INVESTIGATION OF POTENTIAL MOLECULAR MARKERS AND APPROACHES TO CHARACTERIZE AND ISOLATE SPERMATOGONIAL STEM CELLS IN THE NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

Lacerda S.M.S.N., Costa G.M.J., Campos-Jr P.H.A., Rezende-Neto J.V., *Hofmann M-C., França L.R.

Department of Morphology, Federal University of Minas Gerais, Belo Horizonte-MG, 31270-90, Brazil. Fax: +55-31-34992780 email: lrfranca@icb.ufmg.br
*Department of Veterinary Biosciences, University of Illinois at Urbana-Champaign, Illinois, USA.

Introduction:

Undifferentiated spermatogonial population consists of stem and progenitor germ cells, which function to provide the foundation for spermatogenesis. The stem cell component, named spermatogonial stem cells (SSCs), is capable of self-renewal and differentiation. These unique attributes have made them a target for novel technologies to enhance reproductive function in males. First established in mammals, germ cells transplantation is the only functional approach for investigating SSCs biology. In this regard, recently we established successful spermatogonial transplantation in adult Nile tilapia that resulted in donor-derived offspring [1]. In association with in vitro culture, SSCs transplantation have the potential to enhance efficiency of fish production and provide a novel approach to continuously generate transgenic animals, allowing also the preservation of genetic stocks of valuable or endangered fish species. Isolation of SSCs is an essential component for improving the efficiency of these techniques; however, there are no specific molecular markers for SSCs in any vertebrate species, including fish. Therefore, in the current study we aimed to investigate potential molecular markers, particularly those already well established in higher vertebrates [2], which could be properly used to phenotypically characterize and isolate SSCs in the Nile tilapia.

Methods:

Adult tilapia (*O. niloticus*) testes were evaluated by immunohistochemistry and immunofluorescence for the presence of Vasa (germ cell specific marker), GFRα-1 and Notch-1 (undifferentiated spermatogonia surface marker) receptors, and the transcription factor Oct-4 (POU5F1). Reactions were performed according to standard protocols using immunoperoxidase technique and fluorophore-conjugated secondary antibody. For these purposes, the following primary antibodies and the dilutions used were: polyclonal rabbit anti-human DDX/MVH-Vasa (1:200; Abcam), polyclonal rabbit anti-human Notch Homolog 1 (1:100; Lifespan Bioscience), polyclonal goat anti-rat GFRα-1 (1:500; Santa Cruz Biotechnology) and polyclonal rabbit anti-human Oct-4 (1:200; Abcam). Additionally, western blot analyses of protein expression were performed in order to determine the specificity of the antibodies used. The profile and percentage of positive testicular cells were evaluated using flow cytometry (FACS). Using immunomagnetic separation (MACS) we also investigated if some of the surface marker(s) could be used to enrich undifferentiated (SSCs) spermatogonia population.

Results and Discussion:

The flow cytometry analyses showed that the GFRα-1 positive cell fraction comprised approximately 9.5% ± 0.9 (n=3 and three replicates from each fish) of the selected testis cell population evaluated in adult tilapia, whereas Notch-1 positive cells corresponded to 7%±0.8 of the selected testicular cells. Immunohistochemistry and immunofluorescence analysis revealed that GFRα-1 and Notch-1 expression is localized in the seminiferous epithelium, exclusively in single type A spermatogonia (presumptive SSCs). Sertoli cells, differentiating/differentiated germ cells or interstitial cells did not show any evident labeling for these markers. The GFRα-1 and Notch-1 positive spermatogonial cells were found preferentially at the blind ending of the tilapia seminiferous tubules, near to the tunica albuginea, where a high density of type A undifferentiated spermatogonia, characterized by morphological criteria, were previously reported to be located [3]. In addition, these GFRα-1+/Notch-1+ cells showed intense immunoreactivity for anti-Vasa. Using double staining for Notch1 and GFRα-1, we observed that less than one percent of testicular cells were double positive for these markers, suggesting the presence of phenotypically different SSCs populations. The investigation of Oct-4 expression in tilapia testis showed that only type A spermatogonia (presumptively primitive SSCs), also exclusively located at the blind ending of the seminiferous tubules, were positive for this pluripotent marker. Preliminary FACS analyses using double staining for Oct-4 and GFRα-1 showed that the Oct-4 positive cell fraction comprises approximately 6.2% of total selected testicular cells. Interestingly, the entire population of Oct-4 positive cells were also positive for GFRα-1. Considering the promising results related to

GFRα-1 positive staining, we have selected testicular cells for this surfaced marker through immunomagnetic separation. A four-fold relative enrichment of SSCs was obtained in the sorted fraction after MACS separation.

Conclusion:

Taken together, the results found in the present investigation showed that GFRα-1, Notch-1, and Oct-4 are potentially good markers for the SSCs in tilapias. We also demonstrated that an enriched GFRα-1 positive SSCs population from adult tilapia can be obtained using MACS. Therefore, besides providing a better knowledge on SSCs biology in fish, this crucial step represents a very important progress toward the development of new biotechnologies in aquaculture, for instance allowing the generation of transgenic fish using SSCs transfection.

Financial support: FAPEMIG and CNPq

References:

[1]LACERDA, S.M., BATLOUNI, S.R., COSTA, G.M., SEGATELLI, T.M., QUIRINO, B.R., QUEIROZ, B.M., KALAPOTHAKIS, E., FRANÇA, L.R. 2010. A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoS One, 5:e10740.

[2]PHILLIPS, B., GASSEI, K., ORWIG, K.E. 2010. Spermatogonial stem cell regulation and spermatogenesis. Philos. Trans. R. Soc. Lond. B Biol. Sci., 365:1663-1678.

[3]SCHULZ, R. W., FRANÇA, L.R., LAREYRE, J.J., LE GAC, F., CHIARINI-GARCIA H., NOBREGA, R. H., MIURA, T. Spermatogenesis in fish. Gen. Comp. Endocrinol., 167: 390-411.