Theriogenology 86 (2016) 1022–1035

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Conservation of spermatogonial stem cell marker expression in undifferentiated felid spermatogonia

Lindsey M. Vansandt[a,b,1], Janelle L. Livesay[a], Melissa Joy Dickson[a], Lei Li[a], Budhan S. Pukazhenthi[b], Carol L. Keefer[a,*]

[a] Department of Animal and Avian Sciences, University of Maryland, College Park, Maryland, USA
[b] Center for Species Survival, Smithsonian Conservation Biology Institute, Front Royal, Virginia, USA

A R T I C L E I N F O

Article history:
Received 1 December 2015
Received in revised form 18 March 2016
Accepted 19 March 2016

Keywords:
Spermatogonial stem cell
Domestic cat (*Felis catus*)
Cheetah (*Acinonyx jubatus*)
Amur leopard (*Panthera pardus orientalis*)
Testis

A B S T R A C T

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 nonrodent, 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 access this technology in felids is the establishment of molecular markers. This study was designed to evaluate, in the domestic cat (*Felis catus*), the expression both *in situ* and following enrichment *in vitro* of six genes (*GFRA1*, *GPR125*, *ZBTB16*, *POU5F1*, *THY1*, and *UCHL1*) that had been previously identified as SSC markers in other species. Antibodies for surface markers glial cell line–derived neurotrophic factor family receptor alpha 1, G protein–coupled receptor 125, and thymus cell antigen 1 could not be validated, whereas Western blot analysis of prepubertal, peripubertal, and adult cat testis confirmed protein expression for the intracellular markers ubiquitin carboxy-terminal hydrolase 1, zinc finger and BTB domain-containing protein 16, and POU domain, class 5, transcription factor 1. Colocalization 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 after differential plating was also observed. These markers will facilitate further studies in cell enrichment and IVC of felid SSCs enabling both production of transgenic domestic cats and preservation of the male genome from rare and endangered felids.

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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). Spermatogonial stem cells 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 2 years of continuous culture demonstrated

* Corresponding author. Tel.: 301-405-3933; fax: 301-405-7980.
E-mail address: ckeefer@umd.edu (C.L. Keefer).
[1] Present address: Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, Cincinnati, Ohio, USA.

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.03.031

that mouse SSCs could maintain euploid karyotypes, normal methylation patterns, and germline competence during long-term culture [1].

In addition, 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]. Spermatogonial stem cells 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 postthaw 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.

Spermatogonial stem cells are an extremely rare population, estimated to represent only 0.03% of the total germ cell population [12]. Owing to their rarity, a requisite first step to access 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, coexpression of multiple markers in undifferentiated cells occupying the correct morphologic niche is highly suggestive of stemness, as demonstrated in the bull [13].

Although 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, which was demonstrated to be expressed in a rare subpopulation of single spermatogonia in the mouse, was shown by immunolabeling to be expressed in the testes of other mammals, including prepubertal cats [20]. The presence of a partial ubiquitin carboxy-terminal hydrolase 1 (*UCHL1*) transcript in testes of several wild cat has also been demonstrated, but no other candidate markers or comprehensive studies have been reported in the cat [21]. Recently, we demonstrated the expression of several SSC markers (*GFRA1*, G protein–coupled receptor 125 [*GPR125*], Zinc finger and BTB domain-containing protein 16 [*ZBTB16*], POU domain, class 5, transcription factor 1 [*POU5F1*], thymus cell antigen 1 [*THY1*], and *UCHL1*) in cat testis using reverse transcription polymerase chain reaction (RT-PCR) [22], suggesting that SSC markers previously reported in other species [13,19,23–25] are conserved in the cat [22]. However, these experiments were limited to the level of messenger RNA in testicular samples, and thus, it remained unclear whether these transcripts were translated into proteins expressed appropriately in the spermatogonial cells *in situ* or whether after isolation and enrichment, the transcript levels would be increased as expected for markers of undifferentiated spermatogonial cells. Therefore, the objectives of this study were to characterize the protein expression pattern of the candidate SSC markers in felids with regard to (1) levels of expression in prepubertal, peripubertal, and adult individuals; (2) location within the seminiferous tubules in domestic cats; and (3) conservation of markers in two of their wild relatives, the cheetah (*Acinonyx jubatus*) and Amur leopard (*Panthera pardus orientalis*). An additional objective was to determine whether SSC enrichment protocols resulted in higher levels of SSC marker transcripts as would be anticipated, particularly for prepubertal animals which would only have somatic cells and undifferentiated spermatogonial cells with no spermatogonia undergoing differentiation to confound results. Validation of these markers will facilitate further studies in cell enrichment and IVC 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 was not required as domestic cat testes were obtained from local veterinarians following routine neuters and testis tissue from nondomestic felids was obtained postmortem from animals euthanized for health reasons. These are exempt studies under Title 9, Code of Federal Regulations, Subchapter A- Animal Welfare, Parts 1 to 3.

Domestic cat (*Felis catus*) testicles, obtained from local spay-neuter clinics, were stored at 4 °C and were processed within 24 hours of collection, and generally within the first 6 hours. 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 histologic 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 years) and Amur leopard (*Panthera pardus orientalis*, n = 1, 18 years) were collected postmortem following euthanasia for health-related problems. Testis tissue was processed within 6 hours of euthanasia for

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