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Research paper

Characterization of spermatogonial cells and niche in the scorpion mud turtle (*Kinosternon scorpioides*)

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ABSTRACT

Undifferentiated spermatogonia (A_{und}) or spermatogonial stem cells (SSCs) are committed to the establishment and maintenance of spermatogenesis and fertility throughout a male's life and are located in a highly specialized microenvironment called niche that regulates their fate. Although several studies have been developed on SSCs in mammalian testis, little is known about other vertebrate classes. The present study is the first to perform a more detailed investigation on the spermatogonial cells and their niche in a reptilian species. Thus, we characterized A_{und}/SSCs and evaluated the existence of SSCs niche in the *Kinosternon scorpioides*, a freshwater turtle found from Mexico to northern and central South America. Our results showed that, in this species, A_{und}/SSCs exhibited a nuclear morphological pattern similar to those described for other mammalian species already investigated. However, in comparison to other spermatogonial cell types, A_{und}/SSCs presented the largest nuclear volume in this turtle. Similar to some mammalian and fish species investigated, both GFRA1 and CSF1 receptors were expressed in A_{und}/SSCs in *K. scorpioides*. Also, as *K. scorpioides* A_{und}/SSCs were preferentially located near blood vessels, it can be suggested that this niche characteristic is a well conserved feature during evolution. Besides being valuable for comparative reproductive biology, our findings represent an important step towards the understanding of SSCs biology and the development of valuable systems/tools for SSCs culture and cryopreservation in turtles. Moreover, we expect that the above-mentioned results will be useful for reproductive biotechnologies as well as for governmental programs aiming at reptilian species conservation.

1. Introduction

Kinosternon scorpioides is a freshwater turtle found in large areas of Mexico and Central and South America (Vanzolini et al., 1980; Berry and Iverson, 2011). It is reported that its population has recently declined mainly because of hunting and deforestation (Pereira et al., 2007; Carvalho et al., 2010). Therefore, in order to improve the management of this freshwater turtle species, the better understanding of its reproductive biology might support and guide actions aiming at preventing this species from extinction and also enhancing its reproductive capacity in breeding programs (Pereira et al., 2007; Carvalho et al., 2010; da Costa Araújo et al., 2013). In this context, we have recently evaluated and characterized spermatogenesis in adult *K. scorpioides* (Sousa et al., 2014). Particularly, in this study we have investigated the duration and the efficiency of spermatogenesis; however, a detailed spermatogonial cell physiology evaluation was not performed, especially in relation to the more undifferentiated spermatogonia.

The spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and fertility (Mecklenburg and Hermann, 2016; de Rooij, 2017). In mammals, SSCs comprise a small population of undifferentiated type A spermatogonia (A_{und}) located on the basal compartment of seminiferous epithelium. A_{und} can be found as single, paired, and chains of 4–16 cells, designated respectively as A_{single}, A_{pair}, and $A_{aligned}$ (Huckins, 1971; Garbuzov et al., 2018). Using mouse models, this paradigm has been challenged and it has been proposed that the real SSCs are composed by a subpopulation of A_{single} cells only (Hara et al., 2014). Hence, the division of these cells into A_{pair} represents the first step of cell differentiation in spermatogenesis, resulting in sperm production (Huckins, 1971; de Rooij, 1973; Mecklenburg and Hermann, 2016). Recently, studies have identified specific genes expressed on a subset of A_{single} cells, such as *Bmi1*, *Pax7*, *Id4 and Csf1r* (Aloisio et al., 2014; Komai et al., 2014; Helsel et al.,

Abbreviations: A_{dif}, differentiated spermatogonial cells; A_{und}, undifferentiated spermatogonial cells; BV, blood vessel; CSF1R, colony stimulating factor 1 receptor; CT, connective tissue; GDNF, glial cell line derived neurotrophic factor; GFRA1, GDNF family receptor alpha 1; LC, Leydig cells; LS, lymphatic space; SC, sertoli cells; SSC, spermatogonial stem cell

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2017). Multiple reports have described that A_{single} cells express GFRA1, a cell surface receptor for the self-renewal factor GDNF (glial cell line-derived neurotrophic factor), and it is also suggested that SSCs contain a GFRA1-positive population (Meng et al., 2000; Hara et al., 2014). However, other studies have brought new discoveries about spermatogonial markers putting the pursuit of distinctive proteins for the real SSCs under investigation (Buageaw et al., 2005; Nakagawa et al., 2007; Grisanti et al., 2009; Nakagawa et al., 2010; Mutoji and Hermann, 2017).

The SSCs niche exists in specific areas of the seminiferous epithelium where an intrinsically complex coordination of self-renewal or differentiation of spermatogonial cell lineage occurs (de Rooij, 2017). Residing in this environment, SSCs are maintained throughout life favoring the regulation of the spermatogenic process (de Rooij, 2017). Several studies, including investigations from our laboratory, have provided data supporting that the SSCs niche is located preferentially on tubule areas that border the interstitial tissue containing blood vessels (Chiarini-Garcia and Russell, 2001; Chiarini-Garcia et al., 2001, 2003; Yoshida et al., 2007; Campos-Junior et al., 2012, 2014). In addition to Sertoli cells and peritubular myoid cells, some interstitial cells, such as Leydig cells and macrophages, are also key players in the spermatogonial niche. These cells exert influence on SSC fate through production of growth factors, such as GDNF, FGF2 (fibroblast growth factor 2) and CSF1 (colony stimulating factor 1) (Ryan et al., 2001; Oatley et al., 2007; Simon et al., 2007; Hofmann, 2008; Oatley and Brinster, 2008; Campos-Junior et al., 2012, 2014). However, the aforementioned findings were obtained mainly from studies developed in mammalian species, especially rodents. Therefore, to our knowledge, there is no report describing SSCs niche in reptilian species.

In the present study, using morphological tools and phenotypic markers, we aimed to identify and characterize A_{und}/SSCs, as well as to investigate the existence of SSCs niche in *K. scorpioides*. Moreover, these approaches allowed the evaluation of A_{und}/SSCs preferential location in the seminiferous epithelium basal compartment, taking into consideration several components of the intertubular compartment. We expect that the obtained data will be able to stimulate future studies involving SSCs biology and physiology in reptiles.

2. Materials and methods

2.1. Animals and tissue preparation

Nine adult Scorpion mud turtles (*Kinosternon scorpioides*), presenting mean body weight of 325 ± 28 g, testes weight of 0.96 ± 0.17 g and gonadosomatic index (testis mass divided by body weight) of 0.59 ± 0.08%, were used in this study. The animals were obtained from the State University of Maranhão (São Luís, MA; 02°31′48″S, 44°18′10″W), located in the Northeast region of Brazil. The turtles were maintained in captive conditions (MMA/ICMBio/Sisbio – 26136-1/2010), housed at 30–35 °C with 12 h photoperiod, with water and food available *ad libitum*. All procedures were performed in strict accordance with the approved Guidelines for the Ethical Treatment of Animals CEEA/UEMA (Protocol number 011/2010).

The turtles were euthanized with sodium thiopental 2.5% (60 mg/Kg; Cristália). After orchiectomy, testes were separated from epididymis, weighted and cut longitudinally with a razor blade into small fragments. For morphometric analyses, testes were fixed for 24 h by immersion in 4% buffered glutaraldehyde at 4 °C. Tissue samples, 2–3 mm thick, were routinely processed and embedded in glycol methacrylate (Leica Historesin, Heidelberg, Germany). Histological sections, 4 µm thick, were obtained using a glass knife with a rotatory microtome and then stained with toluidine blue solution in 1% sodium borate. For immunostaining analyses, the testes fragments were fixed for 24 h in Bouin's solution and the samples were dehydrated in ethanol, embedded in Paraplast (Sigma), and routinely prepared for evaluation (please see below).

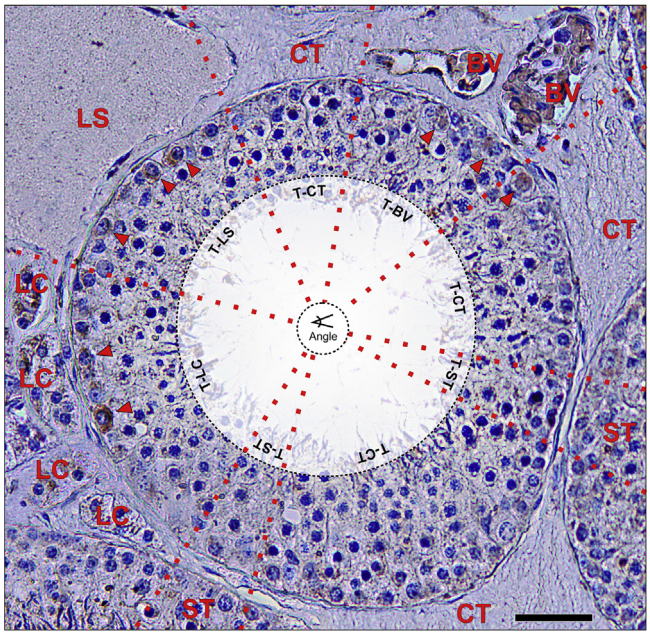

Fig. 1. Schematic illustration regarding the SSCs niche analysis in the *K. scorpioides* turtle. Seminiferous tubules cross-sections, presenting 360°, were fractionated into five different regions according to the area faced by basement membrane in relation to several intertubular compartment components. T-BV, tubular area near the blood vessels (BV); T-LC, tubular region facing Leydig cells (LC); T-LS, tubular area close to lymphatic space (LS); T-T, tubule-tubule contact (ST); and T-CT, tubular contact with connective tissue (CT). A_{und}/SSCs are indicated by arrowheads. Scale bar = 50 µm.

2.2. Spermatogonial cell evaluation

2.2.1. Morphology

The different spermatogonial cell types in Scorpion mud turtles were characterized in detail, complementing the data presented in Sousa et al. (2014). The cell classification/characterization followed morphological nuclear features, such as nuclear shape, presence and disposition of vacuoles, heterochromatin, euchromatin granularity and extent of nucleolus compaction. The nuclear volume of each spermatogonial cell type herein characterized was obtained by measuring the diameter of 30 nuclei per animal (Costa et al., 2012; Campos-Júnior et al., 2012; Sousa et al., 2014). The nucleus heterochromatin intensity for the different spermatogonial cell types was measured in ten randomly chosen nuclei for each animal, using the Adobe Photoshop CS6 (Adobe Systems Software, Mountain View, USA). The images were taken at 100X magnification, using the CellˆF software (Olympus, Massachusetts, USA), converted to grayscale mode and inverted. The average number of pixels was measured in the nucleus area of the cells according to Chiarini-Garcia and Russell (2001).

2.2.2. Immunostaining analyses

Serial sections (5 µm thick) were immunostained using protocols with antibody dilutions previously tested. After dewaxing and rehydration, antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min after boiling in a microwave oven (total period of approximately 10 min). For immunoperoxidase assays, endogenous peroxidase was quenched for 30 min with 3% H2O2 (Sigma Aldrich) in PBS at room temperature. Non-specific binding was blocked with Ultra-V-Block (Thermo Scientific) for 30 min at room temperature. Primary antibodies GFRA1 (goat polyclonal, 1:500, SC-6157, Santa Cruz Biotechnology) and CSF1R (rabbit polyclonal, 1:200, ab61137, Abcam) were applied and the slides were incubated overnight at 4 °C. The slides were exposed to biotinylated rabbit anti-goat (for GFRA1; ab6740, 1:100, Abcam) and goat anti-rabbit (for CSF1R; ab6720, 1:200, Abcam)

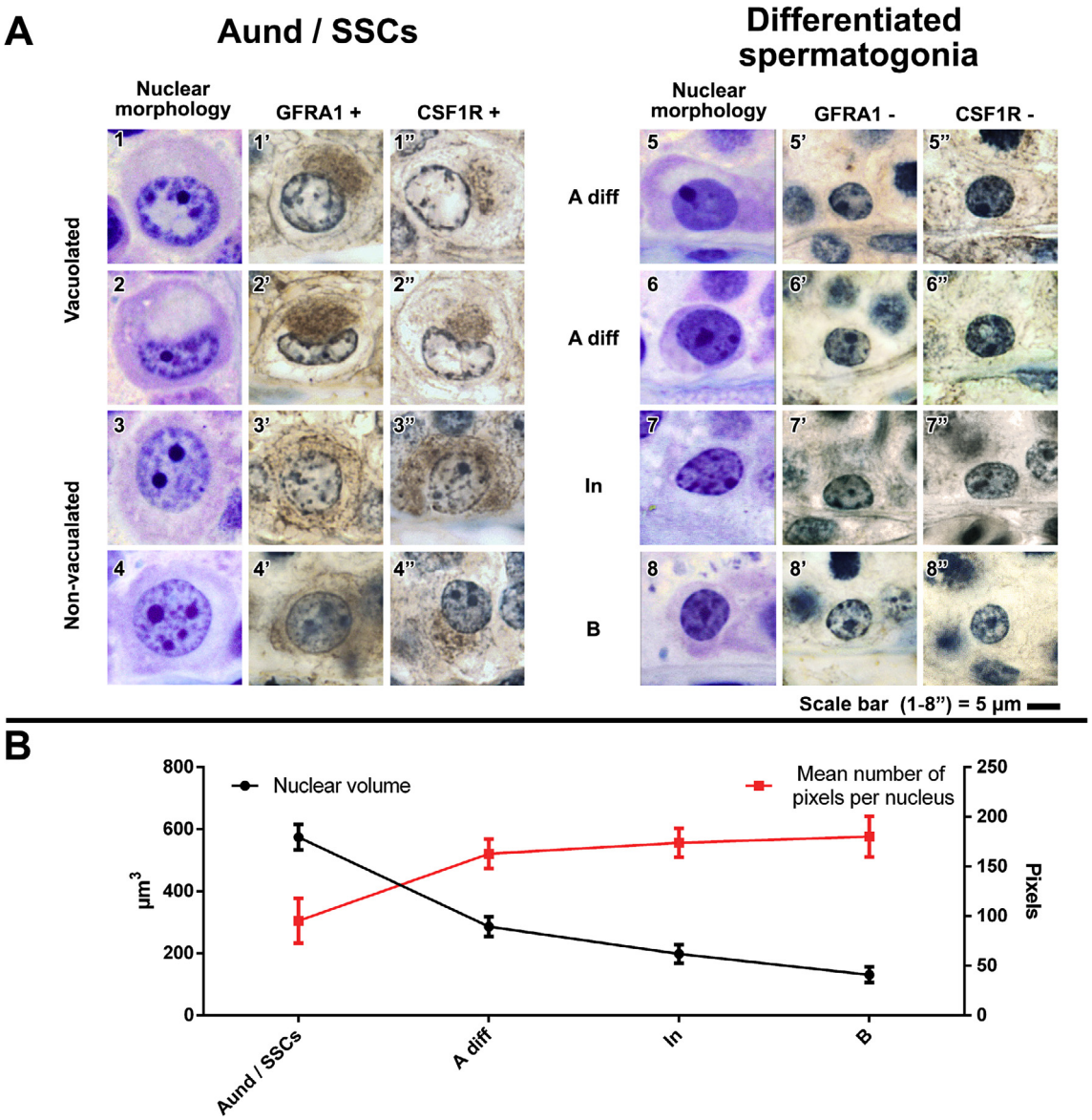

Fig. 2. Morphology of different spermatogonial cell types characterized in the *K. scorpioides* turtle. A) Photomicrographs from the four characterized spermatogonial cell types and their correspondent labelling pattern for GFRA1 and CSF1R in type A undifferentiated (A_{und}/SSCs) (1–4″), type A differentiated (A_{dif}) (5–6″), Intermediate (In) (7–7″) and type B spermatogonia (B) (8–8″). B) Observe that the nuclear volume (black line) of A_{und}/SSCs is strikingly larger than that of A_{dif} and a gradual reduction in this parameter occurs from A_{dif} to type B spermatogonia. On the other hand, the amount of heterochromatin increased from A_{und}/SSCs to type B spermatogonia (red line). Scale bar = 5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunoglobulin G (IgG) antibodies during 60 min at room temperature. Detection of the signal was performed by incubating the sections in streptavidin for 10 min at room temperature, followed by the reaction with peroxidase substrate 3,39-diaminobenzidine (DAB, Sigma Aldrich) and counterstaining with hematoxylin (Merck). Following dehydration, slides were covered with coverslips, mounted with Entellan (Merk) and analyzed.

In order to quantify the labelling pattern of GFRA1-positive and CSF1R-positive in A_{und}/SSCs, thirty cells were classified as i) one cell, two adjacent cells and more than two adjacent cells per seminiferous tubule cross-section. The terminology "adjacent" was used to refer to the cells with internuclear distance of approximately 10 µm apart in a two-dimensional analysis. It is worthy to mention that this analysis does not allow the classification of A_{und} into single, paired or aligned. The latter cells are accurately observed only in three-dimensional evaluations (reviewed by de Rooij, 2017). Moreover, aiming to quantify the percentage of labeled A_{und}/SSCs for GFRA1 and CSF1R, 200 cells, previously characterized according to morphological criteria, were randomly evaluated.

Double-staining immunofluorescence was also performed with the objective of observing whether positive cells for GFRA1 would be also positive for CSF1R or vice versa. Following Ultra-V-Block treatment, the proteins were detected using primary antibodies at the following dilutions: GFRA1 (1:50) and CSF1R (1:100). Next, slides were incubated with Alexa Fluor secondary antibodies [546 anti-goat (1:500) for GFRA1 and 488 anti-mouse (1:500) for CSF1R] for 1 h at room temperature. Confocal images were obtained in a 510 META Laser Scanning Confocal Microscope (Zeiss, Oberkochen, Germany), equipped with 488 and 543 nm lasers. Dual channel images were obtained by sequential scanning.

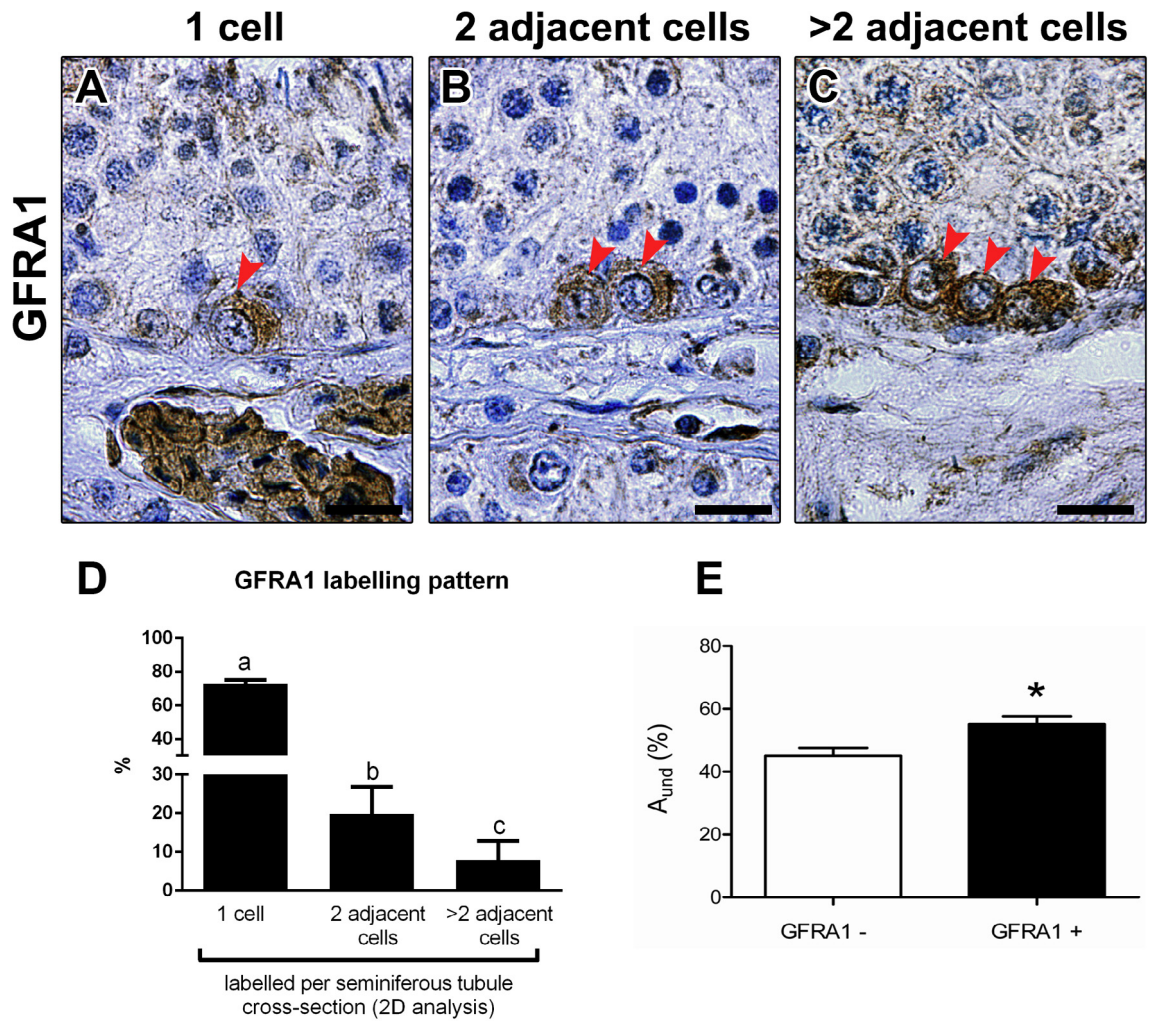

Fig. 3. Immunostaining evaluation of GFRA1 positive Aund/SSCs in the K. scorpioides. Expression of this classical marker was observed exclusively in the membrane/cytoplasm of these cells. In a two-dimensional evaluation, GFRA1-positive Aund/SSCs were observed as one cell (~75%; A and D), two adjacent cells (~20%; B and D) and more than two adjacent cells (~5%; C and D). E) Approximately 55% of Aund/SSCs express this membrane receptor ($p < 0.05$). Scale bar = 10 μm.

2.2.3. A_{und}/SSCs distribution in the seminiferous tubules

In order to investigate the distribution of A_{und}/SSCs, both morphological and immunostaining (GFRA1+ and CSF1R+) evaluations were considered. For this purpose, images from 10 seminiferous tubules cross-sections were obtained for each animal and the seminiferous tubules cross-sections were subdivided into five regions, as follows (Fig. 1): I) adjacent to other tubules (Tubule-Tubule contact or T-T); II) near the interstitial compartment containing Leydig cells (T-LC); III) close to the interstitial compartment containing blood vessels (T-BV); IV) adjacent to the interstitial compartment containing lymphatic space (T-LS); and V) close to the interstitial compartment containing connective tissue (T-CT). Considering that the tubular circumference corresponds to 360°, the numbers of A_{und}/SSCs obtained in the five different regions evaluated were expressed per degree (Costa et al., 2012; Campos-Júnior et al., 2012). Therefore, this approach allowed an estimation of the number of these cells located in each region.

2.3. Statistical analyses

All data were tested for normality and homoscedasticity of the variances. Quantitative data are represented as the mean ± SEM (standard error of the mean). Analyses were conducted using the graphics and statistics program PRISM v5.0 (GraphPad Software, Inc). Data were analyzed by one-way ANOVA for comparisons within groups followed by Newman-Keuls test in case of normal distribution, or by Kruskal-Wallis followed by Dunn's test in case of nonparametric data. Student's T test was used for two-parameter analyses. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Spermatogonial cell morphology and size

According to morphological criteria (Fig. 2A), spermatogonial cells in Scorpion mud turtles were characterized as type A undifferentiated (A_{und}/SSCs), type A differentiated (A_{dif}), intermediate (In) and type B. A_{und}/SSCs exhibited a mottled/granular spherical nucleus with little heterochromatin. Furthermore, these cells exhibited at least four distinct morphologies, i.e. presenting nuclear vacuoles (Fig. 2, A1), cytoplasmic vacuoles (Figs. 2, A1-2), no nuclear vacuoles and presenting up to two large nucleoli (Fig. 2, A3) and no nuclear vacuoles presenting more than two evident nucleoli (Fig. 2, A4). As it can be observed in Fig. 2, all A_{und}/SSCs were found positive for GFRA1 and CSF1R.

The A_{dif} spermatogonia showed a light and finely granular euchromatin (Fig. 2, A5). Although the different subtypes of A_{dif} were not described in the present study, it can be observed that the quantity of heterochromatin in their nuclei tend to increase (Fig. 2, A6). The intermediate spermatogonia presented ovoid nuclei with a little bit more heterochromatin (Fig. 2, A7). Type B spermatogonia showed an increased heterochromatin granularity and the nucleoplasm exhibited a

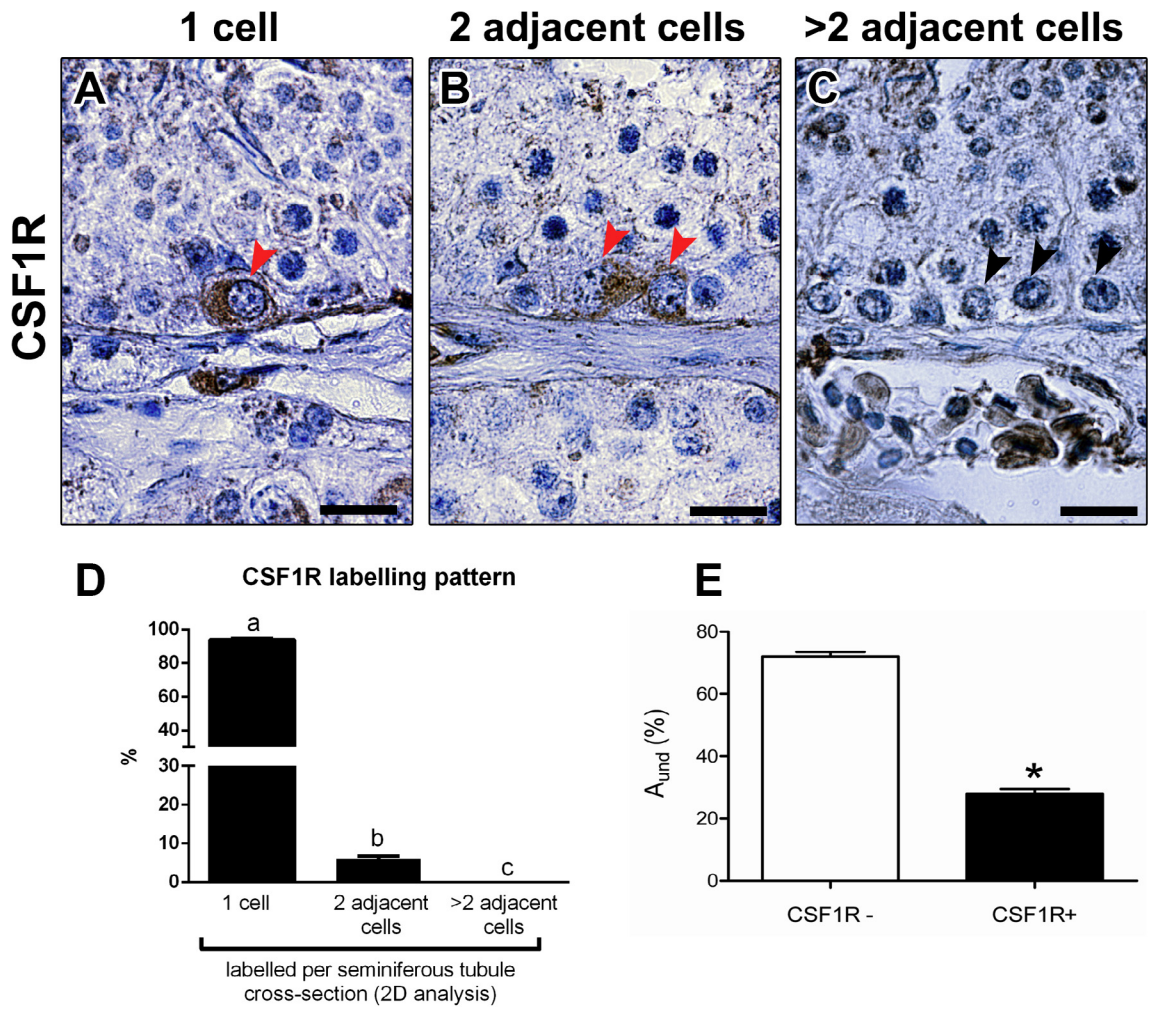

Fig. 4. Immunostaining evaluation of CSF1R positive Aund/SSCs in the K. scorpioides. A-D) As it can be noted, in a two-dimensional evaluation, the expression of this marker was mainly observed as one cell (~95%) (arrowheads). Few Aund/SSCs were CSF1R positive as two adjacent cells (~5%). E) Approximately 22% of Aund/SSCs express this membrane receptor ($p < 0.05$). Scale bar = 10 μm.

granular aspect (Fig. 2, A8). All these differentiated spermatogonia were not labeled for GFRA1 or CSF1R (Fig. 2).

The morphometric analysis (Fig. 2B, black line) indicated that A_{und}/SSCs exhibited the largest nuclear volume, whereas A_{dif} spermatogonia presented almost half of the A_{und}/SSCs nuclear volume which gradually reduced until they became type B spermatogonia. On the other hand, it can be observed that the amount of heterochromatin increased from A_{und}/SSCs to type B spermatogonia (Fig. 2B, red line).

3.2. GFRA1 and CSF1R immunostaining

By means of immunohistochemistry, expression of both receptors GFRA1 (Fig. 3) and CSF1R (Fig. 4) in A_{und}/SSCs were observed exclusively in the membrane/cytoplasm of these cells. In a two-dimensional evaluation, GFRA1-positive spermatogonial cells could be observed as one cell (~75%), two adjacent cells (~20%) and more than two adjacent cells (~5%) (Fig. 3A-D). Quantitative analyses showed that nearly 55% of A_{und}/SSCs expressed GFRA1 protein (Fig. 3E). The CSF1R analysis demonstrated that the majority (~95%) of positive A_{und}/SSCs were present as one cell in a two-dimensional analysis (Fig. 4A-D). In contrast to the data found for GFRA1, only 22% of these cells were positive for CSF1R (Fig. 4E).

In order to better visualize these markers expression and distribution, a qualitative double-labeling immunofluorescence was performed involving both proteins (Fig. 5). In this way, we observed that not all GFRA1-positive cells were positive for CSF1R. Differently, all CSF1R-positive cells were positive for GFRA1. These findings suggest a heterogeneity of protein expression among A_{und}/SSCs (Fig. 5, B3 and C3). Fig. 5D illustrates the different subtypes of A_{und}/SSCs immunolabeled or not for these two markers evaluated in Scorpion mud turtle testes.

3.3. Spermatogonial cell distribution

Taking the five seminiferous tubules regions into account and based on histomorphological features (Fig. 1), it was found that the Scorpion mud turtle A_{und}/SSCs positive for GFRA1 and CSF1R were predominantly located in areas where seminiferous tubule faced the interstitial compartment containing blood vessels (T-BV) ($p < 0.05$; Figs. 6 and 7A, B). Interestingly, GFRA1 positive cells were also observed in high frequency in areas facing the lymphatic space (statistically similar to the previous distribution) (Fig. 6A, C). The frequency of GFRA1+ and CSF1R+ A_{und}/SSCs was significantly reduced in areas facing other seminiferous tubules (T-T) (Figs. 6 and 7A, D) or the interstitial compartment containing Leydig cells (T-LC) (Figs. 6 and 7A, E) and connective tissue (T-CT) (Figs. 6 and 7A, F).

4. Discussion

In comparison to the former report from Sousa et al. (2014), the present study complemented the characterization of *K. scorpioides* A_{und}/

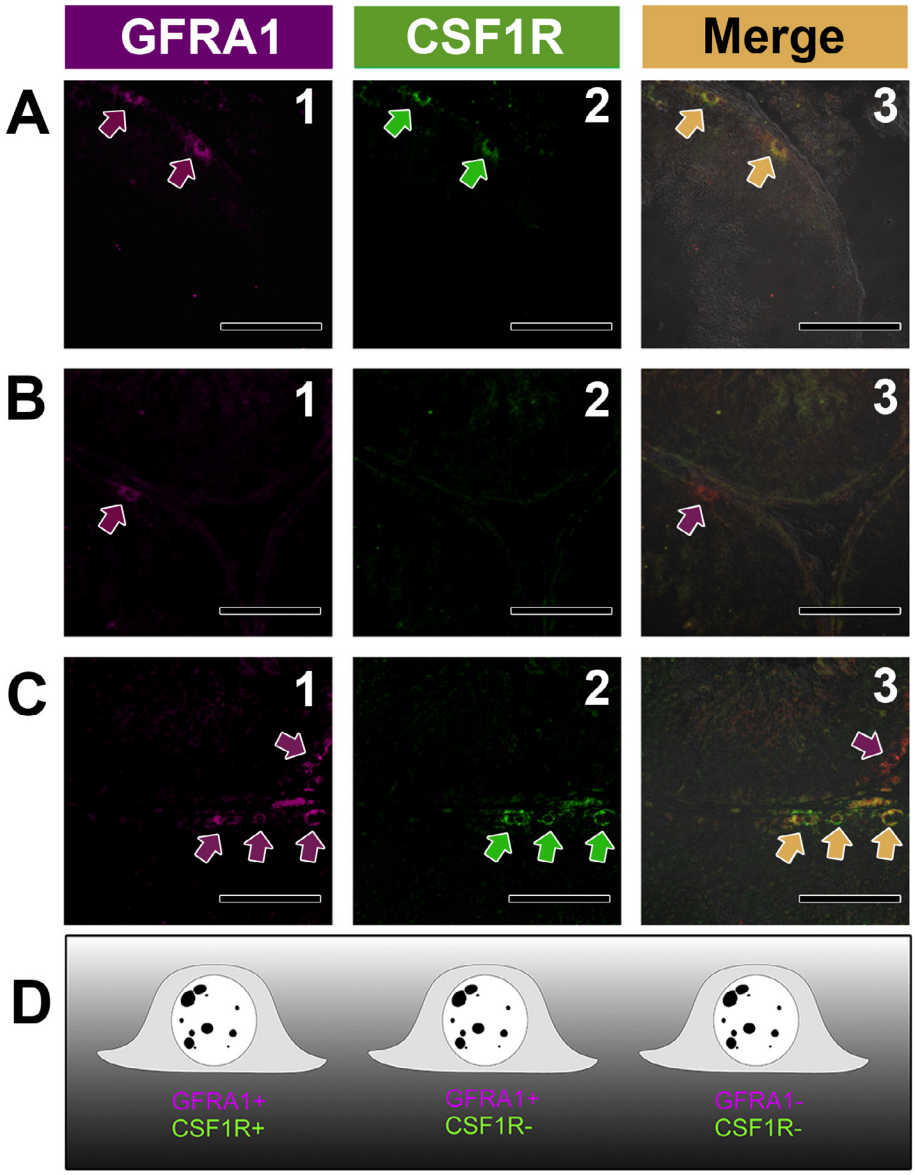

Fig. 5. Co-localization of SSCs markers in the *K. scorpioides* turtle. As illustrated in the merged figure (A3 and C3; orange arrows), GFRA1(+) cells (A1 and C1; purple arrows) can also express CSF1R (A2 and C2; green arrows). However, some GFRA1(+) cells (B1 and C1; purple arrow) do not show the CSF1R marker (B2 and C2). D) Schematic illustration of the presence of GFRA1 and CSF1R in the undifferentiated type A spermatogonia (A_{und}/SSCs). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SSCs. In this regard, their different morphologies, the phenotypic expression of classic molecular markers and their preferential location in the seminiferous epithelium basal compartment were described. Besides providing important data about spermatogonial physiology in reptiles, the present study might bring new clues and contributions to improve our understanding about how early in evolution regulatory features of A_{und}/SSCs are originated and passed on.

Distinct morphologies related or not to nuclear and cytoplasmic vacuoles were evident and described for Scorpion mud turtle A_{und}/SSCs. As observed in mammals (Neuhaus et al., 2017; De Rooij, 2017), these aforementioned characteristics seen among A_{und}/SSCs reinforce recent findings of functional heterogeneity in this particular group of cells. Therefore, the presence of nuclear vacuoles was recently demonstrated in primates (Caldeira-Brant et al., 2018; von Kopylow et al., 2012) and it is speculated that these structures may be involved in RNA processing of long cycling spermatogonia. Lately, different nuclear morphology of A_{und}/SSCs has been associated with stages of cell cycle (Hermann et al., 2010; von Kopylow et al., 2012). In primates, A_{dark} and at least some A_{pale} have been considered as the same cell population but in different stages of the cell cycle (i.e., A_{dark}: G0; A_{pale}: G1/S/G2/M) (Hermann et al., 2010, von Kopylow et al., 2012). Future analysis of the cell cycle might shed light on the relevance of these morphological differences as well as the presence of vacuoles for the turtle SSCs physiology.

In general, the nuclear morphology herein described (i.e., presenting a large volume with reduced quantity of heterochromatin) is similar to that found in the mammalian species already investigated, such as mice, cats, peccaries, donkeys and stallions (Chiarini-Garcia and Russell, 2001; Chiarini-Garcia et al., 2009; Campos-Junior et al., 2012; Costa et al., 2012; Powell et al., 2016). Because *K. scorpioides* A_{und}/SSCs present the largest nuclear volume in comparison to other spermatogonial cell types, specific cell fraction isolation/enrichment techniques based on cell volume and density might be easier to implement for this species; which could provide additional and useful genetic and molecular information about these cells. In relation to differentiated spermatogonia, although we have identified and characterized A_{dif},

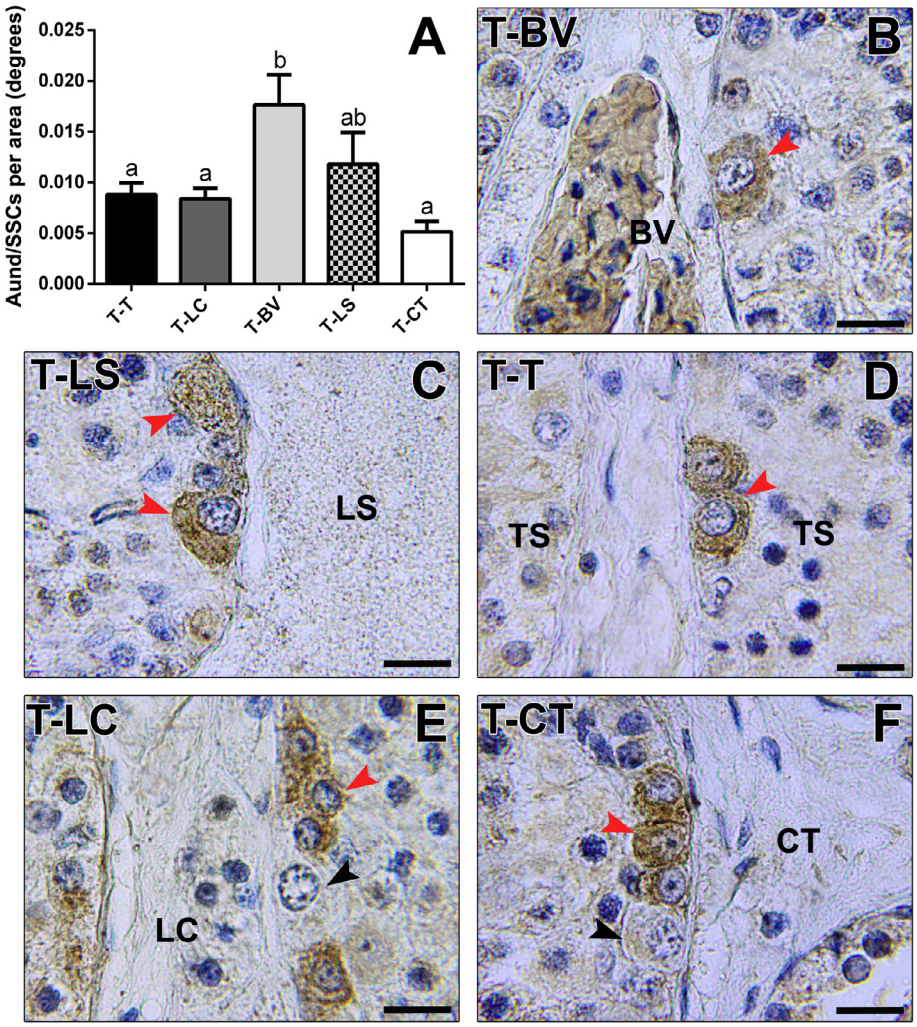

Fig. 6. Distribution of GFRA1 positive type A undifferentiated spermatogonia (A_{und}/SSCs) in the seminiferous tubule cross-sections of *K. scorpioides* turtle. GFRA1 positive cells (red arrowhead) were observed in the five characterized regions of the seminiferous tubules. However, significantly ($p < 0.05$) higher frequency was observed in the region near the blood vessels (A and B, T-BV), and a similar trend was noted for the region in close to the lymphatic space (A and C, T-LS). Lower frequency was found for the A_{und}/SSCs located in the seminiferous tubules areas facing Leydig cells (LC) and connective tissue (CT) (A and D-F). Black arrowhead indicate GFRA1 negative A_{und}/SSCs. T-BV, tubular area near the blood vessels (BV); T-LC, tubular region facing Leydig cells (LC); T-LS, tubular area close to lymphatic space (LS); T-T, tubule-tubule contact (ST); and T-CT, tubular contact with connective tissue (CT). Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intermediate and type B spermatogonial cells, their kinetics and number of generations were not evaluated. In order to better understand the spermatogonial cell physiology, this important aspect surely deserves further investigation.

GFRA1 is considered one of the most important markers of A_{und}/SSCs (Hofmann et al., 2005). This membrane receptor seems to be very well conserved during evolution, being expressed in A_{und}/SSCs of several other species, including fish and mammals (Lacerda et al., 2014, Bellaiche et al., 2014; de Rooij, 2017). In the present study, expression of this protein in A_{und}/SSCs of a reptilian species was for the first time observed. Additionally, similar to other mammals (ex: horses, Costa et al., 2012), following two-dimensional analyses and considering the internuclear distance among A_{und}/SSCs, GFRA1 was observed in one, two adjacent and more than two adjacent spermatogonial cells. However, in order to confirm if these cells could represent the A_{single}, A_{pair}, and $A_{aligned}$ spermatogonial cells, future experiments using whole mounts techniques (Clermont and Bustos-Obregon, 1968) are necessary. Interestingly, although most of A_{und}/SSCs (approximately 55%) express GFRA1 in *K. scorpioides* testis, this number is not as high as those described for some investigated mammalian species, e.g., mice (considering only A_s and A_{pr}; Grisanti et al., 2009), stallions (Costa et al., 2012) and peccaries (Campos-Junior et al., 2012). Considering this substantial percentage difference, the data herein obtained might suggest a different model of self-renewal regulation in reptilian species.

As also observed in mice and stallions (Kokkinaki et al., 2009; Oatley et al., 2009; Costa et al., 2012), the present study reported that only one-third of A_{und}/SSCs express the receptor for colony stimulating factor 1. In the Scorpion mud turtle testis, CSF1R expression was detected mainly in isolated spermatogonial cells according to two-dimensional analysis. Even not using whole mounts samples, the A_{und}/SSCs CSF1R labeling pattern was considerably different from that observed for GFRA1. This data is in accordance with results found for rodents, in which CSF1R expression was observed in a subset of undifferentiated spermatogonial cell population with high self-renewal potential *in vitro* (Oatley et al., 2009; DeFalco et al., 2015). Considering that some GFRA1-positive cells were negative for CSF1R, our data suggest that CSF1R is indeed expressed in a restricted subpopulation of GFRA1 positive A_{single} spermatogonia. According to recent assumptions regarding the hierarchical A_s model (de Rooij, 2017), it can be speculated that CSF1R positive spermatogonia might present higher self-renewal capacity than those expressing only GFRA1. Further studies evaluating the expression of ID4 (Oatley et al., 2011; Chan et al., 2014), PAX7 (Aloisio et al., 2014) and Bmil (Komai et al., 2014) can indicate if CSF1R positive A_{und}/SSCs belong to ultimate or transit amplifying spermatogonia. Moreover, given that colony stimulating factor 1 is secreted by Leydig and peritubular myoid cells in mice and peccaries (Oatley et al., 2009; Campos-Junior et al., 2012), it would be worthy investigating a possible role of these testis somatic cells in the regulation of A_{und}/SSCs in *K. scorpioides*.

Regarding the SSC niche, A_{und}/SSCs population in murine models was observed to be preferentially located on tubule areas near to the interstitial area (Chiarini-Garcia et al., 2001); particularly close to the blood vessels (Yoshida et al., 2007). This preferential location was also demonstrated in other mammalian species, such as peccaries (Campos-

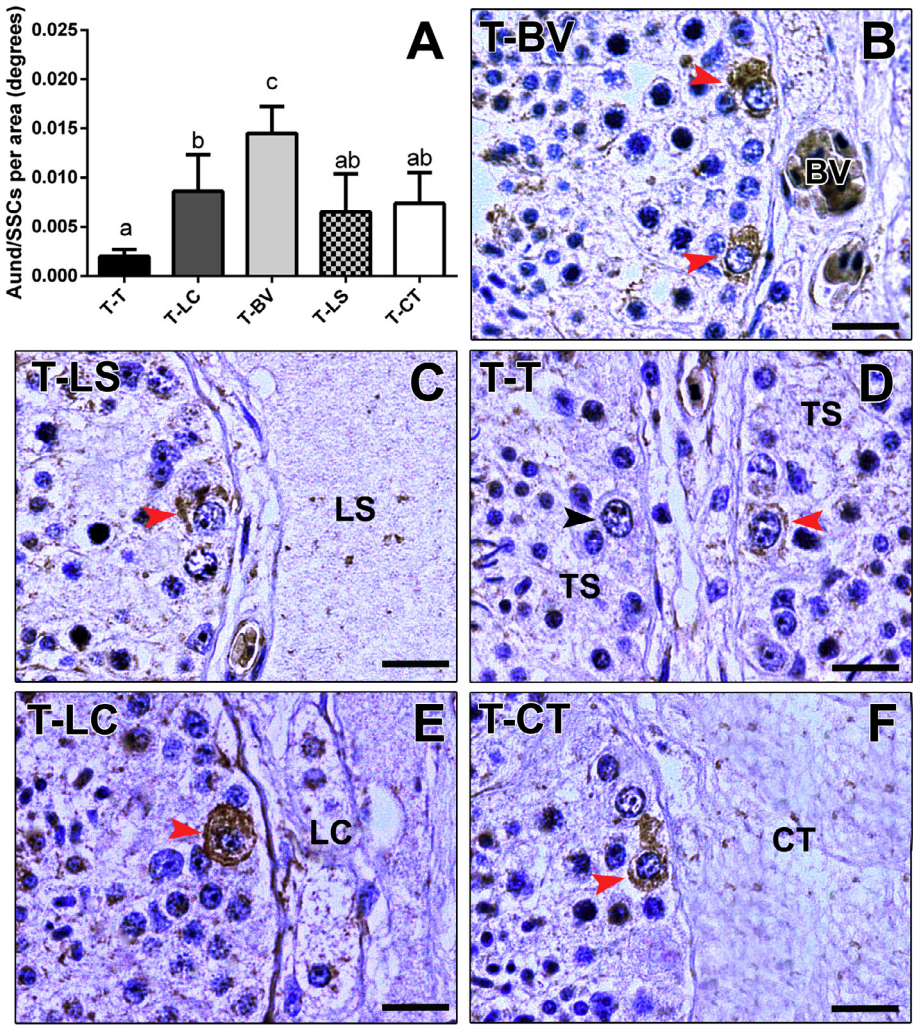

Fig. 7. Distribution of CSF1R positive type A undifferentiated spermatogonia (A_{und}/SSCs) in the seminiferous tubule cross-sections of *K. scorpioides*. CSF1R positive cells (red arrowhead) were observed in all five seminiferous tubules characterized and evaluated regions. Nevertheless, significantly ($p < 0.05$) higher frequency was observed in the region facing the blood vessels (A and B, T-BV). The lowest frequency was found for the A_{und}/SSCs located in the seminiferous tubules areas facing another seminiferous tubule ($p > 0.05$) (A and D, T-T). The regions in contact to the lymphatic space (LS), Leydig cells (LC) and connective tissue (CT) exhibited an intermediate frequency of CSF1R positive A_{und}/SSCs (A, C, E and F). Black arrowhead indicate CSF1R negative A_{und}/SSCs. T-BV, tubular area nearby blood vessels (BV); T-LC, tubular region facing Leydig cells (LC); T-LS, tubular area in association with lymphatic space (LS); T-T, tubule-tubule contact (ST); and T-CT, tubular contact with connective tissue (CT). Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Junior et al., 2012), equids (Costa et al., 2012) and marmosets (Caldeira-Brant et al., 2018). Herein and for the first time, we demonstrated that this distribution pattern is also present in a reptilian species. In this regard, most of the A_{und}/SSCs (GFRA1 or CSF1R positive cells) were also located in areas adjacent to the interstitial compartment containing blood vessels. Interestingly, though in a lesser degree than those observed near blood vessels, GFRA1 positive cells in *K. scorpioides* were present in a high frequency in areas adjacent to the lymphatic spaces/vessels. Considering this cell distribution, it can be speculated that GFRA1 positive cells correspond to spermatogonia cells that are leaving the SSC niche and therefore are more proned to differentiation in comparison to CSF1R positive cells (de Rooij, 2017). Furthermore, the restricted distribution of CSF1R positive cells could suggest that these cells are more primitive than the GFRA1 positive cells (de Rooij and van Beek, 2013).

In relation to the high-volume density of lymphatic space/vessels in the Scorpion mud turtle testis (Sousa et al., 2014), this interstitial component could be independently evaluated and considered, for the first time, as an important player in the spermatogonial cell distribution or niche. On the other hand, lower prevalence of A_{und}/SSCs occurred in areas adjacent to another seminiferous tubule and in regions facing the interstitial compartment containing connective tissue or Leydig cells. These observations are also in agreement with the data obtained in peccaries by Campos-Junior et al. (2012). Therefore, based on these aforementioned data, A_{und}/SSCs distribution in *K. scorpioides* are quite similar to that already described for mammals, suggesting that the A_{und}/SSCs niche presents a well conserved feature during evolution.

In conclusion, to our knowledge, the present study is the first to perform a detailed characterization of spermatogonial cells in *K. scorpioides*, particularly in relation to A_{und}/SSCs morphology and biology. Corroborating the existing data about A_{und}/SSCs niche in other vertebrate species, the presence of this complex and specific microenvironment in a reptilian species was herein also demonstrated. Furthermore, our results suggest that the niche presents peculiar features that are well conserved during evolution. In our understanding, the phenotypical (morphological and molecular) characterization of A_{und}/SSCs in *K. scorpioides* may facilitate their isolation/enrichment, allowing the development of important biotechnological tools useful for the conservation of this species, such as SSCs cryopreservation, culture and transplantation. In order to better understand the A_{und}/SSCs physiology/biology, we expect that the present study will stimulate similar investigations in other reptilian species, allowing the development of comparative reproductive biology studies in vertebrates.

Declaration of interest

There is no conflict of interest in relation to the study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.06.019.

References

Aloisio, G.M., Nakada, Y., Saatcioglu, H.D., Peña, C.G., Baker, M.D., Tarnawa, E.D., Mukherjee, J., Manjunath, H., Bugde, A., Sengupta, A.L., Amatruda, J.F., 2014. PAX7 expression defines germline stem cells in the adult testis. J. Clin. Invest. 124, 3929–3944.

Bellaiche, J., Lareyre, J.J., Cauty, C., Yano, A., Allemand, I., Le Gac, F., 2014. Spermatogonial stem cell quest: nanos2, marker of a subpopulation of undifferentiated A spermatogonia in trout testis. Biol. Reprod. 90 (4).

Berry, J.F., Iverson, J.B. 2011. Kinosternon scorpioides (Linnaeus 1766) – Scorpion Mud Turtle, in: Rhodin, A.G.J., Pritchard, P.C.H., van Dijk, P.P., Saumure, R.A., Buhlmann, K.A., Iverson, J.B., and Mittermeier, R.A. (Eds.). Conservation Biology of Freshwater Turtles and Tortoises: A Compilation Project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group. Chelonian Research Monographs, Mandalay, pp. 063.1–063.15.

Buageaw, A., Sukhwani, M., Ben-Yehudah, A., Ehmcke, J., Rawe, V.Y., Pholpramool, C., Orwig, K.E., Schlatt, S., 2005. GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol. Reprod. 73, 1011–1016.

Caldeira-Brant, A.L., Eras-Garcia, L., Alves-Freitas, D., Almeida, F.R.C.L., Chiarini-Garcia, H., 2018. Spermatogonial behavior in marmoset: a new generation, their kinetics and niche. Mol. Hum. Reprod. 24, 299–309.

Campos-Junior, P.H.A., Costa, G.M., Lacerda, S.M., Rezende-Neto, J.V., de Paula, A.M., Hofmann, M.C., França, L.R., 2012b. The spermatogonial stem cell niche in the collared peccary (*Tayassu tajacu*). Biol. Reprod. 86, 155–161.

Campos-Junior, P.H., Costa, G.M., Lacerda, S.M., Rezende-Neto, J.V., de Paula, A.M., Hofmann, M.C., de França, L.R., 2012a. The spermatogonial stem cell niche in the collared peccary (Tayassu tajacu). Biol. Reprod. 86, 1–10.

Campos-Junior, P.H.A., Costa, G.M.J., Avelar, G.F., Lacerda, S.M.S.N., da Costa, N.N., Ohashi, O.M., Miranda, M.S., Barcelos, L.S., Jorge, É.C., Guimarães, D.A., França, L.R., 2014. Derivation of sperm from xenografted testis cells and tissues of the peccary (*Tayassu tajacu*). Reproduction 147, 291–299.

Carvalho, R.C., Oliveira, S.C.R., Bombonato, P.P., Oliveira, A.S., Sousa, A.L., 2010. Morfologia dos órgãos genitais masculino do jurará *(Kinosternon scorpioides*). Pesqui. Vet. Bras. 30, 289–294.

Chan, F., Oatley, M.J., Kaucher, A.V., Yang, Q.E., Bieberich, C.J., Shashikant, C.S., Oatley, J.M., 2014. Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev. 28, 1351–1362.

Chiarini-Garcia, H., Russell, L.D., 2001. High-resolution light microscopic characterization of mouse spermatogonia. Biol. Reprod. 65, 1170–1178.

Chiarini-Garcia, H., Alves-Freitas, D., Barbosa, I.S., Almeida, F.R., 2009. Evaluation of the seminiferous epithelial cycle, spermatogonial kinetics and niche in donkeys (Equus asinus). Anim. Reprod. Sci. 116, 139–154.

Chiarini-Garcia, H., Hornick, J.R., Griswold, M.D., Russell, L.D., 2001. Distribution of type A spermatogonia in the mouse is not random. Biol. Reprod. 65, 1179–1185.

Chiarini-Garcia, H., Raymer, A.M., Russell, L.D., 2003. Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126, 669–680.

Clermont, Y., Bustos-Obregon, E., 1968. Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "*in toto*". Dev. Dyn. 122, 237–247.

Costa, G.M., Avelar, G.F., Rezende-Neto, J.V., Campos-Junior, P.H.A., Lacerda, S.M., Andrade, B.S., Thomé, R.G., Hofmann, M.C., França, L.R., 2012. Spermatogonial stem cell markers and niche in equids. PloS one 7 (8), e44091.

da Costa Araújo, J., e Rosa, P.V., Palha, M.D.D.C., Rodrigues, P.B., de Freitas, R.T.F., da Silva, A.D.S.L., 2013. Effect of three feeding management systems on some reproductive parameters of Scorpion mud turtles (*Kinosternon scorpioides*) in Brazil. Trop. Anim. Health Prod. 45, 729–735.

de Rooij, D.G., 1973. Spermatogonial stem cell renewal in the mouse. I. Normal situation. Cell Tissue Kinet. 6, 281–287.

de Rooij, D.G., 2017. The nature and dynamics of spermatogonial stem cells. Development 144, 3022–3030.

de Rooij, D.G., van Beek, M.E., 2013. Computer simulation of the rodent spermatogonial stem cell niche. Biol. Reprod. 88, 131–141.

DeFalco, T., Potter, S.J., Williams, A.V., Waller, B., Kan, M.J., Capel, B., 2015. Macrophages contribute to the spermatogonial niche in the adult testis. Cell Rep. 12, 1107–1119.

Garbuzov, A., Pech, M.F., Hasegawa, K., Sukhwani, M., Zhang, R.J., Orwig, K.E., Artandi, S.E., 2018. Purification of GFRα1+ and GFRα1–spermatogonial stem cells reveals a niche-dependent mechanism for fate determination. Stem Cell Reports.

Grisanti, L., Falciatori, I., Grasso, M., Dovere, L., Fera, S., Muciaccia, B., Fuso, A., Berno, V., Boitani, C., Stefanini, M., Vicini, E., 2009. Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27, 3043–3052.

Hara, K., Nakagawa, T., Enomoto, H., Suzuki, M., Yamamoto, M., Simons, B.D., Yoshida, S., 2014. Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14, 658–672.

Helsel, A.R., Yang, Q.E., Oatley, M.J., Lord, T., Sablitzky, F., Oatley, J.M., 2017. ID4 levels dictate the stem cell state in mouse spermatogonia. Development 144, 624–634.

Hermann, B.P., Sukhwani, M., Hansel, M.C., Orwig, K.E., 2010. Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction 139, 479–493.

Hofmann, M.C., 2008. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. Mol. Cell Endocrinol. 288, 95–103.

Hofmann, M.C., Braydich-Stolle, L., Dym, M., 2005. Isolation of male germ-line stem cells; influence of GDNF. Dev. Biol. 279, 114–124.

Huckins, C., 1971. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat. Rec. 169, 533–557.

Kokkinaki, M., Lee, T.L., He, Z., Jiang, J., Golestaneh, N., Hofmann, M.C., Chan, W.Y., Dym, M., 2009. The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. Biol. Reprod. 80, 707–717.

Komai, Y., Tanaka, T., Tokuyama, Y., Yanai, H., Ohe, S., Omachi, T., Atsumi, N., Yoshida, N., Kumano, K., Hisha, H., Matsuda, T., 2014. Bmi1 expression in long-term germ stem cells. Sci. Rep. 4, 6175.

Lacerda, S.M.D.S.N., Costa, G.M.J., de França, L.R., 2014. Biology and identity of fish spermatogonial stem cell. Gen. Comp. Endocrinol. 207, 56–65.

Mecklenburg, J.M., Hermann, B.P., 2016. Mechanisms regulating spermatogonial differentiation. In: Piprek, R.P. (Ed.), Molecular Mechanisms of Cell Differentiation in Gonad Development. Springer, Cham, pp. 253–287.

Meng, X., Lindahl, M., Hyvonen, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J.G., Westphal, H., Saarma, M., Sariola, H., 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287, 1489–1493.

Mutoji, K.N., Hermann, B.P., 2017. Defining the phenotype and function of mammalian spermatogonial stem cells. In: Oatley, J.M., Griswold, M.D. (Eds.), The Biology of Mammalian Spermatogonia. Springer, New York, pp. 67–90.

Nakagawa, T., Nabeshima, Y.I., Yoshida, S., 2007. Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev. Cell 12, 195–206.

Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R.E., Yoshida, S., 2010. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328, 62–67.

Neuhaus, N., Yoon, J., Terwort, N., Kliesch, S., Seggewiss, J., Huge, A., Voss, R., Schlatt, S., Grindberg, R.V., Schöler, H.R., 2017. Single-cell gene expression analysis reveals diversity among human spermatogonia. Mol. Hum. Reprod. 23, 79–90.

Oatley, J.M., Brinster, R.L., 2008. Regulation of spermatogonial stem cell self-renewal in mammals. Annu. Rev. Cell Dev. Biol. 24, 263–286.

Oatley, J.M., Avarbock, M.R., Brinster, R.L., 2007. Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J. Biol. Chem. 282, 25842–25851.

Oatley, M.J., Kaucher, A.V., Racicot, K.E., Oatley, J.M., 2011. Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. Biol. Reprod. 85, 347–356.

Oatley, J.M., Oatley, M.J., Avarbock, M.R., Tobias, J.W., Brinster, R.L., 2009. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136, 1191–1199.

Pereira, L.A., Sousa, A.L., Lemos, J.J.S., 2007. Extrativismo de jurará Kinosternon scorpioides Linnaeus, 1766 (Reptila, Chelonia, Kinosternidae) e avaliação sócio-ambiental dos pescadores no Município de São Bento-MA. In: Silva, A.C., Fortes, J.L.O. (Eds.), Diversidade Biológica Uso e Conservação de Recursos Naturais no Maranhão. Projeto e Ações em Biologia e Química, São Luiz, pp. 269-299.

Powell, R.H., Galiguis, J., Biancardi, M.N., Pope, C.E., Leibo, S.P., Wang, G., Gómez, M.C., 2016. Phenotypic and molecular characterization of domestic cat (Felis catus) spermatogonial stem cells. Biol. Reprod. 95, 20–21.

Ryan, G.R., Dai, X.M., Dominguez, M.G., Tong, W., Chuan, F., Chisholm, O., Russell, R.G., Pollard, J.W., Stanley, E.R., 2001. Rescue of the colony stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1(op)/Csf1(op)) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. Blood 98, 74–84.

Simon, L., Ekman, G.C., Tyagi, G., Hess, R.A., Murphy, K.M., Cooke, P.S., 2007. Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. Exp. Cell. Res. 313, 3090–3099.

Sousa, A.L., Campos-Junior, P.H.A., Costa, G.M.J., França, L.R., 2014. Spermatogenic cycle length and sperm production in the freshwater turtle *Kinosternon scorpioides*. Biol. Reprod. 90 (2).

Vanzolini, P.E., Ramos-Costa, A.M.M., Vitt, L.J., 1980. Répteis das caatingas. Academia Brasileira de Ciências, Rio de Janeiro, pp. 161.

Von Kopylow, K., Staege, H., Spiess, A.N., Schulze, W., Will, H., Primig, M., Kirchhoff, C., 2012. Differential marker protein expression specifies rarefaction zone-containing human Adark spermatogonia. Reproduction 143, 45–57.

Yoshida, S., Sukeno, M., Nabeshima, Y.I., 2007. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317 (2012), 1722–1726.