Phenotypic characterization and in vitro propagation and transplantation of the Nile tilapia (Oreochromis niloticus) spermatogonial stem cells

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Objective: In vertebrates, spermatogonial stem cells (SSCs) play a key role in spermatogenesis, and thus are valuable for the study of spermatogonial physiology. However, in fish, the lack of molecular markers for SSPs limits their identification and purification, making it difficult to apply several biotechnologies in aquaculture. This study aimed to identify potential molecular markers that can be used to phenotypically characterize, culture, and transplant Nile tilapia spermatogonial stem cells.

Methods: Immunolocalization was used to identify potential molecular markers for spermatogonial stem cells (SSPs) in Nile tilapia. The expression of these markers was then used to establish primary culture conditions that allow for the expansion and transplantation of spermatogonial stem cells in vitro.

Results: Immunolocalization revealed that Gfra1 is expressed exclusively in single type A undifferentiated spermatogonia (Aund), presumptive SSPs. Likewise, Nanos2 expression was observed in Aund cells. However, Nanos2-positive spermatogonia were also identified in cysts with two to eight germ cells that encompass type A differentiated spermatogonia (Adiff). Additionally, we established effective primary culture conditions that allowed the Nile tilapia spermatogonia to expand their population for at least one month while conserving their original undifferentiated (stemness) characteristics. The maintenance of Aund spermatogonial phenotype was demonstrated by the expression of early germ cell specific markers and, more convincingly, by their ability to colonize and develop in the busulfan-treated adult Nile tilapia recipient testes after germ cell transplantation. These findings provide the first step in establishing a system that will allow SSPs expansion in vitro, representing an important progress towards the development of new biotechnologies in aquaculture, including the possibility of producing transgenic fish.

Key words: Nile tilapia (Oreochromis niloticus), Spermatogonial stem cells, Gfra1, Nanos2, Spermatogonial transplantation

1. Introduction

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and crucial for transmitting the genetic information to the next generation. In addition, SSCs are unique stem cells that can also be reprogrammed into pluripotent cells with the ability to differentiate into somatic tissues (Conrad et al., 2008; Oatley and Brinster, 2012; Thoma et al., 2011), rendering SSCs into a potential substitute for embryonic stem cells (ES). Moreover, in combination with transplantation techniques, SSCs could be powerful vectors for modified genes (Honaramooz and Yang, 2010).

In mammals, several molecular markers (cell surface markers, RNA-binding proteins, zinc finger proteins, cytokines, cell cycle proteins and others) have been described in order to identify SSCs and their early progeny (Reviewed by Kolasa et al., 2012 and Phillips et al., 2010). Although most molecular mechanisms controlling the onset of spermatogenesis have remained elusive, the roles of some components of these pathways are now being elucidated. For instance, in mice the growth factor GDNF (glial cell-line derived neurotrophic factor) signal emanated from Sertoli cells and the germ cell-intrinsic factor Nanos2 represent key regulators for the maintenance and modulation of SSCs self-renewal (Hofmann 2008; Sada et al., 2009; Suzuki et al., 2009). GDNF acts in the testis through a membrane receptor complex formed by the GDNF family receptor alpha 1 (GFRA1) and the receptor tyrosine kinase RET, both expressed in SSCs of different mammalian species (Gassey et al., 2009, 2010; He et al., 2010; Hofmann et al., 2005), including peccaries (Campos-Junior et al., 2012) and equids (Costa et al., 2012) in studies developed in our laboratory. GFRA1 and RET mutant mice have shown a similar spermatogenic phenotype to that
of the GDNF-null mice, in which SSCs cannot maintain an undifferen-
tiated state that results in germ cell depletion (Buageaw et al.,
2005; Jijiwa et al., 2008; Naughton et al., 2006; Tokuda et al.,
2007). Nanos genes encode evolutionarily conserved zinc-finger
RNA-binding proteins that play important roles for germline stem
cell function (Draper et al., 2007; Jaruzelska et al., 2003; Mochizuki
et al., 2000; Wang and Lin 2004). In adult mouse testes, a hetero-
genreality is clearly observed in the undifferentiated spermatogonia
population and it has been proposed that Gfra1”Nanos2” sperma-
togonia represents the stem cell population (Sada et al., 2012; Su-
zuki et al., 2009). Recently, it was demonstrated that GDFN
signaling is essential to maintain Nanos2 expression in murine
undifferentiated spermatogonia and that overexpression of Nanos2
can alleviate the stem cell loss phenotype caused by the depletion of
the Gfra1 gene. Therefore, Nanos2 is a possible target of GDNF
signaling to suppress differentiation of mice SSCs (Sada et al.,
2012).

Except for some investigations in which early spermatogonial
cells have been more accurately characterized by morphological
criteria (Lacerda et al., 2013; Nóbrega et al., 2010; Schulz et al.,
2010), in teleosts few studies have addressed a more comprehen-
sive phenotypic characterization of undifferentiated spermatogonia.
More specifically, microarray analysis of enriched rainbow trout
(Oncorhynchus mykiss) type A spermatogonia showed that the
receptor notch1 would be a useful molecular marker for this cells (Yano et al., 2009). Also, the lymphocyte antigen 75 (Ly75/
Gfra1) receptor notch1 would be a useful molecular marker for this
putative SSCs in fish (Lacerda et al., 2012, 2013; Okutsu et al.,
2013). Recently, the transcripts showing preferential expression in
rainbow trout type A spermatogonia were isolated by microarray anal-
ysis (Hayashi et al., 2012).

As in mammals, to date germ cell transplantation represents the
only functional bioassay to retrospectively test the stemness of
putative SSCs in fish (Lacerda et al., 2012, 2013; Okutsu et al.,
2006; Takeuchi et al., 2004; Yoshizaki et al., 2012). Therefore, the
appropriate identification and isolation of fish SSCs is essential
not only for a high efficiency of spermatogonial transplantation
techniques, but also for the establishment of effective SSC culture
systems that would offer a better understanding of the prolifera-
tion, self-renewal and differentiation behavior of these very
important cells, as well as to develop valuable reproductive bio-
technologies in aquaculture. In this regard, aiming to develop
long-term culture conditions for propagating SSCs in vitro, as well
as to test their stemness using germ cell transplantation as a func-
tional approach, we investigated in the present study the expres-
sion of Gfra1 and Nanos2 in the testis of adult Nile tilapia.

2. Material and methods

2.1. Animals

Twenty-five sexually mature male Nile tilapia (Oreochromis nil-
oticus) were used in this study. The fish were obtained from the
commercial aquaculture station 3D Aqua Ltda (Morada Nova de
Minas, MG) located in the Southeast region of Brazil. All exper-
imental procedures used were conducted in accordance with the
guidelines approved by the local ethics committee on animal
experimentation – CETEA, UFMG (protocol # 89/2012).

2.2. SDS–PAGE and Western blotting

To qualitatively evaluate the presence of specific markers for
SSCs (Gfra1 and Nanos2) immunoblots were performed using total
protein lysates from Nile tilapia testes (n = 4). For this evaluation,
300 mg of testis parenchyma were immersed in PBS containing
protease inhibitors (SIGMAFAST® Protease Inhibitor Tablets; Sig-
ma Aldrich’s Corp., St. Louis, MO, USA), and the tissues were sub-
mitted to ultrasonic homogenization. After sonication, the lysates
were centrifuged at 14,000g for 30 min. Supernatants were col-
lected, and then frozen at –80 °C. Protein samples were diluted
to 1:2 in a solution of 10% sodium dodecyl sulfate (SDS, Sigma Al-
drich), glycerol, 10% bromophenol blue in 0.5 M Tris buffer, pH 6.8,
and the samples were boiled for 5 min.

Denatured 12% SDS polyacrylamide mini-gels were prepared
and 20 μL samples were loaded into the wells. Protein molecular
weight markers (Thermo Fisher Scientific Inc.) were run parallel
to the samples. Electrophoresis was carried out at 100 Volts con-
stant voltage and separated proteins were then transferred onto
a 0.45 μm PVDF membrane for 60 min. The membranes were
blocked with 1% bovine serum albumin (BSA, Sigma Aldrich) in
PBS for 1 h at room temperature and then blotted with anti-GDNF
receptor alpha 1 antibody (Abcam, ab84106, 1:500), anti-Nanos2
antibody (Abcam, ab76568, 1:200) overnight at 4 °C. After incuba-
tion, the membranes were washed three times with PBS 0.05% Tween-20 (PBS-T) solution and then incubated in biotinylated goat
anti-rabbit IgG antibody (Abcam, ab97049, 1:500) for 60 min. The
membranes were washed three times in PBS-T and incubated in
streptavidin-peroxidase solution (Thermo Scientific, TS-125-HR)
for 15 min. After washing, proteins were revealed with peroxidase
substrate 3, 3-diaminobenzidine (DAB, Sigma Aldrich), 4-cloro-
naphthol and hydrogen peroxide (Sigma Aldrich) for 5 min and
washed in water. Finally, the membranes were scanned in
Epson Perfection 4990 photo scanner. Molecular weights of Nile
tilapia proteins were determined using SignalP 4.1 Server
(www.cbs.dtu.dk/services/SignalP) and ExPASy/ProtParam tool

2.3. Immunostaining analyses

In order to evaluate the in situ expression of proteins analyzed
by Western blotting, we performed immunostaining using the
immunoperoxidase and immunofluorescence methods. Slides
were analyzed by light (BX-60 Olympus) and confocal (510 META
Laser Scanning Confocal, Zeiss) microscopy. The testicular samples
(n = 6) were fixed in 4% paraformaldehyde in PBS and embedded in
paraplast (Sigma Aldrich). Five micrometer thick serial sections
were obtained and used for the immunoperoxidase and immuno-
fluorescence reactions.

Tissue sections were immunostained using protocols specifi-
cally developed for each antigen and antibody dilutions previously
tested. After dewaxing and rehydration, antigen retrieval was per-
formed in 500 ml of citrate buffer (pH 6.0) for 5 min after boiling in
a microwave oven on high power (~1000 watts). Endogenous per-
oxidase was quenched for 30 min with 3% H2O2 (Vetec) in TBS.
Non-specific binding was blocked with 10% normal goat serum in
PBS for 1 h at room temperature and then blotted with anti-GDNF
receptor alpha 1 (Abcam, ab84106, 1:200) and anti-Nanos2 (Ab-
cam, ab76568, 1:40) were applied to slides and incubated at 4 °C
overnight. Biotinylated anti-rabbit IgG antibody (Abcam, ab97049,
1:200) was applied and incubated for 60 min. Detection
of the signal was performed by incubating the section in streptavi-
din-HRP for 15 min, followed by the reaction with DAB and count-
erstaining with hematoxylin (Merck). After dehydration, sections
were mounted and analyzed. For immunofluorescence, antigens
were detected by incubation with Alexa Fluor® 488 Donkey Anti-
Rabbit IgG (Invitrogen, 1:500) for 1 h at room temperature. After
nuclear counterstaining using propidium iodide (Sigma Aldrich),
sections were mounted with Mowiol 4–88 solution and mounted
and analyzed. For immunofluorescence, antigens
were detected by incubation with Alexa Fluor® 488 Donkey Anti-
Rabbit IgG (Invitrogen, 1:500) for 1 h at room temperature. After
nuclear counterstaining using propidium iodide (Sigma Aldrich),
sections were mounted with Mowiol 4–88 solution and counted

per animal \((n = 4)\). Type A undifferentiated spermatogonia were identified according to the criteria proposed by Schulz et al., (2010). The two-parameter analysis was tested for student’s \(T\) test. The analyzes were performed using the GraphPad Prism (version 5) software. All data were expressed as mean ± SD and the significance level considered was \(p < 0.05\).

2.4. Spermatogonia culture

Testes were removed from adult Nile tilapia \((n = 5)\) and dissociated with collagenase, hyaluronidase and DNase (Sigma Aldrich) in Dulbecco Modified Eagle medium/Ham F-12 medium (DMEM/F12 medium; Gibco, Grand Island, NY, USA), and an enriched type A spermatogonia cell suspension was obtained by percoll gradient centrifugation and differential plating \((\sim 2 \times 10^6 \text{cells/testis})\) according to methods previously established for this species (Lacerda et al., 2006, 2010). The cells were then plated on 1% gelatin-coated dishes (Sigma Aldrich) and incubated at 28 °C in an atmosphere of 5% \(\text{CO}_2\). For evaluation of the characteristics of testis cells in culture, multi-well tissue culture plates were employed in order to provide multiple samples of the same preparation for examination at several times after initiation of culture. Culture cells were analyzed and documented in phase contrast and differential interference contrast (DIC) microscopy. The culture medium was DMEM/F12 containing 4.5 g/L glucose buffered with 10 mM Na2-HCO3 (pH 7.3) supplemented with 10,000 U/L penicillin, 10 mg/L streptomycin, glutamine (2 mM), Na-pyruvate (1 mM), Na-selenite (2 nM), nonessential amino acids solution (1%), 2-mercaptoethanol (100 mM), insulin (25 µg/ml), apo-Transferrin bovine (100 µg/ml), human recombinant basic fibroblast growth factor (10 ng/ml), human recombinant epidermal growth factor (100 ng/ml), fetal bovine serum (10%, Gibco), KnockOut Serum Replacement (1%, Life Technologies, Invitrogen), fish serum (1%) from adult Nile tilapia (Hong and Schartl,1996), and tilapia embryo extract (extract from one embryo/ml; Hong and Schartl,1996; Westerfield, 1995). Supplements were purchased from Sigma Aldrich unless otherwise indicated. The cells were replated using StemPro Accutase (Life Technologies, Invitrogen).

For immunocytochemistry, cells were directly fixed with 4% paraformaldehyde in culture dishes for 20 min. Subsequently, they were incubated for 1 h in a blocking solution containing PBS, 5% BSA, and 0.05% Triton-X-100 and then incubated overnight at 4 °C with primary antibodies: anti-DDX4/Vasa (Abcam, ab13840, 1:400) and anti-Oct4 (Abcam, ab18976, 1:100). Secondary Alexa Fluor488 donkey anti-Rabbit IgG (Invitrogen, 1:500 was used for fluorescent detection. Cell nuclei were counterstained with propidium iodide.

In order to observe spermatogonia proliferation in vitro, after 10 days in culture, the cells were incubated for 30 h with 100 nM of 5-bromo-2′-deoxyuridine (BrdU, Sigma Aldrich) in culture medium. Cells were detached, fixed by 70% methanol, and the pellets were resuspended in nuclease-free water and used for cDNA synthesis. RNA concentrations and purity were measured by spectrophotometry (Nanodrop 1000, Pqlab). Total RNA isolated from mature Nile tilapia testes \((n = 2)\) were used as reference for the gene expression pattern. cDNA was synthesized with oligo (dt) primer from total RNA by using M-MuLV Reverse Transcriptase (Thermo Scientific).

Polymerase chain reaction amplification was performed with the primers under the following conditions: 94 °C for 1 min, annealing temperature for 1 min, and 72 °C for 1 min completing a total of 30 cycles. The amount of cDNA per PCR reaction corresponded to 100 ng of total RNA. Constitutively expressed 18S rRNA was used as positive control. For negative control, reactions contained PCR components and specific primers but lacked the cDNA template. Oligonucleotide primers that have been used are listed in Table 1.

2.6. Spermatogonial transplantation

The cultured spermatogonia were maintained for different periods of time and after 30 days in culture were transplanted to the busulfan-treated Nile tilapia recipient testes \((n = 4)\) (Lacerda et al., 2006, 2010). Before transplantation into the testes of sexually mature tilapia, spermatogonia cultivated for 30 days were isolated using StemPro Accutase and then incubated with the fluorescent membrane dye PKH26-Gl (Sigma, St. Louis, MO), which served to identify transplanted cells in the recipient testes. Thus, three weeks after the first busulfan injection, recipient tilapias received the cultivated donor spermatogonia through the common spermatocyst duct according to techniques previously established (Lacerda et al., 2006, 2010). Following the germ cell transplantation, the recipient tilapia was maintained for 3–4 weeks, and their testes were then analyzed by confocal microscopy. Confocal images were obtained using a 20x objective and 488 nm Argon laser. The ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/) software was used for the image analysis. Laser power and acquisition settings were adjusted to produce submaximal pixel values in the testis tissue. Background subtraction and contrast/brightness enhancement (up to 20% enhancement using the maximum slider in Image J) were identically performed for all the images in the same experiment.

3. Results

3.1. Expression of Gfra1 in the adult Nile tilapia testis

To study Gfra1 expression we used a commercial antibody developed against human GFRα1 peptide sequence that shows similarities with predicted Nile tilapia Gdnf receptor alpha 1 protein (GenBank: XP_003438304.1). The human and Nile tilapia Gfra1 amino acids sequence alignment is presented in Supplementary Fig. 1. In all fish investigated here, immunoreactivity of anti-GFRα1, evaluated in histological serial sections, was found exclusively in type A differentiated spermatogonia, which are large single cells showing a large nucleus and a prominent nucleolus (Fig. 1A–I). As it can be also evidenced in Fig. 1, early cysts of 2 to 8 spermatogonial cells, comprising type A differentiated spermatogonia \((A_{\text{diff}})\), did not show any evident labeling for Gfra1 \((\text{Fig. 1G–I})\). More advanced germ cells such as type B spermatogonia, spermatocytes, spermatids and spermatooza, as well as testicular somatic cells

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including Sertoli and Leydig cells likewise did not show immunoreactivity. Immunofluorescent analysis of dissociated testicular cells clearly demonstrated the expected membrane-associated labeling of Gfra1 in the Nile tilapia Aund spermatogonia (Fig. 1J–L), whereas other smaller testicular cells showed no staining (Fig. 1J′ and K′). The specificity of the GFRAl antibody to recognize Nile tilapia Gfra1 protein was demonstrated by Western blot analysis using total lysates from adult testes. Therefore, the samples containing testicular protein showed a predominant band at the expected molecular weight (≈50 kDa) of predicted Nile tilapia Gfra1 protein (SignalP 4.1 Server and ExPaSy/ProtParam) (Fig. 1M and Supplementary Fig. 2). Quantitative analysis using morphological criteria and Gfra1 immunolabeling showed that 96 ± 1.6% (p < 0.05) of Aund spermatogonia in the adult Nile tilapia testes were Gfra1-positive (Fig. 1N).

Analysis of transverse sections from the Nile tilapia testis submitted to immunofluorescence and immunoperoxidase reactions allowed us to observe that a high density of Gfra1-positive spermatogonial cells were present at the distal regions of the seminiferous tubules, near to the tunica albuginea (Fig. 10–P). Although at lower magnification these spermatogonial cells appeared as a group of cells, in fact they were single cells individually enveloped by Sertoli cells (Fig. 10–P′). Serial section images of distal regions of seminiferous tubules showing detailed single Aund spermatogonia enveloped by Sertoli cells are presented in Supplementary Fig. 3.

3.2. Expression of Nanos2 in the adult Nile tilapia testes

To evaluate the Nanos2 protein distribution in the adult tilapia testes we performed immunofluorescence and immunoperoxidase analysis using a commercial antibody developed against an immunogenic sequence of human Nanos homolog 2 protein. Sequence alignment of human and predicted Nile tilapia Nanos2 protein (GenBank: XP_003439364.1) is shown in Supplementary Fig. 4. In all fish investigated, using histological serial sections, immunoreactivity for anti-Nanos2 was frequently observed in clusters of single type Aund spermatogonia located at the distal regions of the seminiferous tubules, near to the tunica albuginea (Fig. 2A–C). Nanos2 expression was also found in single Aund cells situated along the seminiferous tubules, set aside a certain distance from the tunica albuginea (Fig. 2D–E). Besides that, we could also observe Nanos2 positive spermatogonia along the tubules in cysts with germ cell clones of two (Fig. 2F), four (Fig. 2G) and eight spermatogonial cells (Fig. 2H), but not in larger cysts. As it can be also observed in Fig. 2 no evident labeling was found in more advanced germ cells such as type B spermatogonia, spermatocytes, spermatids and spermatozoa, or in somatic cells such as Sertoli and Leydig cells. Using the Nanos2 antibody, Western blot analysis of Nile tilapia testes lysates showed a predominant band of approximately 18 kDa, which corresponds to the expected size of this protein previously determined (SignalP 4.1,ProtParam) (Fig. 2) and Supplementary Fig. 2). Quantitative analysis using morphological criteria and Nanos2 immunolabeling indicated that 94.5 ± 1.5% (p < 0.05) of single Aund spermatogonia in the adult tilapia testis were positive for Nanos2 (Fig. 2K).

3.3. Nile tilapia spermatogonia culture

Cells were harvested from adult tilapia testes and spermatogonia were enriched from dissociated testicular cells suspension using percoll gradient and differential plating (Lacerda et al., 2010). After 12 h in culture, the non-adherent cells, which usually form clumps (Fig. 3A), were seeded in gelatin-coated tissue culture plates in the specific culture medium. Until 2 days in culture, these cells were found to only barely attach to the gelatin-coated dish. However, increased cell attachment was observed at day 3 (Fig. 3B), and most of the attached cells displayed strong staining for DDX4/Vasa (Fig. 3C), a RNA helicase specifically expressed in the germ cells lineage of large number of animal taxa (Supplementary Fig. 7)( Gustafson and Wessel, 2010; Fujimura et al., 2011). DDX4/Vasa corresponding sequence alignment is presented in Supplementary Fig. 5. Besides that, these cultured cells also expressed the transcription factor Pou5f1/Oct4 (Fig. 3D), a specific pluripotency gene present in ES, ES-derived germ cells and primordial germ cells (PGC) of mammals and fish (Hong et al., 2004; Sánchez-Sánchez et al., 2010; Wang et al., 2011; Onichtchouk 2012; Takehashi et al., 2012, Froschauer et al., 2013) (Supplementary Figs. 6 and 7). The remaining negative cells were heterogeneous in morphology showing epithelioid and fibroblast-like appearance (Fig. 3C and D). Validation of DDX4/Vasa and Oct4 antibodies is shown in Supplementary Fig. 2. After 7 days (Fig. 3E), spermatogonial cells actively proliferate as demonstrated by BrDU incorporation (Fig. 3F). The nuclear BrDU labeling revealed the perinuclear chromatin distribution and a large nucleus that is a typical characteristic of fish early spermatogonial cells (Nóbrega et al., 2010; Schulz et al., 2010). At ten days, tilapia spermatogonia continued to propagate in vitro (Fig. 3G) and the immunostaining showed that these cells retained the expression of the Gfra1 receptor (Fig. 3H), as well as Nanos2 protein (Fig. 3I), which are potential good markers of Aund spermatogonial cells.

Because fish ES cells and spermatogonia are able to differentiate in vitro by enhanced cell–cell interactions (Hong et al., 1996, 2004), we kept the cells at high confluence, without subculture, in order to investigate the tilapia spermatogonia behavior in our cultivation system. Therefore, at 26 days of culture in high confluence, tilapia spermatogonia generated large colonies as shown in Fig. 4A and C. To further analyze the identity of the colony-forming cells we investigated the presence of the vasa, sox2, gfra1 and dmc1 gene transcripts in these groups of cells. Similar to vasa, the gene sox2 is expressed in tilapia germ cells from spermatogonia to spermatocytes (Kobayashi et al., 2002; Pfennig et al., 2012), whereas dmc1 is exclusively expressed in meiotic cells (Kajura-Kobayashi et al., 2005). RT-PCR analysis confirmed that cultivated cells transcribed the germ line-specific genes vasa, sox2 and the gfra1 gene, but not the meiotic marker dmc1 (Fig. 4B). Similar expression patterns of

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<td>dmc1</td>
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<td>407 pb</td>
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* Pfennig et al. (2012).
* Cnaani et al. (2007).
* Present work.
Fig. 1. Immunohistochemical localization of Gfra1 protein-positive spermatogonial cells in the adult Nile tilapia testis. (A–I) Gfra1 protein is detected in single type Aund spermatogonial cells that are morphologically characterized as larger cells showing a large nucleus and a prominent nucleolus (black arrowhead). In contrast, Gfra1 immunoreactivity was not detected in cysts of type A differentiated spermatogonia (Aund; red arrowhead in G–I). Figure F shows at higher magnification a Gfra1-negative Aund cyst containing 2-cells. Similar to Aund, type B spermatogonia (SpgB), spermatocytes (Spc), spermatids (Spt) and spermatozoa (Sptz), as well as somatic cells such as Sertoli (Sc) and Leydig cells (Lc), did not show any evident labeling. Gfra1-negative Aund spermatogonia are rarely observed and a negative control is shown in F′ (arrowhead). J–L and J′–L′ represent respectively bright field and fluorescent microscopy images. In these figures, immunofluorescence staining of isolated testicular cell evidencing the membrane-associated labeling of Gfra1 receptor in the Nile tilapia Aund spermatogonia is observed (large white arrowhead), whereas other smaller testicular cells show no staining (small arrowhead). (M) The suitability of GFRA1 antiddody to recognize the Nile tilapia relative protein (molecular weight: ~50 kDa) in the adult testes evaluated was validated through immunoblotting analyses. (N) Quantitative analysis demonstrated that over 95% of Aund spermatogonia are Gfra1-positive cells in the Nile tilapia testis (*p < 0.05). (O–P) Transversal (dorsoventral) sections of the Nile tilapia testis show that a high density of individual Gfra1-positive spermatogonial cells (arrowhead and white circle in P′) is found at the distal region of the seminiferous tubules, near to the tunica albuginea (TA). The insert at higher magnification in O′ shows that each individual Gfra1-positive cell (arrowhead) is enveloped by Sertoli cells (Sc). Green labeling indicates the Gfra1/Alexa Fluor488, whereas red denotes the propidium iodide. Bar: 10 μm in A–I′ and O–P′; and 50 μm in O–P.
mRNAs in adult testis strongly suggested that tilapia spermatogonial population was able to expand in vitro while conserving their original characteristics. Using immunofluorescence labeling we could observe in situ that at 30 days of culture the cells present in the colony also expressed Gfra1 protein (Fig. 4D). Thus, we could assume that the colony-forming cells contained tilapia Aund spermatogonial cells.

In order to exclude eventual chromosomal changes, which are indicative of cell transformation, we further evaluated herein the DNA content of 30 days-cultivated cells, which also allow us to observe the germ cells progression through spermatogenesis. As a control, fresh dissociated adult testicular cells were used and the flow cytometry histogram displayed three peaks corresponding to haploid (c), diploid (2c) and tetraploid (4c) DNA content (Fig. 4E). In comparison to fresh dissociated cells, we found that the DNA content pattern of cultivated spermatogonia remained relatively unchanged once both histograms showed similar values of mean fluorescence intensity (x-axis; Fig. 4F). In addition, as expected for diploid cells in active proliferation, 30 days-cultivated cells exhibited prominent peaks of diploid (2c) and S-phase fraction (S), besides a modest tetraploid (4c) DNA content (Fig. 4F). Thus, we conclude that tilapia Aund cells remained euploid for at least 30 days under culture conditions and did not differentiate or formed haploid cells.

Fig. 2. Immunohistochemical localization of Nanos2 protein-positive spermatogonial cells in the adult Nile tilapia testis. (A and B) Immunoreactivity of anti-Nanos2 is frequently found in clusters of single type Aund spermatogonia (arrowheads) located near to the tunica albuginea (TA) either through immunoperoxidase (A) or immunofluorescence (B) reactions. Figure C is a representation at higher magnification of Figure A showing that each individual Nanos2-positive cell is enveloped by Sertoli cells (Sc). (D-I) Nanos2-positive spermatogonia (arrowheads) can also be observed along the seminiferous tubules as single cells (Aund; D and E), as well as in cysts of 2 (Aund; F), 4 (Aund; G) and 8-cells (Aund; H-I). The insert in G illustrates at higher magnification a cyst of Aund spermatogonia that did not express Nanos2 protein. All type B spermatogonia (SpgB; H and I), spermatocytes (Spc; D-F), spermatids (Spt; D and H), spermatooza (Sptz; G), Sertoli cells (SC; D, E and H) and Leydig cells (LC; F) did not show any evident labeling. A negative control is shown in F. (J) The specific expression of the Nanos2 protein (~18 kDa) in the testis of two adult Nile tilapia was confirmed by immunoblotting. (K) Quantitative analysis demonstrated that almost 95% of Aund spermatogonia in the Nile tilapia testis are Nanos2-positive cells (p < 0.05). Bar: 50 μm in A; and 10 μm in B-F.
Fig. 3. Evaluation of cultured Nile tilapia spermatogonial cells. (A) After differential plating within 12 h in pre-culture to deplete testicular somatic cells, germ cells remain in suspension and tend to form clumps (black arrowhead). (B) Germ cells attachment to the dish initiated within 3 days. Most of the attached cells are Vasa-positive (C; in green) and also express Pou5f1 (D; in pink). The remaining negative cells exhibit epithelioid and fibroblast-like appearance (C, D; white arrowhead). (E) At 7 days of culture, spermatogonial cells actively proliferate, as evidenced by cells confluence and BrdU incorporation (F; arrowhead). The insert in F shows the pattern of the nuclear BrdU-labeled chromatin distribution in two proliferating spermatogonia. (G) After 10 days of culture, attaching spermatogonia retain the expression of Gfra1 (H; in green) and Nanos2 (I; in green) proteins as demonstrated by fluorescent and brightfield DIC merged images (Figure H and I). The cell nuclei are stained with propidium iodide (PI; in red). Inserts in H and I show phase contrast images of 10 days-cultured cells evidencing the morphological details of Gfra1 and Nanos2-positive spermatogonia, which are not clearly observed in DIC images. Bar: 100 µm in A; and 20 µm in B–I.

Fig. 4. Maintenance and propagation of the Nile tilapia spermatogonia in vitro. (A) At 26 days of culture, under high confluence, tilapia spermatogonia form large colonies (arrowhead). (B) Gene expression pattern evaluated by RT-PCR in the adult Nile tilapia testis and in colony-forming cells (Cf cells). As it can be observed, in comparison to the adult testis, cultivated cells expressed vasa, sox2 and gfra1 genes, but not the meiotic marker dmc1. 18Sr mRNA is used as internal control for RT-PCR amplification, whereas the H2O lane represents a negative control containing no cDNA template. (C and D) At 30 days of culture, immunofluorescence labeling shows that the cells present in the colony (C) expressed the Aund spermatogonia marker Gfra1 (D; in green) and that their nuclei are stained with propidium iodide (PI; in red). (E and F) Flow cytometry analysis of DNA content of the adult (E) and 30 days-cultured (F) testicular cells. In addition to diploid and tetraploid peaks (F; 2c and 4c), adult testicular cells histogram show a prominent haploid peak (E: c). After 30 days in culture, tilapia spermatogonial cells apparently maintain their identity (ploidy) as well as their proliferation pattern, showing diploid (F; 2c) and tetraploid peaks (F; 4c). The S-phase of the cell cycle (S) is observed in both histograms. Bar: 60 µm.
3.4. Transplantation of cultivated spermatogonia

Fish SSCs are only retrospectively identified by their activity to colonize recipient seminiferous tubules after germ cell transplantation. We previously established in our laboratory all the necessary techniques for spermatogonial transplantation in the adult Nile tilapia and demonstrated that transplanted SSCs were able to colonize, proliferate and produce functional sperm in the recipient tilapia testes (Lacerda et al., 2010). Taking advantage of this system, we examined whether Gfra1-positive cultured spermatogonia maintained their ability to colonize the recipient tilapia gonads. Thus, these cells were maintained under optimized conditions for at least 1 month, labeled with the PKH26 fluorescent dye (Fig. 5A–C) in order to trace them in the recipients and subsequently transplanted into de testis of adult busulfan-treated recipient fish (Fig. 5D). Three (Fig. 5E and G) to four weeks (Fig. 5I and K) after transplantation, evaluation of the testes in toto revealed that PKH26-positive cultured SSCs were incorporated into the recipients gonads. Therefore, many clusters/colonies of fluorescent-positive cells were observed in the seminiferous tubules of all transplanted tilapias (Fig. 5E, G, I, K), but not in the gonads of non-transplanted ones (Fig. 5F, H, J, L). We also observed that the quantity of donor cells present in the fluorescent clusters/colonies increased from three to four weeks post-transplantation, indicating therefore the functional viability of the transplanted SSCs to survive, colonize and proliferate in the recipient testes.

4. Discussion

The lack of SSC molecular markers and the small number of SSCs in the testis have been a great hurdle to identify or purify SSCs in fish. Therefore, to date the SSCs biology in this group of vertebrate is poorly known and the promising applications of SSCs in several valuable biotechnologies involving fish production are...
not yet well explored. In the present study we report the establishment of reliable markers for single type A undifferentiated spermatogonia in the Nile tilapia, the Gfra1 receptor, as well as another useful marker for early spermatogonia, the protein Nanos2. Our data also demonstrate the development of effective culture conditions for the propagation of the Nile tilapia Aund spermatogonia for at least 1 month. The spermatogonial identity in culture was characterized by several evidences that included the Aund spermatogonial phenotype, expression of germ cell markers and, more convincingly, the ability of these cells to colonize the recipient testis when transplanted into the adult Nile tilapia.

The Gfra1 membrane receptor is considered one of the most useful markers of Aund cells in mammals (Hofmann et al., 2005) and its expression on these germ cells was already described in mice (Baloh et al., 2000; Dettin et al., 2003; Hofmann et al., 2005; Jing et al., 1996; Von Schönfeldt et al., 2004), rats (Gassei et al., 2009), hamster (Sato et al., 2011), pigs (Kuijk et al., 2009), domestic cats (Powell et al., 2012; Silva et al., 2012), peccaries (Campos-Junior et al., 2012), equids (Costa et al., 2012), rhesus monkeys (Gassei et al., 2010; Hermann et al., 2010) and humans (Gassei et al., 2010, He et al., 2012). Different from fish, in which Aund spermatogonia are characterized as single cells surrounded by Sertoli cells (Leal et al., 2009; Nóbrega et al., 2010; Schulz et al., 2010), in most investigated mammalian species Aund spermatogonia correspond to a group of cells comprising A single (A1), A paired (Ap2) and A aligned (Aa2) spermatogonia, the latter usually being clones of 4, 8, or 16 cells (De Rooij and Russell, 2000). Although in mammals Gfra1 expression is observed in all Aund spermatogonial types, its expression gradually decreases in mice as spermatogonial clones become larger (Nakagawa et al., 2010; Suzuki et al., 2009). In the present investigation, even using histological serial sections, we could not observe cysts of 2 or more spermatogonial cells immunolabeled for Gfra1, but rather single cells (Aund) were found to express Gfra1 exclusively in the testis of adult Nile tilapia, contrasting therefore with the expression pattern observed in mammals. Also, single Aund spermatogonia do not uniformly express Gfra1 in the Nile tilapia testis. We still do not know the functional significance of this finding. However, Gfra1-negative Aund spermatogonia are less abundant, accounting for only about 4% of the total number of Aund spermatogonia. In rodents, morphometric analysis demonstrated that about 10% of A1 spermatogonia did not express Gfra1 but were able to form colonies after germ cell transplantation assays (Grisanti et al., 2009). So, it remains controversial as to whether the heterogeneous expression of markers in spermatogonia in fact reflects different temporal stage (e.g. phases of cell cycle) within a functionally homogenous cellular population and not functionally distinct populations (Izadyar et al., 2002; Lok et al., 1982; Schulz et al., 2010).

It is currently widely accepted that A1 spermatogonia represents the actual SSC population in mammalian testes (Yoshida et al., 2007; Oatley et al., 2011). Considering this aspect, we could speculate that Gfra1-positive spermatogonia, or at least a large fraction of this population, represent the SSCs in the adult Nile tilapia testis. While the expression of other candidate molecules has been reported in fish, including L775 (Nagasawa et al., 2010, 2012), Notch1 (Yano et al., 2009), Pzf (Ozaki et al., 2011), Pou5f1 (Sanchez-Sanchez et al., 2010) and SCSA1 (Kobayashi et al., 1998), to our knowledge Gfra1 represents the first described molecular marker to distinguish Aund cell from other progenitor spermatogonia in the teleost testis. Therefore, since Gfra1 receptor is a selective surface marker it might be feasible to employ it as molecular target in immunomagnetic or immunofluorescent based cell sorting in order to isolate or obtain enriched Aund spermatogonia population in Nile tilapia or other fish species.

Nanos2 is a key stem cell regulator that is expressed in self-renewing SSCs and maintains the stem cell state during murine spermatogenesis (Sada et al., 2009; Suzuki et al., 2009). The GDNF/Gfra1 signaling pathway is one of the candidates to induce or maintain Nanos2 expression in the mouse testis (Sada et al., 2012). In fish, Nanos2 protein has been previously demonstrated in zebrafish PGCs at early stages of sex differentiation (Beer and Draper, 2012) and in medaka (Oryzias latipes) spermatogonia and oogonia (Aoki et al., 2009). Similar to the pattern observed in rodents in which A1 to Aa2 undifferentiated spermatogonia are Nanos2-positive (Sada et al., 2009, 2012; Barrios et al., 2010; Suzuki et al., 2009), in the testis of the adult Nile tilapia Nanos2 was found to be expressed in both type Aund and Aa2 spermatogonia up to the 8-cell clone. Using double immunostaining, a previous study showed that in mouse about two-thirds of Nanos2-positive A1 to Aa2 cells co-expressed Gfra1. However, all of the Gfra1-positive cells were also Nanos2-positive (Suzuki et al., 2009). Although we did not perform this kind of analysis in the present investigation, based on the high percentage of Gfra1-positive (~96%) and Nanos2-positive (~94%) single Aund observed here, we could

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Fig. 6. Illustration of Gfra1 and Nanos2 protein expression in the different types of spermatogonial cells in the Nile tilapia testis. As it can be noticed, the expression of Gfra1 (in red) is restricted to single Aund spermatogonia (Aund1 and Aund2) that contain the spermatogonial stem cell population, which supposedly present the capacity of self-renewing (curved arrow) or differentiation (straight arrow). Although Nanos2 (in green) is observed in both single Aund spermatogonia, its expression is also found in type A differentiated spermatogonia (Adef) that form clones/cysts of 2–8 germ cells. Nanos2 expression is no longer observed after the type Adef divide to give rise to type B spermatogonia (Bearly). However, it is not yet known whether the Nile tilapia Aa2 spermatogonia (2 to 8-cell clones) possess the ability to dedifferentiate (7; long arrows) and become single cells with stemness capacity.
expect that, similar to mice, in the Nile tilapia Adiff spermatogonia, from A2 to Aα (8-cell clones), maintain their stemness status, being able to differentiate into subsequent spermatogonial clones (Aβ or becoming a new Aα) by fragmentation of the spermatogonial clone (Nakagawa et al., 2010). Considering that in the murine spermatogenic stem cell compartment Aβ to Aα show reversibility and potential stemness capacity, it would be of great value to investigate if in Nile tilapia Adiff spermatogonia (2 to 8-cell clones), Nanos2-positive cells show such a reversibility as well, or if they are definitely committed to differentiation. Based on the results found here, Fig. 6 summarizes the expression pattern of the two proteins evaluated in the Nile tilapia spermatogonial cells.

In the present study we also developed functional in vitro culture conditions for supporting the survival, mitotic activity and self-renew of tilapia Aund spermatogonia. So far, the basic techniques for the long-term in vitro culture of spermatogonia have been established only for zebrafish (Kawasaki et al., 2012), rainbow trout (Shikina et al., 2008; Shikina and Yoshizaki, 2010) and medaka (Hong et al., 2004). Indicating the maintenance of the undifferentiated status of the spermatogonial cells, we observed that after 26 days of culture and under high confluence the Nile tilapia spermatogonia formed large colonies of Gfra1-positive cells. In contrast, at full confluence without subculture, medaka spermatogonial cells underwent meiosis and spermiogeneration, generating motile sperm in vitro (Hong et al., 2004). Similar to zebrafish and mammalian spermatogonial culture systems (Aponte et al., 2008; Kala et al., 2012; Kawasaki et al., 2012; Mirzapour et al., 2012; van der Wee et al., 2001), some testicular somatic cells derived from dissociated testicular cells could be observed in our culture system, acting as a feeder layer for tilapia Aund spermatogonia. Although we have observed Gfra1 receptor expression in cultured spermatogonia in different time periods, the recombiant mammalian growth factor GDNF was not used as a supplement in our culture medium. Shikina and Yoshizaki (2010) have previously reported no effect of rat GDNF or rat GFRα1-Fc fusion protein on the proliferation of rainbow trout type A spermatogonia, whereas recombinant human GDNF was shown to enhance zebrafish spermatogonia proliferation in vitro. However, in comparison to mouse SSC cultures, a 5- to 10-fold higher concentration of this growth factor was used for zebrafish cells (Kawasaki et al., 2012). Surely, this important aspect deserves a better investigation in our culture system in future studies.

It is widely accepted that only SSCs are able to colonize the available niches and re-establish spermatogenesis in the recipient testis. Therefore, germ cell transplantation provides a functional approach to study the stem cell niche in the testis and to unequivocally detect SSCs (Nagano et al., 2013; Tang et al., 2012). Using germ cell transplantation assay, we demonstrated that the Nile tilapia Aund spermatogonia, maintained for at least 1 month in culture, were able to colonize busulfan-depleted seminiferous tubules of recipient fish. While further studies are still necessary to determine whether normal sperm and offspring can be obtained from cultured cells, the wide spread of PHK26-positive colonies/clusters observed 3–4 weeks after transplantation functionally demonstrate the regenerative capacity of cultured Aund spermatogonia, which also suggest their stemness potential.

Based on our findings, it is reasonable to assume that important aspects related to SSCs physiology are phylogenetically conserved among vertebrates, or at least in teleosts and mammals. However, further investigations are still required to determine if any differences from patterns already known in mammals do exist, which would allow discovering potentially novel (fish specific?) signaling pathways. Despite that, besides providing a better knowledge of fish SSCs biology, the findings reported here represent crucial steps for the progress toward the development of new biotechnologies in fish production. Therefore, we expect that these results will be valuable tools furthering the development of better methods for in vitro SSCs expansion, which represent an important prerequisite for SSC-based genetic manipulations in fish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.06.013.

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