being difficult to cryopreserve; a problem no doubt compounded by the high prevalence of teratospermia seen among felids [8-11]. Thus, cryopreservation of SSCs could help alleviate these species-specific issues.

SSCs are an extremely rare population, estimated to represent only 0.03 percent of the total germ cell population [12]. Due to their rarity, a requisite first step to accessing this technology is the establishment of molecular markers. Identification of SSCs in cats presents a challenge because markers used to identify SSCs in mice—the most studied species—may not be valid for felids. As an additional hurdle, none of the candidate SSC markers are expressed exclusively by the stem cells. Many are expressed by other undifferentiated spermatogonia that have already lost their stem cell activity and some markers are expressed in somatic cells. Nevertheless, co-expression of multiple markers in undifferentiated cells occupying the correct morphological niche is highly suggestive of stemness, as demonstrated in the bull [13].

While limited information regarding cat SSC markers has been presented at scientific meetings [14-16], few full reports exist. In regard to protein expression *in situ*, glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1) expression by a subset of undifferentiated spermatogonia in the domestic cat testis was demonstrated by immunohistochemical studies [17,18], consistent with reports in other species for which GFRA1 is used as an SSC marker[19]. Also Paired Box 7 (PAX7), which was demonstrated to be

88 expressed in a rare subpopulation of single spermatogonia in the mouse, was shown by
89 immunolabeling to be expressed in the testes of other mammals, including prepubertal cats [20].
90 The presence of a partial ubiquitin carboxy-terminal hydrolase 1 (*UCHL1*) transcript in testes of
91 several wild cat has also been demonstrated, but no other candidate markers or comprehensive
92 studies have been reported in the cat [21]. Recently, we demonstrated the expression of several
93 SSC markers (*GFRA1*, G protein-coupled receptor 125(*GPR125*), Zinc finger and BTB domain-
94 containing protein 16 (*ZBTB16*), POU domain, class 5, transcription factor 1(*POU5F1*), thymus
95 cell antigen 1 (*THY1*), and *UCHL1*) in cat testis using RT-PCR [22], suggesting that SSC
96 markers previously reported in other species [13,19,23-25] are conserved in the cat [22].
97 However, these experiments were limited to the level of mRNA in testicular samples and thus, it
98 remained unclear whether these transcripts were translated into proteins expressed appropriately
99 in the spermatogonial cells *in situ* or whether following isolation and enrichment, the transcript
100 levels would be increased as expected for markers of undifferentiated spermatogonial cells.
101 Therefore, the objectives of this study were to characterize the protein expression pattern of the
102 candidate SSC markers in felids with regard to 1) levels of expression in prepubertal,
103 peripubertal, and adult individuals; 2) location within the seminiferous tubules in domestic cats;
104 and 3) conservation of markers in two of their wild relatives, the cheetah (*Acinonyx jubatus*) and
105 Amur leopard (*Panthera pardus orientalis*). An additional objective was to determine whether
106 SSC enrichment protocols resulted in higher levels of SSC marker transcripts as would be
107 anticipated, particularly for prepubertal animals which would only have somatic cells and
108 undifferentiated spermatogonial cells with no spermatogonia undergoing differentiation to
109 confound results. Validation of these markers will facilitate further studies in cell enrichment and

in vitro culture of felid SSCs enabling both generation of transgenic domestic cats and preservation of the male genome from rare and endangered felids.

2. Materials and methods

2.1 Collection of testes

Approval from an Institutional Animal Care and Use Committee (IACUC) was not required as domestic cat testes were obtained from local veterinarians following routine neuters and testis tissue from non-domestic felids was obtained post-mortem from animals euthanized for health reasons. These are exempt studies under Title 9, Code of Federal Regulations, Subchapter A-Animal Welfare, Parts 1-3.

Domestic cat (*Felis catus*) testicles, obtained from local spay-neuter clinics, were stored at 4°C and were processed within 24 h of collection, and generally within the first 6 h. A portion of each testis was fixed in 4% formaldehyde and embedded in paraffin, as described in Section 2.3. Donor age was recorded if known or estimated by the attending veterinarian. Age group was then confirmed by histological analysis of hematoxylin and eosin-stained testis samples for progression through spermatogenesis.

Testis tissue samples from adult cheetah (*Acinonyx jubatus*, n = 2, 8 and 10 y) and Amur leopard (*Panthera pardus orientalis*, n = 1, 18 y) were collected post-mortem following euthanasia for health-related problems. Testis tissue was processed within 6 h of euthanasia for one cheetah and within 24 h for the other two donors. Samples were donated by the Smithsonian's National Zoological Park (cheetahs) and Bronx Zoo (Amur leopard).

2.2 Immunoblotting

Testes from prepubertal (n = 3), peripubertal (n = 3), and adult (n = 3) domestic cats were used for western blot analysis. While adult male cats are able to produce sperm throughout the year, the literature is currently divided on whether season can have an effect on the quality and rate of spermatogenesis [26-29]. Therefore, to reduce variability due to season, only samples collected between June and August 2013 were used. The tunica albuginea was removed and small pieces of testis were flash frozen in liquid nitrogen and stored at -80°C. Total protein was extracted using NP40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) NP40) and protein concentrations were determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Forty micrograms of protein per sample were heated to 98°C in a dry bath for 10 min in 6X SDS Sample Buffer (375 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 60% (v/v) glycerol, 0.06% (w/v) bromophenol blue; 5% (v/v) 2-mercaptoethanol). Total protein lysates were subjected to SDS-PAGE and western blot protocols according to Bio-Rad Mini-PROTEAN 3 Cell instructions (Bio-Rad Laboratories). Blocking solution, which contained 5% (w/v) nonfat dry milk in Tris-buffered saline (20 mM Tris-HCL pH 7.6, 137 mM NaCl) and 0.05% (v/v) Tween-20 (TBST) for all probes except ZBTB16 for which TBST containing 3% (w/v) bovine serum albumin (Sigma, St. Louis, MO, USA) was used, was applied to the membranes for 2 h. Primary antibodies from at least three different sources for each gene product were assessed by immunoblot and immunostaining for the gene products of SSC markers (GFRA1, GPR125, THY1, POU5F1, ZBTB16, and UCHL1). See Table 1 for a complete list of the validated antibodies and concentrations subsequently used in these studies for POU5F1, ZBTB16, and UCHL1. See supplementary information for the antibodies tested for GFRA1, GPR125, and THY1 but were

not used in these studies as they could not be validated (Table S1). Membranes were washed three times for 5 min in TBST and then incubated with a species- and isotype-specific horseradish peroxidase-conjugated secondary antibody (Table 1) in blocking solution for 1 h at room temperature. Membranes were washed again in TBST, and antigen detection was performed using the Supersignal West Pico chemiluminescent substrates (Thermo Scientific, Wilmington, DE, USA) and visualized using a ChemiDoc XRS system (Bio-Rad Laboratories). Immunoblots were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) according to manufacturer's instructions and reprobed for beta-actin as described above, except that the secondary antibody was only incubated for 45 min at room temperature. Immunoblot images were processed for figures using Photoshop CS6 (Adobe Systems, San Jose, CA, USA). Intensities of immunoblot bands were quantified from unmodified immunoblot images using Image Lab software (Bio-Rad Laboratories). Relative expression levels were calculated by normalizing band intensities to their corresponding beta-actin band intensity. Comparisons were performed between each of the juvenile groups and the adult group using Dunnett's test from the MIXED procedure of SAS statistical software (SAS Institute, Cary, NC, USA). Data for bar graphs were transformed by setting the prepubertal group to one. However, statistics were performed on untransformed data. As a negative control, additional immunoblots were performed for each antibody which included a predicted negative tissue or cell line (Supplementary Fig. 1).

2.3 Fluorescent immunohistochemistry

Testes were fixed in 4% (v/v) methanol-free formaldehyde (Polysciences Inc., Warrington, PA, USA) for 1 h at room temperature or overnight at 4°C, dehydrated through a series of graded alcohols, and embedded in paraffin wax (Fisher Scientific, Fair Lawn, NJ, USA) at 60°C. Five

micron thick sections were dewaxed using xylene and rehydrated through a series of graded alcohols. Samples were permeabilized with 0.2% (v/v) Triton-X 100 and 0.1% (v/v) Tween-20 diluted in 1X Dulbecco phosphate-buffered saline (DPBS, Cellgro, Corning, NY, USA) for 9 min at room temperature. Sections were blocked with MAXblock Blocking Medium (Active Motif, Carlsbad, CA, USA) for 2 h at room temperature. Primary antibodies were diluted in MAXbind Staining Medium (Active Motif) and incubated overnight at 4°C. See Table 1 for a complete list of the immunoblot-validated antibodies and concentrations used in these studies. Slides were washed three times for 5 min at room temperature in immunohistochemistry (IHC) washing buffer (DPBS and 0.05% (v/v) Tween-20) and then incubated with a species- and isotype-specific fluorescent-conjugated secondary antibody (Table 1) in MAXbind Staining Medium solution for 2 h at room temperature. Hoescht 33342 (1 µg/ml; Sigma) was used to visualize cell nuclei. Images were acquired using a Carl Zeiss AxioObserver Z1 inverted microscope with a black and white Zeiss AxioCam MRm camera and AxioVision, release 4.6 software (Carl Zeiss Microimaging, Thornwood, NY, USA). Images were pseudocolored in AxioVision and exported to Photoshop CS6 for image processing. In negative controls, sections were incubated with non-immune serum and the primary antibody was omitted (Supplementary Figures 2 and 3).

2.4 Fluorescent immunocytochemistry

Testes were enzymatically digested and plated onto Lab-Tek two-well glass chamber slides (Nalge Nunc International Corporation, Rochester, NY, USA) pre-coated with 0.1% (v/v) poly-L-lysine (Sigma). Cells were fixed with 4% (v/v) methanol-free formaldehyde for 15 min at room temperature, gently washed three times with IHC wash buffer, and permeabilized with 0.2% (v/v) Triton-X 100 and 0.1% (v/v) Tween-20 diluted in DPBS for 9 min at room

temperature. Blocking, antibody incubation, mounting, and imaging were performed as described in the fluorescent IHC section. See Table 1 for a complete list of antibodies and concentrations used. Negative controls performed by incubating sections with non-immune serum and omitting the primary antibody showed minimal immunoreactivity.

2.5 RT-qPCR Analysis

Differential plating was performed on domestic cat testis tissue as previously described [22]. Cells immediately following dissociation (no enrichment) and after differential plating were stored in RLT lysis buffer (Qiagen, Valencia, CA, USA) for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression of five SSC genes (E-cadherin (*CDH1*), *POU5F1*, *ZBTB16*, *THY1*, and *UCHL1*), one Sertoli cell gene (vimentin (*VIM*)), and three reference genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L17 (*RPL17*), and tryptophan 5'-monooxygenase activation protein zeta isoform (*YWHAZ*)) were measured with RT-qPCR. Total RNA was extracted using RNeasy Midi kits (Qiagen), according to manufacturer's instructions. RNA concentration was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One microgram of total RNA from each sample was used in the reverse transcription reactions. For each sample, a second set of reverse transcription reactions were performed without reverse transcriptase enzyme, as a control for genomic DNA contamination. First strand cDNAs were synthesized according to manufacturer's instructions using the QuantiTect® Reverse Transcription kit (Qiagen). The cDNA was diluted 1:20 for use in RT-qPCR reactions.

Primers (Integrated DNA Technologies, Coralville, IA, USA) used in the RT-qPCR reactions were designed using the Primer3 software (<http://bioinfo.ut.ee/primer3/>) to have a melting temperature (T_m) of 58 to 60°C, GC content of 40 to 60%, length of 18 to 22 nucleotides,

and amplicon length of 100 to 180 base pairs. Designs were based on transcript sequences in the National Center for Biotechnology Information cat genome database (<http://www.ncbi.nlm.nih.gov/genome?term=felis%20catus>). Primers were designed to span an exon junction and/or primers pairs were separated by at least one intron whenever possible. Endpoint RT-PCR and gel electrophoresis were conducted to ensure that each primer pair produced a single amplicon of predicted size. For each set of primers, PCR products were sequenced to confirm identity. Primer pair efficiencies were determined by performing a five-fold serial dilution of cDNA (0.016 to 50 ng cDNA) pooled from prepubertal and adult testes, before and after differential plating. A linear standard curve was generated by plotting quantification cycle (Cq) against the log of template concentration. Primer pair efficiency was calculated with the equation $E = 10^{-1/\text{slope}}$ as described by Pfaffl [30]. Reference gene stability was determined using the geNorm software in qBase^{PLUS} [30]. Primer sequences and amplification efficiencies are listed in Table 2.

The PCR reaction consisted of 7.5 µl of iQ SYBR Green Supermix (Bio-Rad Laboratories), 300 nM forward and reverse primers, and 2 µl diluted cDNA (5 ng) in a total volume of 15 µl. All PCR reactions were performed in triplicate, with the exception of RT-minus (pooled across each set of samples) and no template controls, which were performed in duplicate.

Cycling was performed in CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Following thermocycling, a dissociation protocol was performed to confirm that a single PCR product was amplified, and was absent in the RT-minus and no template controls.

3. Results

3.1 Confirmation of protein expression for SSC markers

Western blot analysis was performed on whole cat testis from three age groups: prepubertal juvenile cats (14 to 17 wk, $n = 3$), peripubertal juvenile cats (6 mo, $n = 3$), and adult cats (11 to 12 mo, $n = 3$). A single band at approximately 27 kDa was detected for UCHL1 (Fig. 1A). No significant difference was detected between the prepubertal and adult cats ($P = 0.709$) or between the peripubertal and adult cats ($P = 0.392$) (Fig. 1B). ZBTB16 displayed an immunoreactive band at approximately 115 kDa (Fig. 1C). Analysis of the band intensities revealed a significantly higher expression of ZBTB16 for the prepubertal group as compared to adults ($P = 0.018$). Expression of ZBTB16 was also higher ($P = 0.0502$) in peripubertal compared with adult donors (Fig. 1D). An approximately 44 kDa band was detected for POU5F1 (Fig. 1E). The expression pattern was highly variable for this protein, both between and within age groups. As such, no significant difference of POU5F1 expression levels was found between the two juvenile and adult groups (Fig. 1F). While a minimum of two antibodies were assessed for each of the surface markers (GFRA1, GPR125, and THY1), none of them demonstrated clearly identifiable bands of the correct size (Table S1).

3.2 SSC markers are expressed on a subset of cells adjacent to the basement membrane

Our next aim was to characterize which cells of the testis expressed the three potential SSC markers. To this end, fluorescent IHC was used to localize positive cells within prepubertal (8 to 14 wk, $n = 5$) and adult (11 mo to 4 y, $n = 6$) cat testis. In the prepubertal testis, UCHL1 staining appeared specific to spermatogonia, as identified by their low cytoplasmic to nuclear ratio and

location within seminiferous tubules (Fig. 2A-C). Single or paired UCHL1-positive cells were randomly distributed between the non-staining cells of the seminiferous tubule, adjacent to the basement membrane. In the adult testis, UCHL1 staining was also limited to spermatogonia (Fig. 3A-C). UCHL1-positive cells could be found as single, paired, or aligned spermatogonia in a single layer contiguous with the basement membrane of the seminiferous tubules. To investigate if UCHL1 expression is restricted to spermatogonia in the cat, double-immunolabeling was performed with UCHL1 and vimentin, an intermediate filament specifically expressed by Sertoli cells [31]. Double-labeled IHC sections demonstrate that Sertoli cells, as identified by vimentin expression, do not co-express UCHL1 (Fig. 4A-D). Because of the high number of cells in close proximity within the seminiferous tubules, it is possible that double-positive cells could escape detection. Therefore, immunocytochemistry was performed on dissociated testis cells so that individual cells could be evaluated (Fig. 4E-H). Of the approximately 1,000 testicular cells assessed per individual ($n = 3$), no cell expressed both markers, confirming that UCHL1 expression is specific to spermatogonia in the cat.

Single ZBTB16-positive cells were found interspersed among the non-staining cells of the seminiferous tubules in prepubertal testis (Fig. 2D-F). In adult testis, ZBTB16 displayed an even more restricted expression pattern than UCHL1, with only a subset of spermatogonia staining positive (Fig. 3D-F). ZBTB16 expression was localized to the nucleus, and positive cells were typically found as single spermatogonia located adjacent to the basement membrane.

In prepubertal cat testis, POU5F1 was expressed in single or paired cells within the seminiferous tubules (Fig. 2G-I). Similar to ZBTB16, POU5F1 showed a restricted expression pattern in the adult limited to a subset of spermatogonia along the basement membrane (Fig. 3G-

L). Although the majority of POU5F1-positive cells in the cat displayed a nuclear staining pattern, a subset of cells (less than five percent) also expressed POU5F1 in the cytoplasm.

To determine whether the positive staining cells represented the same subpopulation, triple-immunolabeling was performed. In the prepubertal testis, the same subset of cells expressed all three markers (Fig. 5A-D). In the adult testis, ZBTB16- and nuclear POU5F1-positive cells also expressed UCHL1 (Fig. 5E-H). However, several UCHL1-positive cells did not express ZBTB16 or POU5F1. The majority of cells expressing ZBTB16 also expressed POU5F1; however a subset of ZBTB16-positive, POU5F1-negative cells were also identified in the adult testes. Taken together, these data demonstrate that UCHL1, ZBTB16, and POU5F1 reliably identify a small subpopulation of undifferentiated spermatogonia in the domestic cat, likely including SSCs, particularly in prepubertal individuals in which spermatogenesis has not commenced, and therefore no differentiating spermatogonial cells are present to confound results. Protein localization for the surface markers (GFRA1, GPR125, and THY1) could not be detected with any of the antibodies tested (Table S1).

3.3 SSC Markers are conserved in wild felids

To investigate the utility of the domestic cat as a model for SSCs in rare and endangered felids, testis tissue from three wild cats were examined: one sample was obtained from an Amur leopard and two from cheetahs. Western blot analysis showed that SSC markers UCHL1, ZBTB16, and POU5F1 are also expressed in wild felid testis, and immunoreactive bands are present at similar molecular weights to the domestic cat (Fig. 6). Moreover, expression levels appeared quite similar to that of the adult domestic cat (Fig. 1). Fluorescent IHC analysis also paralleled the

domestic cat expression pattern. UCHL1 was the most abundant marker, with several positive basement membrane-associated spermatogonia per tubule cross-section (Fig. 7A, B, G, and H). ZBTB16 and POU5F1 expression also appeared limited to spermatogonia bordering the basement membrane, and a more restricted expression pattern was seen (Fig. 7C, D, I, and J for ZBTB16 and Fig. 7E, F, K, and L for POU5F1 expression). UCHL1 could be detected in both the nucleus (Fig. 7G and H) and cytoplasm (Fig. 7A and B) whereas ZBTB16 and POU5F1 generally remained localized to the nucleus (Fig. 7C-F and I-L). Overall, fewer positive cells were noted per tubule cross-section in the wild felid testes as compared to the adult domestic cat; this is likely due to an age-related reduction of SSCs in the wild cats tested, whose ages were 8, 10, and 18 years old. Co-localization studies could not be performed with the wild felid samples due to a high level of autofluorescence noted when visualizing the slides. This autofluorescence was likely due to post-mortem removal of the testes and the increased time between euthanasia and tissue processing. As a result, IHC studies could only be performed using secondary antibodies in the far red spectrum (i.e., Alexafluor 647), precluding co-localization analysis.

3.4 Enrichment of candidate markers following differential plating

Differential plating is an established method for enrichment of SSCs as non-germ cells preferentially stick to the plastic surfaces [32]. Differential plating was performed using dispersed cells from freshly isolated seminiferous tubules of prepubertal domestic cats (3 to 4.5 mo, n = 4). Following differential plating, 15.5 ± 4.6 percent of total tubular cells were recovered. Cell viability of the enriched fraction (92.8 ± 1.4 percent) was comparable to the input cells (95.7 ± 0.4 percent), as determined by trypan blue dye exclusion. The gene expression

profiles of the two fractions were compared with RT-qPCR. Five SSC marker genes (*UCHL1*, *ZBTB16*, *POU5F1*, *CDH1*, and *THY1*) were evaluated and *VIM* was used to assess the presence of Sertoli cells. The results are shown in Figure 8. Marker genes *UCHL1*, *POU5F1*, and *CDH1* were all significantly increased in the post-differential plating fraction, indicating a higher number of undifferentiated spermatogonia as compared to the unenriched fraction. Genes *ZBTB16* and *THY1* also trended upwards, but were not statistically significant. Sertoli cell marker *VIM* expression appeared similar between the two fractions. The p-values for all prepubertal gene comparisons can be found in Figure 8.

4. Discussion

The present study examined the protein expression pattern and localization of three key SSC markers in the domestic cat: *UCHL1*, *ZBTB16*, and *POU5F1*. This extends the findings in our previous study [22], by 1) confirming the SSC marker transcripts are being translated to protein, 2) determining that positive cells are limited to a subset of spermatogonia within the appropriate *in situ* niche, and 3) that the protein expression patterns of these markers within undifferentiated spermatogonia are conserved in two of their wild relatives, the cheetah (*Acinonyx jubatus*) and Amur leopard (*Panthera pardus orientalis*). These results represent the first detailed report of SSC markers in either the cheetah or Amur leopard and the most comprehensive in the domestic cat. Furthermore, increased expression of candidate SSC markers in the enriched fraction was detected, suggesting the utility of this technique and these markers for subsequent studies seeking to improve enrichment and *in vitro* culture protocols for felid SSCs.

It was critical to ascertain specificity of the antibodies by Western blot analyses, as none had previously been verified in the cat. The antibodies against UCHL1, ZBTB16, and POU5F1 detected specific bands consistent with the previously reported sizes (Fig. 1A, C, and E). Interestingly, ZBTB16 expression was significantly reduced in the adult testis, as compared to either juvenile group (Fig. 1C and D). SSCs are rarer in the adult testis due to the progressive ageing of the SSC niche [33,34] and dilution by meiotic and post-meiotic germ cells [12]. As ZBTB16, also known as promyelocytic leukaemia zinc finger (PLZF), is regarded as a more specific marker than UCHL1 [13], the reduced expression noted in the adult testis suggests that it is a more specific marker in the cat as well.

Fluorescent IHC analyses demonstrated that all three markers label a subpopulation of spermatogonia located adjacent to the basement membrane (Fig. 2 and 3), the ideal expression pattern for an SSC marker. ZBTB16 and POU5F1 appear to be more specific than UCHL1, consistent with reports in other species. Co-immunofluorescent studies confirmed that the same small subpopulation of undifferentiated spermatogonia express all three of these intracellular markers (Fig. 5). We were unable to demonstrate protein expression for surface markers GFRA1, GPR125, or THY1 either by western blot or fluorescent ICC/IHC (Table S1). This is disappointing as these markers are commonly employed in rodent species for antigen-based enrichment, such as fluorescent- or magnetic-activated cell sorting. Harkey et al.[35] reported a similar issue in the dog, which might suggest an absence of these surface SSC markers in carnivores. Given that we had previously detected mRNA coding for these proteins in cat testis [22], the lack of signal is more likely due to several alternatives; 1) posttranslational modifications including alterations in the glycosylation patterns which would affect the antigenicity of the marker, 2) the decreased sensitivity of IHC for cell surface antigens [36] and

3) the paucity of suitable antibodies available for non-traditional lab animals such as the dog or cat (see Table S1 for antibodies tested against the surface proteins).

Unlike other members of the UCH family, UCHL1 expression is limited to neuronal tissue and gonads [37]. In the mouse, both Sertoli cells and spermatogonia express UCHL1 [38]. However, in higher order mammals such as the bull [39], pig [25], and monkey [40], UCHL1 expression is exclusive to spermatogonia. We are confident that UCHL1 is also a spermatogonia-specific marker in the cat. This conclusion is based on our fluorescent IHC studies which revealed that UCHL1 expression was restricted to spermatogonia, as identified by their morphological appearance and location within the seminiferous tubules (Fig. 2A-C and Figure 3A-C). Moreover, our co-immunofluorescent studies demonstrated that vimentin-positive Sertoli cells do not express UCHL1, either *in situ* (Fig. 4A-D) or in dissociated individual cells (Fig. 4E-H).

POU5F1 expression appeared highly variable in the cat testis, with differences in expression levels noted between and within the three age groups in our study (Fig. 1E and F). In fact, one individual in the prepubertal group did not display an immunoreactive band for POU5F1 (Fig. 1E, lane 1). However, a strong immunoreactive band for beta-actin was detected following reprobing of the blot; thus it is unlikely that a loading error contributed to the lack of a POU5F1-reactive band. More likely, this individual's POU5F1 expression level was below the detectable threshold. In other species, POU5F1 expression is regulated by post-translational modifications [41]. Therefore, the highly variable expression pattern in cat testis could be due to the dynamic nature of the POU5F1 protein. A similar phenomenon of differential POU5F1 expression in mouse testis prior to the initiation of spermatogenesis has been reported [42]. Whether the variability observed in cat testis is a normal phenomenon due to age-critical

regulation at the transcriptional, translational, or post-translation level; or due to a particular sensitivity of the POU5F1 protein to processing remains to be determined.

Also intriguing is the cytoplasmic POU5F1 staining pattern noted in a small subset of the spermatogonia (Fig. 2G-I and Fig. 3G-L). POU5F1 is a transcription factor and hence, is predicted to translocate to the nucleus to regulate gene expression via its DNA binding domain. POU5F1 has a well-established pattern of nuclear localization in the mouse embryo [43]. In contrast, a more diffuse (i.e., cytoplasmic and nuclear) distribution of the POU5F1 signal has been reported in pig [44], bull[44,45], and human [46] embryos. It has been suggested that localization of the POU5F1 signal is determined by the protein isoform [47]. Currently, only one *POU5F1* transcript has been characterized in the cat [48]; however, other isoforms are likely to exist. The antibody used in our studies was raised against human Oct4A isoform (Millipore, catalog number MAB4401). Studies in human preimplantation embryos and ESCs demonstrated that the OCT4A isoform is nuclear-localized and promotes self-renewal of ESCs [49,50]. Additional experiments will be required to determine the functional significance of the shift in POU5F1 localization in cat spermatogonia. Nevertheless, the majority of POU5F1-positive cells in the cat displayed a nuclear staining pattern indicative of a stem cell.

Of particular interest is the expression of these markers in wild felids. The Amur leopard and cheetah are two diverse members of the family Felidae, and are estimated to have phylogenetically diverged from the domestic cat 10.8 million and 7.2 million year ago, respectively [51]. The Amur leopard is a rare subspecies of leopard that has been listed as critically endangered since 1996. The cheetah has been listed as vulnerable since 1986 [52]. We were able to demonstrate that UCHL1, ZBTB16, and POU5F1 display a similar expression pattern in the testis of these species (Fig. 6 and 7). While interpretations made on such a small

sample size could be deceptive, conservation of SSC markers among these three species, representing three distinct felid lineages and including the oldest (*Panthera*) and youngest (*Felis*) genera, is highly suggestive that these markers are conserved across all members of family Felidae.

SSC technology has the potential to impact the conservation of felids in several ways. It offers an alternative approach to generate spermatozoa (either *in vitro* or *in vivo* following xenotransplantation), particularly for prepubertal animals whose genotypes might otherwise be lost due to premature death. Domestic cat testes are capable of supporting ocelot SSCs [17], which suggests that domestic cats may also be suitable hosts for other members of the family Felidae. Indeed, existing evidence suggests that the genome, as well as physiological mechanisms, is relatively well-conserved across felids [51,53]. SSC-based technologies could also provide an alternative to somatic cell nuclear transfer (SCNT) for the production of transgenics thus overcoming the problems associated with transgenic SCNT [54-56]. However, to take advantage of these technologies in non-rodent species, improved methods for isolating and culturing SSCs must be established. In species such as the domestic cat, prepubertal testes provide an excellent source for such studies as these tissues are discarded following routine neutering. Moreover, prepubertal seminiferous tubules contain only two cell types: gonocytes (SSCs) and Sertoli cells. Thus, cells obtained following differential plating, an established method used to enrich SSCs in a variety of non-rodent species [25], should display increased expression of any proposed SSC markers. Indeed, increased level of transcripts for the SSC markers *UCHL1* and *POU5F1* were observed following enrichment for prepubertal cat gonocytes. While only a tendency toward enrichment of *ZBTB16* and *THY1* transcripts was

detected, this was likely due to high variability which may be resolved following further refinement and increased sample number.

5. Conclusions

This study reports the conservation of UCHL1, ZBTB16, and POU5F1 in the undifferentiated spermatogonia of three distantly related felids: domestic cat, cheetah, and Amur leopard. These proteins may be used as markers to evaluate cell enrichment strategies and monitor the success of *in vitro* culture systems. Conservation of these proteins, which have essential roles in SSC self-renewal in other species, suggests that these self-renewal mechanisms may be conserved among felids as well. This study validates the domestic cat as a model for rare and endangered felids, in addition to its role as an important biomedical model.

Declaration of interest: Conflicts of interest: none

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Figure captions

Figure 1 Quantitative analysis of SSC marker expression by western blot. Representative western blots of UCHL1 (A) and ZBTB16 (C) protein expression in prepubertal (lane 1), peripubertal (lane 2), and adult (lane 3) domestic cat testis. (E) Western blot of POU5F1 protein expression in domestic cat testis. Number denotes age group: prepubertal (1), peripubertal (2), and adult (3). Blots were visualized with Supersignal West Pico chemiluminescent substrate. Expression levels of UCHL1 (B), ZBTB16 (D), and POU5F1 (F) were quantified using Image Lab software. Values represent relative density normalized to beta-actin. * $P = 0.018$; ** $P = 0.0502$.

Figure 2 Distribution of SSC markers in prepubertal domestic cat testis. Representative fluorescent immunohistochemistry images with Hoechst 33342 to mark cell nuclei (A, D, and G) and magenta labeling (Alexa Fluor® 647) of UCHL1 (B), ZBTB16 (E), and POU5F1 (H) protein with merged image (C, F, and I). Arrows indicate positive cells. Arrowhead indicates nuclear localization of POU5F1 protein. Seminiferous tubule borders are outlined in white. Scale bars; 20 μm .

Figure 3 Distribution of SSC markers in adult domestic cat testis. Representative fluorescent immunohistochemistry images with Hoechst 33342 to mark cell nuclei. (A, D, and G) and magenta labeling (Alexa Fluor 647®) of UCHL1 (B), ZBTB16 (E), and POU5F1 (H) protein with merged images (C, F, and I). Insets within each image display higher magnification of red dotted regions. Higher magnification (J, K, and L) of yellow dotted region in G, H, and I

respectively. Both a nuclear staining pattern (red dotted region) and the less common cytoplasmic staining pattern (yellow dotted region) were observed. Seminiferous tubule borders are outlined in white. Scale bars; 50 μ m.

Figure 4 Localization of vimentin and UCHL1 in domestic cat testis. (A- D) Representative fluorescent immunohistochemistry images with blue (Hoechst 33342) staining to mark cell nuclei (A, E), green (Alexa Fluor® 488) labeling of vimentin (B, F), and magenta (Alexa Fluor® 647) labeling of UCHL1 (C, G) with merged images (D, H) demonstrating that the proteins were located in different cells in the adult cat testis (A-D) and that no cells in the dissociated adult cat testis co-expressed the two proteins (E-H). Seminiferous tubule borders in A-D are outlined in white. Arrows indicate UCHL1-positive cells. White arrowheads indicate vimentin-positive cells. Red arrowhead indicates cells negative for both UCHL1 and vimentin. Scale bars; 50 μ m.

Figure 5 Co-localization of SSC markers in domestic cat testis. Representative fluorescent immunohistochemistry images with green (Alexa Fluor® 488) labeling of ZBTB16 (A and E), red labeling (Alexa Fluor® 594) of UCHL1 (B and F), and magenta (Alexa Fluor® 647) labeling of POU5F1 (C and G) demonstrating that the proteins localized to the same cells in prepubertal (A- D) and adult (E-H) cat testis. Merge of previous images with blue (Hoechst 33342) to mark cell nuclei (D and H). Seminiferous tubule borders are outlined in white. Arrows indicate positive cells. Scale bars; 50 μ m.

Figure 6 Western blot analysis of SSC marker expression in domestic cat and wild felids.

Western blots of UCHL1, POU5F1, and ZBTB16 in the testes of an adult domestic cat (lane 1) as the positive control, Amur leopard (lane 2) and cheetah (lanes 3 and 4). For a loading control, blots were stripped and reprobed for beta-actin. Blots were visualized with Supersignal West Pico chemiluminescent substrate.

Figure 7 Distribution of SSC markers in wild felid testis. Representative fluorescent immunohistochemistry images with magenta (Alexa Fluor® 647) labeling of UCHL1 (A and G), ZBTB16 (C and I), and POU5F1 (E and K) protein in cheetah and Amur leopard testis, respectively. Merge of respective images with Hoechst 33342 to mark cell nuclei (B, H, D, J, F, and L). Seminiferous tubule borders are outlined in white. Arrows indicate positive cells. Scale bars; 20 μ m.

Figure 8 RT-qPCR analysis of SSC (*UCHL1*, *ZBTB16*, *POU5F1*, *CDH1*, and *THY1*) and Sertoli cell (*VIM*) gene transcripts using mRNA isolated from prepubertal cat testis before and after differential plating. Four animals were used and analyses were performed in triplicate. Errors bars represent $\pm$ SEM. Relative mRNA expression for *UCHL1*, *POU5F1*, and *CDH1* are significantly different ($P \leq 0.05$) as indicated by p-values displayed over bars.

Supplementary Figure 1 Negative control western blots. To confirm specificity of antibodies, western blots were performed using predicted negative tissue and cell lines. Peripubertal

domestic cat testis (6 mo) was included as a positive control. Forty micrograms of total protein were loaded for each sample. Blots were visualized with Supersignal West Pico chemiluminescent substrate. UCHL1 was expressed in both cell lines. However, no expression was noted in the uterus, consistent with previous reports that *in corpora* UCHL1 expression is limited to neuronal and gonadal tissues. POU5F1 was strongly expressed in HEK293 cells, and to a lesser degree, in uterine tissue. No band was detected for cat fetal fibroblasts. Similarly, ZBTB16 expression was high in HEK293 cells, lower in uterus, and absent in cat fetal fibroblasts. CFF, cat fetal fibroblasts.

Supplementary Figure 2 Fluorescent immunohistochemistry single-label negative controls.

Control slides incubated with non-immune serum in which the primary antibody against UCHL1 (B), ZBTB16 (E), and POU5F1 (H) were omitted showed minimal immunoreactivity with secondary antibody. (A, D, and G) Hoechst 33342 to mark cell nuclei. (C, F, and I) Merge of previous two images. Scale bars; 50 μ m.

Supplementary Figure 3 Fluorescent immunohistochemistry co-localization negative controls.

Control slides incubated with non-immune serum in which the primary antibody against ZBTB16 (B and F), UCHL1 (C and G), and POU5F1 (D and H) were omitted showed minimal immunoreactivity with secondary antibodies. (A and E) Hoechst 33342 to mark cell nuclei. (A-D) Prepubertal domestic cat testis. (E-H) Adult domestic cat testis. Scale bars; 50 μ m.

Table 1 antibodies used

Table 1
List of antibodies used in western blot analysis (WB) and immunofluorescence (IF).

Primary Ab	Isotype	Source	Dilution	Secondary Ab	Source	Dilution
POU5F1 (OCT4)	Mouse IgG ₁	Millipore	1500(WB) 100 (IF)	Horse anti-mouse IgG (WB), Goat anti- mouse IgG (IF)	Cell Signaling Technology (WB), Molecular Probes (IF)	1000 (WB), 400 (IF), 200 (triple-label IF)
ZBTB16 (PLZF)	Goat IgG	Santa Cruz	500 (WB) 50 (IF)	Donkey anti-goat IgG (WB, IF)	Jackson Immunoresearch Laboratory (WB), Molecular Probes (IF)	2500(WB), 400 (IF)
UCHL1	Rabbit IgG	Novus	4000 (WB) 200 (IF)	Goat anti-rabbit IgG (WB, IF)	Cell Signaling Technology (WB) Molecular Probes (IF)	2500 (WB), 400 (IF)
Vimentin	Mouse IgG ₁	Abcam	1000 (IF)	Goat anti-mouse IgG (IF)	Molecular Probes (IF)	400 (IF)
Beta-actin	Mouse IgG _{2a}	Sigma	2500 (WB)	Horse anti-mouse IgG (WB)	Cell Signaling Technology (WB)	5000 (WB)

Table 2 primers used

Table 2
Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Accession Number	Primer Sequence	Amplicon (bp)	Efficiency
<i>Spermatogonial stem cells</i>				
CDH1	XM_003998189.1	F: 5'-GCTGTGTCTTCAAATGGGCA-3' R: 5'-CGTCGTCATCTGCATCTGTG-3'	178	97%
POU5F1 (OCT4)	NM_001173441.1	F: 5'-CTGGAGCAAAACACCGAGGAG-3' R: 5'-TTTGGCTGAACACCTTCCCAAA-3'	160	113%
ZBTB16 (PLZF)	XM_003992367.1	F: 5'-ATCCCTACGAGTGTGAGTTCTG-3' R: 5'-TTCTCACCTGTATGCACCCTATAG-3'	169	96%
THY1	XM_003992443.2	F: 5'-CAAGGACGAGGGGATGTACA-3' R: 5'-TATGCCCTCACACTTGACCA-3'	112	105%
UCHL1	XM_003985462.1	F: 5'-CCTGTGGTACCATCGGACTG-3' R: 5'-CATCATGGGCTGCCTGAATG-3'	168	98%
<i>Sertoli cells</i>				
VIM	XM_003988131.1	F: 5'-GGGAGAAGTTGCAGGAGGAG-3' R: 5'-CAAGGTCAAGACGTGCCAAA-3'	105	101%
<i>Reference genes</i>				
GAPDH	NM_001009307.1	F: 5'-AGAACATCATCCCTGCTTCTACT-3' R: 5'-CTTCTTGATGTCATCGTATTTGGC-3'	170	87%
RPL17	NM_001128842.1	F: 5'-CCCGACAAAATCATGCAAATCAAG-3' R: 5'-GATACTTGGTGGCTTTTCGGATAT-3'	113	100%
YWHAZ	XM_004000022.1	F: 5'-GCCCTTAACTTCTCTGTGTTCTAT-3' R: 5'-TTATTAGCGTGCTGTCTTTGTAGG-3'	142	98%

Figure 1 analysis by western blot

Figure 2 IHC of SSC markers_prepubertal

Figure 3 IHC of SSC markers_adult

Figure 4 Localization of vimentin and UCHL1

Figure 5 Co-localization of SSC markers

Figure 6 revised western blot

Figure 7 Distribution of SSC markers in wild felid testis

Figure 8 RT-qPCR analysis