Isolation, Identification, Culture, Cryopreservation, Genetic Transformation and Transplantation of Catfish Germline Stem Cells

by

Mei Shang

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 14, 2013

Keywords: Catfish Germline Stem Cells, Isolation, Identification, Culture, Genetic Transformation, Transplantation

Approved by

Rex A. Dunham, Chair, Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Elizabeth Lipke, Associate professor, Chemical Engineering
Covadonga R. Arias, Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Eric J. Peatman, Assistant Professor, School of Fisheries, Aquaculture, and Aquatic Sciences

Abstract

The hybrid of channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*Ictalurus furcatus*) ♂ (C × B) is the best catfish for pond culture. The current technology, artificial fertilization and handstripping is labor intensive, time consuming, and requires sacrifice of mature 5 ~ 6 year old blue catfish males. Xenogenic catfish produced by transplanting blue catfish stem cell into the gonads of triploid channel catfish could reduce the generation interval to produce blue catfish and might result in channel catfish males that are able to produce blue catfish sperm, and increase the efficiency of C × B hybrid catfish embryo production.

Gonadal cell size and type of 90 ~ 100 day - old fry (TL: 5 ~ 6 cm), two - year old juvenile (TL: 25 ~ 30cm) and mature adults (TL: 65 ~ 70 cm) blue catfish was examined. Gonads of all ages of fish contained oogonia (12 ~ 15 μm diameter, distinct nucleus 7 ~ 8 μm) and spermatogonia (12 ~ 15 μm, distinct nucleus 6 ~ 7.5 μm). Male juvenile blue catfish, which had suitable size and higher percentage of germline stem cells, were used for stem cell isolation for further study. After testes were dissected from the peritoneal cavity, germline stem cells were isolated with discontinuous density gradient centrifugation to enrich the percentage of spermatogonia. Four distinct cell bands were generated after centrifugation. It was estimated that 55% of the total cells in top - first bands, cell band I, were type A spermatogonia (diameter 12 ~ 15 μm) and type B spermatogonia (diameter 10 ~ 11 μm), 35% were spermatocytes (diameter 5 ~ 9 μm), 10% were spermatids (diameter < 1 μm). In the second band - cell bands II, most of the cells were spermatocytes (60%, diameter 5 ~ 9 μm), some were type B spermatogonia (30%), a few were spermatids (10%) and cell clusters could also be observed. The third band ~ cell band III contained most of the spermatids (95%) and very few secondary

spermatocytes (5%, diameter 5 ~ 8 μm). Red blood cells (95%) and a few spermatids (5%) were the predominant cells in the fourth cell band - cell band IV.

Different bands generated from Percoll density gradient centrifugation were used for germ cell marker identification studies. Expression of seventeen genes (pfkfb4, urod, Oct4, Plzf, sycp3, SOX2, Integrin6, neurogenin3, ID - 4, integrinV, Thy1, GFRα, CDH1, Smad, Pum2 and Prdm14 and Kit) from cells of different bands were analyzed by qRT - PCR in both channel and blue catfish. pfkfb4, urod, Plzf, Integrin6, ID - 4, integrinV, Thy1 and CDH1 genes, which showed the same expression change pattern in different types of testicular germ cells of both channel and blue catfish, were identified as spermatogonia marker. SOX2 gene, which was up - regulated in spermatocytes and even higher up - regulated in spermatids, was identified as spermatids marker.

Blue catfish testicular and ovarian germ cell *in vitro* cultures were studied. When testicular tissues were used as the initial culture materials, testicular germ cells migrated outward the edges of tissues and formed monolayers during the first two - week culture. Cultured testicular germ cells were fibroblast - like, growing fast after the third passage. For ovaries, ovarian germ cell isolated after trypsin digestion was used as the initial culture material, and the cells attached to the plate during the first week of culture. Cells from ovaries were also fibroblast - like in culture.

Electroporation was used for both channel and blue catfish germ cell genetic transformation. Both blue catfish ovarian germ cells and channel catfish testicular germ cells expressed green florescence protein (GFP) for a long time period after cells were transformed by the expression construct FRMwg driven by the carp β - actin promoter.

Two transplantation techniques, surgery and catheterization, were studied for transplanting spermatogonian stem cell enrichment and testicular germ cells into triploid channel catfish gonads. Ten months after the surgery transplantation, eight fish were recaptured; all three recipient fish which were transplanted by surgery were proved containing blue catfish cells; four out of five recipient fish which were transplanted by

catheterization contained blue catfish cells. Both surgery and catheterization were efficiency transplantation method and had a very high transplantation ratio.

Acknowledgments

I would fist like to thank everyone in Dr. Dunham's genetics group. I would like to thank Baofeng Su, my husband, for his love, his suggestions and help; without him, I cannot image how I could finish my PhD study overseas. I would also like thank Dayan Perera, Michael Fobes, Donny Wade, Yun Li, Zhenkui Qin, Qi Cheng, Ahmed Alsaqufi, Zhi Ye, and Chia - Chen Weng Chen for their help in the greenhouse and laboratory.

I would also like to thank everyone in Dr. Lipke's research group. I would like to especially thank Dr. Lipke for being patient with me and giving me so many wonderful suggestions on my project. I would also like thank Dr. David Dunn, Aaron Seeto, Samuel Chang, Shantanu Pradha and Alex Hodge for their help, no matter when I asked, they always did everything they could to help me.

I would like to thank Dr. Eric Peatman, Dr. Gemerlyn Garcia and Dr. Sehriban Cek for their help throughout my research. I would also like to thank Mr. Karl Hayden and Ms. Theresa Wood for their help in the Disease laboratory, for hands - on experience and skills. I would like to express my gratitude to Dr. Zunchun Zhou, from Liaoning Ocean and Fisheries Science Research Institute, for his suggestions on how to start preparation work for cell culture and Dr. Songlin Chen from Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Science, for one of his very useful books as a gift and reference. I would like give a special thanks to Chao Li for his help on sequence searching. I would also want to thank my friends, Jun Yao, Jiaren Zhang, Chen Jiang and Ailu Chen, who have brought so much happiness in my research at Auburn.

I would also like to thank everyone in Fisheries. I am grateful to Dr. Covadonga Arias, Dr. William Daniels, Dr. Stephen Bullard and Dr. Yolanda Brady, who gave me so much knowledge during my study. I would also like to thank Ms. Karen Booker and Ms. Susan Smith, who gave so much love and help.

Finally, I would like to thank my parents and my whole family for their countless love, sacrifices, support, and encouragement during my whole life. My gratitude also goes to Daniel Douty's whole family, for all of their love.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

GSC	Germline Stem Cell
EDTA	Ethylenediamine Tetraacetic Acid
PGC	Primordial Germ Cell
TL	Total length,
SSC	Spermatogonial Stem Cell
MACS	magnetic activated cell sorting
FACS	fluorescence activated cell sorting
NBF	Neutral Buffer Formalin
MS - 222	Tricaine Methanesulfonate (MS - 222)
HBSS	Hanks' Balanced Salt solution
L - 15	Leibovitz's - 15 medium
DMEM	Dulbecco's Modified Eagle Medium,
GFP	Green Florescence Protein
BFP	Blue Florescence Protein

CHAPTER ONE

Introduction

The U.S. Catfish Industry: Decline of a Major Agricultural Industry

Catfish farming is the largest freshwater food fish aquaculture industry in the United States. Because of catfish's wide market acceptability and high adaptability to different environmental culture conditions, commercial catfish farming has been established since the 1930s (Dunham and Smitherman 1984). The early catfish farming research was mostly conducted by Dr. H. S. Swingle and his co - workers at Auburn University, thereby providing the impetus for the first significant growth spurt of the industry in the 1960's. The catfish industry was highly developed by the 1980s. Now, annual production of farm - raised catfish has the highest economic value of any aquaculture industry in the United States. In 2011, the total value of catfish sales reached to 423 million dollars, which was up 5.2% from 2010. However, this number dropped to 341 million dollars in 2012, which was down 20% from the previous year. A total of 300 million pounds of farm - raised catfish were processed during 2012, 10% below the 334 million pounds processed in 2011. The average price paid to producers in 2012 was 0.98 dollar per pound, up 19 cents from 2011 (NASS, 2013), but the price dropped to 0.82 in 2013. The top four States (Mississippi, Alabama, Arkansas, and Texas) accounted for 95% of the United States total sales.

However, due to the increasing cost of feed, the high cost of fuel, production inefficiencies and competition of inexpensive imported Chinese and Vietnamese catfish, domestic production of farm - raised catfish has declined by half since its peak a decade ago. The water surface acres used for catfish production declined to 83,000 acres in 2012,

which was down 58% from 196,590 acres in 2001. Although the United States Congress passed a law preventing the imported fish from being labeled as catfish in early 2001 and the Department of Commerce (DOC) and the U.S. International Trade Commission (ITC) issued antidumping orders for frozen catfish fillets in 2003, the importation of the Vietnamese catfish has not been effectively prevented (Frame, 2013). Vietnamese catfish - basa (*Pangasius bocourti*) fish took up more than 75% of the U.S. market in 2012. Since China has developed their own aquaculture programs for North American channel catfish, *Ictalurus punctatus*, catfish imports from China have grown rapidly. According to the U.S. Department of Agriculture (USDA, 2012) report, China's total cultured catfish production was estimated at 610,000 tonnes (1.35 billion lbs) in 2012 (USDA GAIN: China Fishery Products Annual 2012). Approximately, 0.1%, 1.32 million pounds, was exported to U.S. The U.S. domestic catfish industry is highly threatened.

By 2030, aquaculture will need to increase by 365% worldwide to cover the impending seafood shortage, with an additional 21 million tonnes of fish will be needed for human consumption (FAO 2012). In the US, there is a trade deficit of more than $10 billion for fisheries products (NOAA 2012). The US is not in position to take advantage of the opportunity of the expanding global market of aquaculturally grown fish. In reality, if US farmers do not become more efficient, the industry will continue to decline and die, and the quantity of aquatic food that we import will continue to grow, which opens up another Pandora's Box of food safety, food security and national security issues. The major regions of US catfish production can be characterized as rural, agricultural, high poverty and with high unemployment levels. The continued decline in the catfish industry will exacerbate these conditions.

The hybrid resulting from the mating of channel catfish ♀ × blue catfish, *I. furcatus*, ♂ is the best catfish for pond culture (Dunham et al. 2008) and in pond raceway culture (Brown et al. 2011). This fish is one of the keys for the survival, recovery and growth of the catfish industry as indicated by its performance as well as the fact that it a key to the success of new improved culture systems beginning to be employed in the catfish industry.

Hybrid Catfish

Production

Selection and breeding programs with the goal of enhancing the genetic potential of catfish have been carried out since the 1960s. The hybrid catfish, channel catfish ♀ × blue catfish ♂ (C × B) is the only commercially valuable hybrid among 42 interspecific hybrids of major North American catfish species, and has superior performance compared to the commonly grown channel catfish (Dunham et al. 2012).

Small quantities of hybrid catfish were occasionally produced in the catfish industry beginning in the 1980s, but were first produced on a regular basis by Gold Kist Inc. in Inverness, Mississippi beginning in 1999 with an initial crop of around 500,000 hybrid fry. Eagle Aquaculture, began commercial production of hybrid catfish using Auburn University spawning technology in 2005. Immediately hybrid fry production dramatically increased, and has increased every year since with an estimated output of 170 million fish in 2013 (Brian Bosworth, personal communication). This increased application of the hybrid in commercial settings verified the research results that this fish has improved performance.

Performance

The C × B hybrid catfish has a higher growth rate, especially at higher densities, than the parent species. Growth rate of the C × B hybrid catfish was 35% higher than the channel catfish and the growth improvement through interspecific hybridization was three times higher than through mass selection of channel catfish (Dunham et al. 1999). The C × B hybrid consumed more feed with a better feed conversion ratio and more weight gain when compared to NWAC 103 channel catfish (Li et al. 2004).

The C × B hybrid catfish also had a higher survival rate than channel catfish and blue catfish in several studies (Dunham et al. 1987, 1990, Li et al. 2004, Dunham et al. 2008). The C × B hybrid catfish was more tolerant of low dissolved oxygen than the parent species. In earthen ponds, only 7.5% of the hybrid catfish died, but 50.5% of the channel catfish died because of hypoxia (Dunham et al. 1983). In cage culture, 51.0% of

hybrids and 87.5% of channel catfish died due to low dissolved oxygen. No channel catfish survived in concrete tanks, but 67% of the hybrids survived under the same conditions.

The C × B hybrid catfish was more resistant to disease infection than the channel catfish. When challenged with an immersion bath of *Edwardsiella ictaluri*, 73.8% hybrid catfish survived, but only 62.0% channel catfish survived (Wolters et al. 1996). Recently, hybrid catfish were shown to be more resistant to *Flavobacterium columnare* BGFS - 27 (genomovar II) than channel catfish and blue catfish (Arias et al. 2012). However, the C × B hybrid catfish did not show improvement for channel catfish virus disease resistance (Chappell. 1979, Dunham et al. 1990).

The C × B hybrid had a higher dress - out and fillet percentage than the channel catfish (Argue et al. 2003). Additionally, the hybrid had higher dress - out yield, nugget yield, fillet moisture, protein and lower level of fillet fat compare to channel catfish, but there was no difference in shank fillet yield (Li et al. 2004). When meat yield and meat quality traits among NWAC 103 line channel catfish, Norris line channel catfish and hybrids catfish were compared, hybrid catfish had higher dress - out and fillet yields than channel catfish strains, and there were only minor differences for fillet quality (Bosworth et al. 2004). Based on the carcass characteristics, chemical, microbiological and sensory qualities of fillets, the hybrid and the channel catfish were similar (Huang et al. 2004). However, hybrids had significantly higher visceral fat than channel catfish.

The C × B hybrid was also much easier to catch by seining as well as by hook and line (Tave et al. 1981; Dunham et al. 1986; Dunham and Argue 1998; Masser and Dunham, 2012). The relative position difference between C × B hybrid and channel catfish in the water column might be related to the differences in seinability (Dunham et al. 1983).

Artificial fertilization is effective for large - scale production of hybrid fry, but it depends on hormones, induced ovulation of channel catfish females, manual stripping of eggs, and artificial fertilization of the eggs with blue catfish sperms (Dunham et al. 2000, Perera. 2012). Blue catfish need to be sacrificed to obtain sperms. Channel catfish

females require 3 - 4 years to become sexually mature, and blue catfish males 5 - 6 years. Although the current technology is effective, the artificial fertilization process and the generation interval of the parent species, especially that of the blue catfish, presents problematic inefficiencies for production. Xenogenesis, a method of reproduction, in which successive generations differ from each other, is a potential tool that might be used to address these inefficiencies.

Germline Stem Cells

In multicellular organisms, germ cell is a general term for all the cells that can produce offspring, and it includes primordial germ cell (PGCs), gonocyte and mature gamete (sperm and egg). PGC is the earliest germ cell, which is morphologically, genetically and epigenetically different from somatic cell. PGC, which can self - renew or differentiate into either spermatogonia or oogonia after gonadal sex differentiation, is considered to be stem cell (Yoshizaki, 2002, 2003). In fish, PGCs undergo three major events: early formation in the blastula stage of the embryo; migration to the genital ridge; proliferation and late differentiation in the genital ridge. After gonadal sex differentiation, testes are formed in males and spermatogonia continue with mitosis, Cells enter meiosis at a later developmental stage. Ovaries are formed in females and oogonia in the female gonads commence meiosis immediately (Okutsu et al. 2010). There are four major types of germ cells - spermatogonia A, committed spermatogonia B, spermatocytes and spermatozoa in testes, and three major types of germ cells - oogonia, oocytes and eggs in ovaries (Evans and Claiborne. 2006). Spermatogonia A, which are also called spermatogonial stem cells (SSCs), are the only cell type that are formally considered to be stem cells in testes, because they can self - renew and have directional potential to differentiate into functional gametes. In fish, PGCs and SSCs are the common germline stem cells that are used for xenogenesis. Even though information about the plasticity of the ovarian germ cells after sex differentiation is limited, oogonia function as germline stem cells (GSCs) and have been also used for xenogenesis study (Yoshizaki et al. 2010).

Xenogenesis

Xenogenesis is a method of reproduction in which successive generations differ from each other (Dunham, 2011). A xenogenic organism contains elements typically foreign to its species. One process to accomplish xenogenesis in fish involves transplanting diploid germline stem cells (GSCs), such as primordial germ cells (PGCs) and spermatogonial stem cells (SSCs) of donor fish into triploid recipient fish, resulting in the host producing gametes of the donor. If the GSCs are transplanted into the sterile recipient at the blastula stage or around the time of hatching, they have the potential to migrate to the genital ridge and develop into gonads that only contain the donor germ cell type (Ciruna et al. 2002; Okutsu et al. 2006; Saito et al. 2008, 2010). If the GSCs are transplanted into a recipient's matured but compromised gonad, they can develop into gametes of the donor fish in a short period (Lacerda et al. 2012). Potentially, xenogenesis could be used to reduce the generation interval of the donor species or as a mechanism to improve the efficiency of hybridization.

PGC Transplantation

Zebrafish (*Danio rerio*) PGCs have been isolated from donor embryos and transplanted into blastula stage embryo of the same species (Ciruna et al. 2002). Before transplantation, the host embryos had been microinjected with a *dead end* gene antisense morpholino oligonucleotide (MO) to block the endogenous PGC development. The donor's PGC completely replaced the host germ line. PGCs have also been transplanted interspecifically. A single PGC of pearl danio (*Danio albolineatus*), goldfish (*Carassius auratus*) and loach (*Misgurnus anguillicaudatus*) were transplanted into sterilized blastula - stage zebrafish embryos (Saito et al. 2008) and the single PGC developed into a normally sized testis in the recipient fish. A blastomere and single PGC were used as two different donor materials in a transplantation efficiency study (Saito et al. 2010). Blastomeres from transgenic zebrafish, pearl danio, goldfish, and loach were transplanted into blastula - stage of normal zebrafish embryos; the transplantation efficiency was low because the somatic cells, which were derived from donor blastomeres, formed aggregates in the host embryos. But in the case of single PGC transplantation, no species

difference has been reported regarding donor PGC colonization and migration toward the gonadal region of the host embryo at a comparatively high rate.

PGCs were also transplanted into newly hatched fish embryos. After GFP - labeled PGCs were sorted by flow cytometry from the transgenic rainbow trout (*Oncorhynchus mykiss*), they were transplanted into newly hatched embryos of wild type fish (Takeuchi, 2003). PGCs were able to migrate to the recipient's gonadal ridge, proliferate and develop into function gametes

SSC Transplantation

Testicular cells containing SSCs, which were isolated from adult male *vasa* - GFP transgenic rainbow trout, were transplanted into the peritoneal cavity of newly hatched embryos of wild rainbow trout (Okutsu et al. 2006). The donor's testicular germ cells differentiated into spermatozoa in male recipients and fully functional eggs in female recipients. Furthermore, the donor - derived spermatozoa and eggs obtained from the recipient fish were able to produce normal offspring spermatogonia from *vasa* - GFP transgenic rainbow trout were transplanted into newly hatched sterile triploid masu salmon (*Oncorhynchus masou*). Two years after transplantation, triploid salmon recipients only produced trout sperm and eggs and those gametes resulted in rainbow trout offspring (Okutsu et al. 2007).

SSCs were also transplanted into adult Nile tilapia (*Oreochromis niloticus*) gonads. Percoll gradient centrifuge enriched Nile tilapia type A spermatogonia were labeled with the fluorescent lipophilic dye PKH26 - GL and transplanted into adult recipient whose endogenous spermatogenesis was suppressed by the cytostatic drug, busulfan (1, 4 - butanediol dimethanesulfonate) and high temperature (35 ℃). PKH26 labeled germ cells were found in the lumen of the seminiferous tubules of recipient's testes (Lacerda et al. 2006). Another study also demonstrated complete spermatogenesis in a tilapia recipient 8 ~ 9 weeks after transplantation (Lacerda et al. 2010).

Oogonia Transplantation

In an oogonia transplantation study, oogonia from 6 ~ 9 month old p*vasa* - *Gfp* transgenic rainbow trout were transplanted into hatching - stage fry of normal rainbow trout (Yoshizaki et al. 2010). Two years after transplantation, the transplanted oogonia had differentiated into functional eggs in female recipients and sperm in male recipients.

Objectives

The first set of objectives was to isolate and enrich GSCs by 1) identification of GSCs in both blue and channel catfish using histology, 2) standard cell isolation procedures, 3) Percoll density gradient centrifugation for SSCs enrichment. The second major objective was to identify SSCs specific makers by determining gene expression in different types of testicular germ cells of both channel and blue catfish. The third set of objectives was to 1) establish an *in vitro* cell culture system for both blue and channel GSCs and 2) study germ cell cryopreservation conditions. The last objectives were to use electroporation to transfer green florescence protein expression construct - FRMwg, and blue florescence protein expression construct - FRM2bl, into catfish germline cells and obtain expression of both of those genes. The other transformation goal was to produce xenogenic channel catfish by interspecific transfer of enriched SSCs from diploid donor blue catfish into the gonads of triploid channel catfish host.

Reference

Arias, C. R., Cai, W., Peatman E., Bullard S. A. 2012. Catfish hybrid *Ictalurus punctatus* × *I. furcatus* exhibits higher resistance to columnaris disease than the parental species. Diseases of Aquatic Organisms, 100(1): 77 - 81.

Argue, B. J., Liu, Z., Dunham, R. A. 2003. Dress - out and fillet yields of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and their F1, F2, and backcross hybrids. Aquaculture, 228: 81 - 90.

Cressey, D. 2009. Aquaculture: Future fish. Nature, 458, 398 - 400.

Bosworth, B. G., Wise, D. J., Terhune, J. S., Wolters, W. R. 2003. Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish × blue catfish backcross hybrids. Aquac Res 34: 569 - 57.

Bosworth, B. G., Wolters. W. R., Silva, J. L., Chamul, R. S. and Park, S. 2004. Comparison of production, meat yield, and meat quality traits of NWAC103 line channel catfish, norris line channel catfish, and female channel catfish × male blue catfish F1 hybrids. North American Journal of Aquaculture, 66 (3): 177 - 183.

Chappell, J. A. 1979. An evaluation of twelve genetic groups of catfish for suitability in commercial production. Doctoral dissertation. Auburn University, AL.

Ciruna, B., Weidinger, G., Knaut, H. , Thisse, B. , Thisse, C., Raz, E., Schier, A. 2002. Production of maternal - zygotic mutant zebrafish by germ - line replacement. Proceedings of the National Academy of Sciences, USA, 99: 14919 - 14924.

Dunham, R. A., Smitherman, R. O., Webber, C. 1983. Relative tolerance of channel × blue hybrid and channel catfish to low oxygen concentrations. Progressive Fish - Culturist, 45: 55 - 57.

Dunham, R. A., Smitherman, R. O., Goodman, R. K. 1987. Comparison of mass selection, crossbreeding, and hybridization for improving growth of channel catfish. Progressive Fish - Culturist, 49: 293 - 296.

Dunham, R. A., Brument, R. E. Ella, M.O., Smitherman, R.O. 1990. Genotype - environment interactions for growth of blue, channel and hybrid catfish in ponds and cages in varying densities. Aquaculture, 85: 143 - 151.

Dunham R. A., Brummett, R. E. 1999. Response of two generations of selection to increased body weight in channel catfish, *Ictalurus punctatus*, compared to hybridization with blue catfish, *I. furcatus*, males. Journal of Applied Aquaculture, 9: 37 - 45.

Dunham R. A., Argue, B.J. 2000. Reproduction among channel catfish, blue catfish, and their F1 and F2 hybrids. Transactions of the American Fisheries Society, 129: 222 - 231.

Dunham R. A. 2011. Aquaculture and Fisheries Biotechnology: Genetic Approaches, 2nd Edition 93.

Evans, D. H., Claiborne, J. B. 2006. The Physiology of Fish. CRC Press, Taylor & Francis Group, p. 349 - 359.

Fan, L., Moon, J., Wong, T., Crodian, J., and Collodi, P. 2008. Zebrafish primordial germ cell cultures derived from *vasa*::RFP transgenic embryos. Stem Cells and Development, 17: 585 - 598.

FAO Fisheries and Aquaculture Department. 2012. The State of World Fisheries and Aquaculture. http://www.fao.org/docrep/016/i2727e/i2727e.pdf.

Frame, A. 2013, Catfish Industry Flounders Despite Congressional Support, Food Safety news.

Graham, K., 1999. A review of the biology and management of blue catfish. American Fisheries Society Symposium, 23: 37 - 49.

Hong, Y. H., Liu, T. M., Zhao, H.B., Xu, H.Y., Wang, W.J., Liu,R., Chen, T.S., Deng, J.R., Gui, Lacerda, S., Batlouni, S. R. Silva, S. B. G., Homem, C. S. P. 2006. Germ cells transplantation in fish: the Nile - tilapia model. Animal Reproduction Science, 3: 146 - 159.

Huang, Y. W., R. T. Lovell, R. A. Dunham. 1994. Carcass characteristics of channel and hybrid catfish, and quality changes during refrigerated storage. Journal of Food Science, 59: 64 - 66.

Lacerda, S., Batlouni, S. R., Costa, G. M. J., Segatelli, T. M. 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.

Lacerda, S., Costa, G. M., Campos - Junior, P. H., Segatelli, T. M., Yazawa, R., Takeuchi, Y., Morita, T., Yoshizaki, G.,França, L. R. 2013. Germ cell transplantation as a potential biotechnological approach to fish reproduction. Fish Physiology and Biochemistry, 39: 3 - 11.

Li, M. H., Robinson, E. H., Manning, B. B., Bosworth, B. G., Wolters, W. R. 2001.Comparison of growth, processing yield, and body composition of USDA103 and Mississippi ‘‘normal’’ strains of channel catfish *Ictalurus punctatus* fed diets containing three concentrations of protein. Journal of the World Aquaculture Society, 32: 402 - 408.

Li, M. H., Robinson, E. H., Manning,B. B., Yant, D. R., Chatakondi, N. G., Bosworth, B. G., Wolters, W.R. 2004. Comparison of the channel catfish, *Ictalurus punctatus* (NWAC103 Strain) and the channel X blue catfish, *I. punctatus* X *I. furcatus*, F1 hybrid for growth, feed Efficiency, processing yield, and body composition. Journal of Applied Aquaculture, 15: 63 - 71.

Lilyestrom, C. G., Wolters, W. R., Bury, D., Rezk, M., Dunham, R. A., 1999. Growth, carcass traits and oxygen tolerance of diploid and triploid catfish hybrids. North American Journal of Aquaculture, 61: 293 - 303.

Majhi, S. K., Hattori, R. S., Yokota, M., Watanabe, S., Strüssmann, C. A.. 2009. Germ cell transplantation using sexually competent fish: An approach for rapid propagation of endangered and valuable germlines. PLoS ONE, 4(7): e6132.

Masser, M., Dunham, R. A. 2012. Production of hybrid catfish. SRAC Publication No. 190.

Okutsu, T.,Yano, A.,Nagasawa, K., Shikina,S. ,Kobayashi, T., Takeuchi, Y., Yoshizaki, G. 2006. Manipulation of fish germ cell: visualization, cryopreservation and transplantation. Journal of Reproductive, 52: 685 - 693.

Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., Yoshizaki, G. 2006. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proceedings of the National Academy of Sciences, USA, 103(8): 2725 - 9.

Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y.,Yoshizaki, G. 2007. Production of trout offspring from triploid salmon parents. Science, 317: 1517

Perera, D. A. 2012. Studies for improvement of reproductive biotechnology for production of channel catfish (*Ictalurus punctatus*) female X blue catfish (*Ictalurus furcatus*) male hybrid embryos. PhD thesis. Auburn University, AL.

Saito, T., Goto - Kazeto, R., Arai, K., Yamaha, E. 2008. Xenogensis in teleost fish through generation of germ - line chimeras by single primordial germ cell transplantation. Biology of Reproduction, 78: 159 - 166.

Saito, T., Goto - Kazeto, R., Fujimoto, T., Kawakami, Y., Arai, K., Yamaha, E. 2010. Inter - species transplantation and migration of primordial germ cells in cyprinid fish. The International Journal of Developmental Biology, 54: 1481 - 1486.

Sakai, N. 2006. In vitro male germ cell cultures of zebrafish. Methods, 39: 239 - 245.

Shikina, S., Ihara, S., Yoshizaki, G. 2008. Culture conditions for maintaining the survival and mitotic activity of rainbow trout transplantable type A spermatogonia. Molecular Reproduction and Development, 75: 529 - 537.

Shikina S, Yoshizaki G. 2010. Improved in vitro culture conditions to enhance the survival, mitotic activity, and transplant ability of rainbow trout type A spermatogonia. Biology of Reproduction, 83: 268 - 276.

Takeuchi, Y. 2003. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biology of Reproduction, 69: 1142 - 1149.

Tave, D., McGinty, A., Chappell, J., and Smitherman, R. 1981. Relative harvestability by angling of blue catfish, channel catfish, and their reciprocal hybrids. North American Journal of Fisheries Management 1: 73 - 76.

Wolters, W. R., Wise, D. J., Klesius, P. H. 1996. Survival and antibody response of channel catfish, blue catfish, and channel catfish female × blue catfish male hybrids after exposure to *Edwardsiella ictaluri*. Journal of Aquatic Animal Health, 8: 249 - 254.

Yoshizaki, G., Takeuchi, Y., Kobayashi, T., Ihara, S., Takeuchi, T. 2002. Primordial germ cells: the blueprint for a piscine life. Fish Physiology and Biochemistry, 26: 3 - 12.

Yoshizaki, G., Takeuchi, Y., Kobayashi, T., Takeuchi, T. 2003. Primordial germ cell: a novel tool for fish bioengineering. Fish Physiology and Biochemistry, 28: 453 - 457.

Yoshizaki, G. 2005. Green fluorescent protein labeling of primordial germ cells using a nontransgenic method and its application for germ cell transplantation in *Salmonidae*. Biology of Reproduction, 73: 88 - 93.

Yoshizaki, G., Ichikawa, M., Hayashi, M., Iwasaki, Y., Miwa, M., Shikina, S., Okutsu, T. 2010. Sexual plasticity of ovarian germ cells in rainbow trout. Development, 137: 1227 - 123.

CHAPTER TWO

Germline Cell Morphology and Spermatogonial Stem Cell Isolation and Enrichment for Blue Catfish (*Ictalurus furcatus*)

Abstract

Blue catfish (*Ictalurus furcatus*) gonad morphological structure was studied histologically. The 90 ~ 100 - day - old fry (TL: 5 ~ 6 cm), two - year - old juvenile (TL: 25 ~ 30cm) and matured adult (TL: 65 ~ 70 cm) were examined by histological staining and light microscopy. Gondal tissue was dissected free from both male and female fish, and prepared for histology with hemotoxylin and eosin stain. Histology slides were observed under the microscope and the size of the germ cells was measured. Oogonia (12 ~ 15 μm diameter, distinct nucleus 7 ~ 8 μm) were found in all ages of female fish, while spermatogonia (12 ~ 15 μm, distinct nucleus 6 ~ 7.5 μm) were found in all ages of male fish. Germline stem cells were more numerous in younger blue catfish of both sexes. Spermatogonia and Sertoli cells were the only type of cells in the testes of male fry. The percentage of spermatogonia decreased progressively as the animals aged. Less than one percent of the cells of adult testes were spermatogonia. Male two - year - old juvenile blue catfish were chosen for purposes of developing a reliable isolation and purification scheme for gonadal cell lines. To determine the enzymatic efficiency in testicular tissue trypsinization, two different concentrations of trypsin were compared. The 0.25% Trypsin - EDTA showed higher efficiency and a higher amount of cell isolation than 0.05% trypsin - EDTA enzymatic digestion. A discontinuous density gradient of 70%, 45% and

35% of Percoll was used to enrich spermatogonia yields. Four distinct cell bands were generated after the centrifugation. From top of the centrifuge tube, the four cell bands were named as cell band I, cell band II, cell band III and cell band IV, successively. The cell band I was present on the top of 35% Percoll; cell band II was present in the middle of the 35% Percoll, while cell band III was present at the interface of 70% and 45% Percoll; cell band IV was the pellet on the bottom of the centrifuge tube. Microscopical observation showed that some of the spermatogonia, spermatocytes and spermatids adhered to each other and formed cell clusters in cell band I and cell band II. It was estimated that 55% of the total cells in cell band I were type A spermatogonia (diameter 12 ~ 15 μm) and type B spermatogonia (diameter 10 ~ 11 μm), 35% were spermatocytes (diameter 5 ~ 9 μm), and 10% were spermatids (diameter < 1 μm). In cell band II, most of the cells were spermatocytes (60%, diameter 5 ~ 9 μm), some were type B spermatogonia (30%), and a few were spermatids (10%); cell clusters could also be observed. The band III contained most of the spermatids (95%) and very few secondary spermatocytes (5%, diameter 5 ~ 8 μm). Red blood cells (95%) and a few spermatids (5%) were the predominant cells in the cell band IV.

Keywords: blue catfish, germline stem cell, spermatogonial stem cell, density gradient centrifugation, cell enrichment

Introduction

Germline Stem Cell Isolation

The following is a general methodology for the isolation of fish germline stem cells, as described by Saito (2008) and Lacerda (2010), which has eight steps, as follows: 1) In order to isolate the germline cell, the investigator must sacrifice the fish. The gonads are paired and locate in the posterior body cavity immediately ventral to the trunk kidney and swim bladder. 2) Gonadal tissues are dissected by scalpel from the fish peritoneal cavity. 3) Connective tissues are manually dissected away from the gonads. 4) The resulting cleaned tissues are rinsed in PBS (phosphate buffered saline) or HBSS (Hank's balanced salt solution) and 5) minced into small pieces by blade for purposes of for increasing the surface to volume ratio, which increases exposure of cellular surfaces to enzymes in the next stage of isolation. 6) Proper enzymatic digestion is the most important step in GSC isolation. Trypsin, collagenase or hyaluronidase must be added to ensure maximum dispersion of the gonadal tissues (Saito et al. 2008; Lacerda et al. 2010); fortunately these treatments do not affect cell morphology or viability. Appropriate pipetting and stirring during the enzymatic digestion can increase digestion efficiency. 7) Chelating agents, such as ethylenediamine tetraacetic acid (EDTA), bind divalent cations of extracellular matrix, thereby decoupling cell - cell connections and allowing the cells to be homogeneously dispersed. In the cell dissociation process, 8) DNase is often added as well to prevent the re - adhesion of already dissociated cells caused by released DNA.

For primordial germ cell (PGC) isolation, early stage embryos or genital ridges from fry, which were newly hatched or a few days old, were dissociated by enzyme treatment (Yoshizaki et al. 2003; Saito et al. 2008), and followed by gentle pipetting to generate cell suspension. For GSC isolation, the gonads were dissected from juvenile or adult fish and cleaned by eliminating the connective tissues. Gonadal tissues were minced into small pieces, and enzymatically digested by one or multiple enzymes (Trypsin, collagenase, actinase E, DNase, etc.) (Yoshizaki et al. 2005). Trypsin enzymatic dissociation was terminated by adding serum or culture medium with serum. Single cell

suspension was generated by filtering the digested tissues through a 40 ~ 50μm nylon mesh (Takeuchi et al. 2002; Majhi et al. 2009).

Primordial Germ Cell Enrichment

Flow cytometry sorting, including magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS), is a marker - based - method which requires cells having a known and manipulable surface marker or fluorescent signal. In mammals and birds, PGCs have been successfully isolated by MACS, which used antibodies against cell surface markers expressed specifically on the PGCs (Pesce and De Felici, 1995; Durcova - Hills et al. 1999; Mozdziak et al. 2005). But in fish, non PGC - specific cell surface markers have not yet been found.

FACS became a successful and efficient method used in PGCs isolation via transgenics in several fish species, such as medaka, zebrafish (Fan et al. 2008) and rainbow trout (Takeuchi et al. 2002). In these transgenic fish embryo, green or red fluorescent protein (GFP / RFP) was expressed specifically in PGCs, which directed the visualization of PGCs in developing embryos and during migration to genital ridge, and which also made PGCs available to be sorted by flow cytometry.

In zebrafish, the red fluorescent protein (RFP), under the control of the PGC - specific *vasa* promoter was highly expressed in PGCs since the 26 - somite stage of transgenic embryos (Fan et al. 2008). Gonadal regions from 1 - day - old embryos were treated by trypsin solution (0.5% trypsin and 1mM EDTA) followed by occasional gentle pipetting for 2 h to dissociate the cells. After filtering through 42 μm nylon mesh, cells were sorted by flow cytometry. The percentages of RFP - positive cells increased to 95% from initial 0.5% after flow cytometry sorting.

After the fusion construct of green fluorescent protein (*gfp*) and zebrafish *nos* 1 3' - untranslated region (UTR) mRNA was microinjected into 1 ~ 4 cell stage embryo of zebrafish, all the resulting embryos showed specific GFP labeling in PGCs, which continued to be visible for a short time period (Goto - Kazeto et al. 2010). At the somite stage, embryos were first placed in Ringer's solution and yolks were removed by excising the abdomen with fine forceps, and then embryos were treated by 200 ~ 400 U/mL

collagenases for 10 min at room temperature. After filtered through 42 μm nylon mesh, cells were sorted by flow cytometry.

During the observation of transgenic trout embryo, which carried a p*vasa* - GFP construct (the green fluorescent protein gene driven by the rainbow trout *vasa* - like gene regulatory regions), green fluorescent protein (GFP) was first expressed specifically in PGCs at the eyed stage. In this system, the expression level greatly increased at the hatching stage (Yoshizaki et al. 2000; Takeuchi et al. 2002). Cells from genital ridges of hatching embryos consisted of GFP - positive PGCs and GFP - negative somatic cells were sorted by flow cytometry based on fluorescence intensity. The percentages of GFP - positive cells, initially 0.5% of the total, increased to 99% of all of the cells in the preparation after flow cytometry sorting.

Spermatogonial Stem Cell Enrichment

In teleost fish, spermatogenesis takes place in cysts within the seminiferous tubules (Pudney. 1995; Schulz et al. 2005). The spermatogenic cyst is formed when Sertoli cells enclose single primary spermatogonium, which can self - renew and divide into differentiated germ cells. The germ cells divide synchronously to constitute an isogenic germ cell clone, bordered by the cytoplasmic extensions of a single layer of Sertoli cells (Schulz et al. 2005). The concentration of spermatogonial stem cells (SSCs) in the testis of adult fish is extremely low (< 1%) because of the numbers of differentiated spermatogonia generations are much higher in the seminiferous tubules compared to mammals.

Based on the density, size and adherent ability difference of spermatogonia, primary spermatocytes, diplotenic spermatocytes, secondary spermatocytes, spermatids, and testicular somatic cells, density gradient centrifugation and adherent difference sorting are the common methods used in the SSC enrichment.

Lacerda et al. (2006) used discontinuous Percoll density gradient centrifugation to enrich spermatogonia from sexually mature Nile - tilapia (Oreochromis niloticus). Four distinct cell bands were observed after the centrifugation. Cells in each band were histologically visualized. The two upper bands, which contained a high concentration of

spermatogonia, were used for transplantation. For loach (*Misgurnus anguillicaudatus*), a discontinuous density gradient (65%, 50%, 40%, 36%, 33%, 30%, 25%, and 20% Percoll solution in PBS) centrifugation was performed to enrich the early spermatogonia (Yoshikawa et al. 2009). Approximately 60% cells in the 36% ~ 30% Percoll fractions were type A and early type B spermatogonia. For carp spermatogonia enrichment, Ficoll gradient centrifugation by layering 4 mL carp (Labeo rohita) testicular cell suspension on the top of 3 mL Ficoll - Paque PLUS was carried out in a 15 mL centrifuge tube (Panda et al. 2011). After centrifugation, cells that have the similar size stayed in the same density of Percoll and from a cell layer. The germ cell populations containing early spermatogonia had lower density and were at the top and middle layers, whereas sperm and other somatic / blood cells were in the pellet. Density gradient centrifugation method is simple and easy method, but the SSCs may mix with differentiated cells which have the similar size after centrifugation.

Adherent difference sorting is also used in spermatogonia enrichment. It is based on cells different adhesion characteristics. Dissociated testicular cells suspension from 8 ~ 10 months old transgenic rainbow trout were cultured in 0.1% gelatin coated plates for 30 ~ 40 hr (Shikina et al. 2008; Shikina and Yoshizaki. 2010). The somatic cells first attached to the culture plate, whereas spermatogonia were suspended or weakly attached to the somatic cells or culture plates. Then the suspended spermatogonia were passaged into a new gelatin coated plate for another 30 ~ 40 hr. After two or three passages, the percentage of spermatogonia was > 95%, and the enriched spermatogonial population, containing few residual testicular somatic cells, was used as the initial material for spermatogonia culture studies.

The forward light - scatter (FS) and the side light - scatter (SS) are two indicators for cell size and granularity in application of flow cytometry sorting. Type A spermatogonia have distinctive FS and SS properties compared with other types of testicular cell (Kise et al. 2012; Yano et al. 2009). Based on this characteristic, type A spermatogonia have been previously isolated and enriched by flow cytometry in rainbow trout. FACS was also used to enrich SSCs in rainbow trout (Okutsu et al. 2006), However,

Okutsu and his colleagues did not follow through to make transgenic individuals or transgenic lines.

The objective of this research was to study the morphology of blue catfish gonads, and to identify the different gonadal cell type histologically in the different ages / sizes of blue catfish, thus confirm the best age of blue catfish for GSC isolation. Another objective was to enrich the SSCs of blue catfish by using Percoll discontinuous density gradient centrifugation. Enriched SSCs will be used for marker identification and transplantation studies.

Materials and Methods

Germline Stem Cells Morphology Study

Experimental Fish

Blue catfish fry that were 90 ~ 100 day - old (total length, TL: 5 ~ 6 cm), two - year - old juveniles (TL: 25 ~ 30 cm) and four - year - old mature adults (TL: 65 ~ 70 cm) were collected from the Fish Genetics Research Unit, School of Fisheries, Aquaculture and Aquatic Sciences (Auburn University, AL). All blue catfish were euthanized with tricaine methanesulfonate (MS - 222, Finquel) at 300 mg/L and left on ice prior to dissection. For fry, the whole fish were first fixed in Davidson's Fixative directly for 12 ~ 24 hr, and then the head, tail and the dorsal sections (above the vertebra) were removed. Only the ventral parts which contained gonadal regions and the associated connective tissue were placed in histology cassettes (VWR) and submerged in 10% neutral buffer formalin (NBF). Gonads of juveniles and adults were excised and placed into cassettes individually and immediately fixed in 10% NBF for more than 24 hr. The smaller juvenile testes from an individual fish were first wrapped in paper and then placed into a cassette to avoid losing the tissue during the dehydration process. All cassettes were labeled by a solvent resistant marker (MarkeLab).

Histology

Tissue - containing cassettes were gently transferred to 70% ethanol (Fisher Scientific) for 12 ~ 24 hr. Cassettes were then placed into Tissue Tek VIP model E150 automatic dehydrator (Sakura Finetek, Torrance, CA). Tissue dehydration was processed automatically through a series of ethanol gradients, 100% ethanol, a xylene - replacement clearing agent (HistoClear), and finally liquid paraffin (60 ℃, Thermo Scientific). Briefly, the dehydration process was as follows: 70% ethanol for 40 min; 95% ethanol for 40 min; 95% ethanol for 1 hr; 100% ethanol for 1 hr; 100% ethanol for 1 hr; 100% ethanol for 1.5 hr; xylene for 30min; xylene for 40min; liquid paraffin indefinitely till further processing. The cassettes were transferred to a Tissue - Tek Tissue Embedding Console System

(Sakura Finetek, Torrance, CA), and the tissues were embedded in paraffin. The tissue blocks were then carefully trimmed by removing excess paraffin to make sure that the tissue sample fit easily on the slide. Before microdissection, silane coated slides were prepared. Glass slides were cleaned with 70% ethanol, wiped with tissue paper (VWR) and air dried; then slides were placed in a glass slide holder (staining rack); 4 glass trays (Coplin jar) were filled with 2% silane solution (2.0 mL silane (Triangle Biomedical) dissolved in 100.0mL acetone), pure acetone, pure acetone and ddH_2O, respectively; the slide holders containing slides were submerged in the first and second glass trays in order for 1 min, followed by rinsing in acetone and in ddH_2O, finally left in a fume hood to let them air dry.

Tissue blocks were sectioned into 5 μm thick ribbons with an American Optical "820" Spencer Microtome. The ribbons were transferred to an adjacent Fisher Tissue Prep Flotation Bath model 135 set at 45 ℃. Selected tissue sections were then cut with a metal wire coated with Histosol (a xylene substitute; Fisher Scientific) and placed on silane coated slides. All slides were labeled with a solvent resistant marker. Slides were set vertical to air dry and then placed on a Fisher Slide Warmer set at 45 ℃ until totally dry. Before staining, slides were heated in a 600 ℃ oven for 15 to 30 minutes to remove the paraffin. The slides were then stained using hematoxylin and eosin stain by the method of Shehand and Hrapchak (1980). Stained slides were mounted with a Surgipath Micromount and covered with a coverslip. Slides were observed using an Olympus BX41 Fluorescence Microscope and imaged using a Nikon Coolpix 5.1 megapixel camera.

Spermatogonial Stem Cells Enrichment

Cell Isolation

Twelve male blue catfish (juveniles, TL: 25 ~ 30cm, two year old) were harvested and anesthetized with tricaine methanesulfonate (MS - 222, Finquel), then all catfish were incubated on ice and transferred to the lab. Twelve male fish were divided into two groups for enzyme digestion, and each group was divided into two replicates (three fish per replicate). Testicular tissues of group one were digested by 0.25% trypsin - EDTA, and testicular tissues of group two were digested by 0.05% trypsin - EDTA.

The surface of the fish was sterilized with 70% ethanol. The testes were removed from the peritoneal cavity, taking care to avoid the connective tissue, including the peritoneum and blood vessels. Testes of three fish from one replicate were measured and then placed in 5 mL of anti - agent medium (Hanks' Balanced Salt solution, HBSS, with 1.0 μg/mL $NaHCO_3$, 100 unit/mL penicillin and 100 μg/mL streptomycin) in a one 15 mL centrifuge tube whose weight had been measured before the testes were introduced. The weight of the centrifuge tube containing the testes was also measured. Weight of the testes was calculated by using the weight of the centrifuge tube containing the medium and testes minus the initial weight of the centrifuge tube containing only medium. Thereafter, the tubes were transferred into a biosafety cabinet (Labconco, Kansas City, MO) to undergo cleaning and sterilization.

Within the biosafety cabinet, testes from one tube were transferred into a plastic Petri dish and any connective tissue and blood was removed using a pair of scalpel blades #10 (Feather). The testes were rinsed three times by 1 mL anti - agent medium, and then soaked in 5 mL of 0.5% bleach solution (prepared fresh at 0.5% in ddH_2O from a newly opened bottle) for 2 min, followed by three rinses with HBSS. The testes were then minced into small pieces with a pair of sterilized blades. Minced testicular tissues of each replicate were transferred into one 50 mL autoclaved glass flask which contained a stir bar. The samples were treated as follows: two replicates in group one were treated by 0.25% trypsin - EDTA (2.5 g/L trypsin and 0.38 g/L EDTA; Gibco), and the volume of the trypsin - EDTA was 50 times of the weight of the testicular tissue for each replicate. All the conditions were the same for group two, but the concentration of trypsin - EDTA was 0.05% (0.5 g/L trypsin and 0.2 g/L EDTA; Gibco). All the samples were incubated on ice for 30 min followed by 1 hr at 22 ℃ with a magnetic stirrer to achieve higher digestion efficiency. The cell suspension from each replicate was then filtered using a 40 μm cell strainer (nylon mesh, Falcon) and centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet resuspended in 2 mL HBSS. Five μL of cell suspension were gently mixed with 45 μL Trypan blue. The concentration and viability of cells was measured by hemocytometer.

Density Gradient Centrifugation

A discontinuous density gradient was made by using three concentrations of Percoll, 2 mL of 70%, 45% and 35% each in HBSS, in a fetal bovine serum (FBS, Atlanta Biologicals) coated 15 mL centrifuge tube. One ~ 2 mL of 6 ~ 10 × 10^8 /mL cell suspension was placed on top of the Percoll gradient and centrifuged at 800 g for 40 minutes at 4 ℃. After centrifugation, the same cell bands came from the same replicate of fish were pooled together into 50 mL centrifuge tube. Two times volume of the cell suspension of HBSS was added into each tube. Centrifugation was carried out at 500 g for 5 min. Cell pellets were resuspended in 2 mL catfish GSC culture medium (Leibovitz's - 15 medium, Lonza) supplied with 25 mM HEPES, 100 unit/mL penicillin, 100 μg/mL streptomycin, 1.0 μg/mL $NaHCO_3$, 0.3 μg/mL L - glutamine, 20% FBS, 5% catfish serum and 1 ng/mL bFGF). Cell suspension - 5 μL of was gently mixed with 45 μL Trypan blue for cell concentration and viability measurement by hemocytometer. Cells from each band were observed using an inverted Nikon Eclipse TS100 microscope and imaged using a Nikon D40X DSLR camera.

Results

Germline Stem Cell Morphology

GSC in Blue Catfish Fry

The gonads of blue catfish located in the posterior body cavity, ventral to the truck kidney and swim bladder. They were attached to the mesenterium, connecting with parietal peritoneum, which in turn covered the ventral surface of the trunk kidney (Fig.1 A and C). In the ovaries of 90 ~ 100 day - old blue catfish fry, the basement lamina and oogonial nests were formed (Fig. 1B). Morphologically, the oogonia (Fig. 1B, arrow) still retained the PGC characteristics: large size (diameter: 12 ~ 15 μm) and large, distinct nucleus (7 ~ 8 μm). Early stage primary oocytes were in high number, and vitellogenesis was already started in some of the oocytes. Anaphase of the oogonia meiosis could easily be recognized in Fig. 1B (asterisk). In testes, type A undifferentiated spermatogonia and Sertoli cells were the only types of cells (Fig. 1D). Spermatogonia (Fig. 1D, arrow) had diameters of 12 ~ 15 μm and centrally located nuclei. The nucleolus had a prominent, centrally located nucleus and diameters of 6 ~ 7.5 μm.

GSCs Morphology of Juvenile Blue Catfish

In the ovaries of two - year - old juvenile blue catfish, oogonial nest was surrounded by a monolayer of granulose cells and filled with lamina (Fig. 2A). Oogonia were found in "nests" associated with the lining of the ovarian lumen (Fig. 2B, arrow). Oogonia had the same characteristics as those in blue fry (diameters of 12 ~ 15 μm, and centrally located nuclei, 6 ~ 7.5 μm). Subsequently, in the oogonial nest, primary oocytes and follicular secondary oocyte were the main cell types.

In male juveniles, testicular structures were elongated masses of villiform lobes (Fig. 2C). The whole tissue was solid, and a lumen hid not yet formed. Type A spermatogonia (Fig. 2D, arrow) were the largest cells. Type B spermatogonia had a little bit smaller cell size, oval to round shape, and clearly defined nuclei (Fig. 2D, asterisk). The density of primary spermatocytes was 172 cells / mm^3.

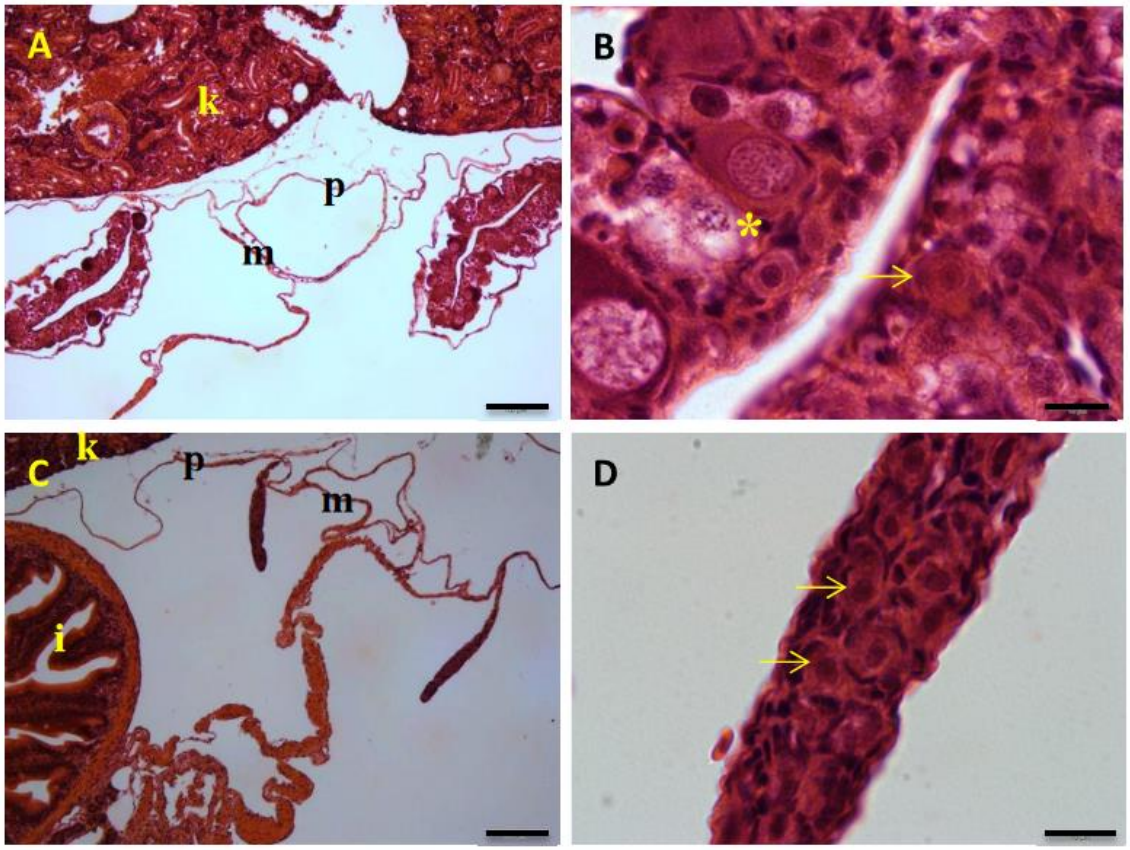

Fig. 1 Morphology of oogonia and type A spermatogonia in the gonads of 90 ~ 100 day - old blue catfish (*Ictalurus furcatus*) fry histologically. All figures were cross section. A) Paired ovaries of 90 ~ 100 day - old female blue catfish fry; ovaries were attached to mesenterium (m); the ventral surface of the trunk kidney (k) was covered by the parietal peritoneum (p). B) The magnification of 90 ~ 100 day - old blue catfish ovarian tissue; oogonia (arrow, →) and anaphase of the oogonia meiosis (asterisk, *) were observed. C) Paired testes of 90 ~ 100 day - old male blue catfish fry; testes were attached to mesenteries (m); the ventral surface of the trunk kidney (k) was covered by the parietal peritoneum (p); the intestine (i) was also showed. D) The magnification of 90 ~ 100 day - old blue catfish testicular tissue section; type A spermatogonia (arrow, →) were observed. Scale Bars A and C = 100 μm, B and D = 10 μm.

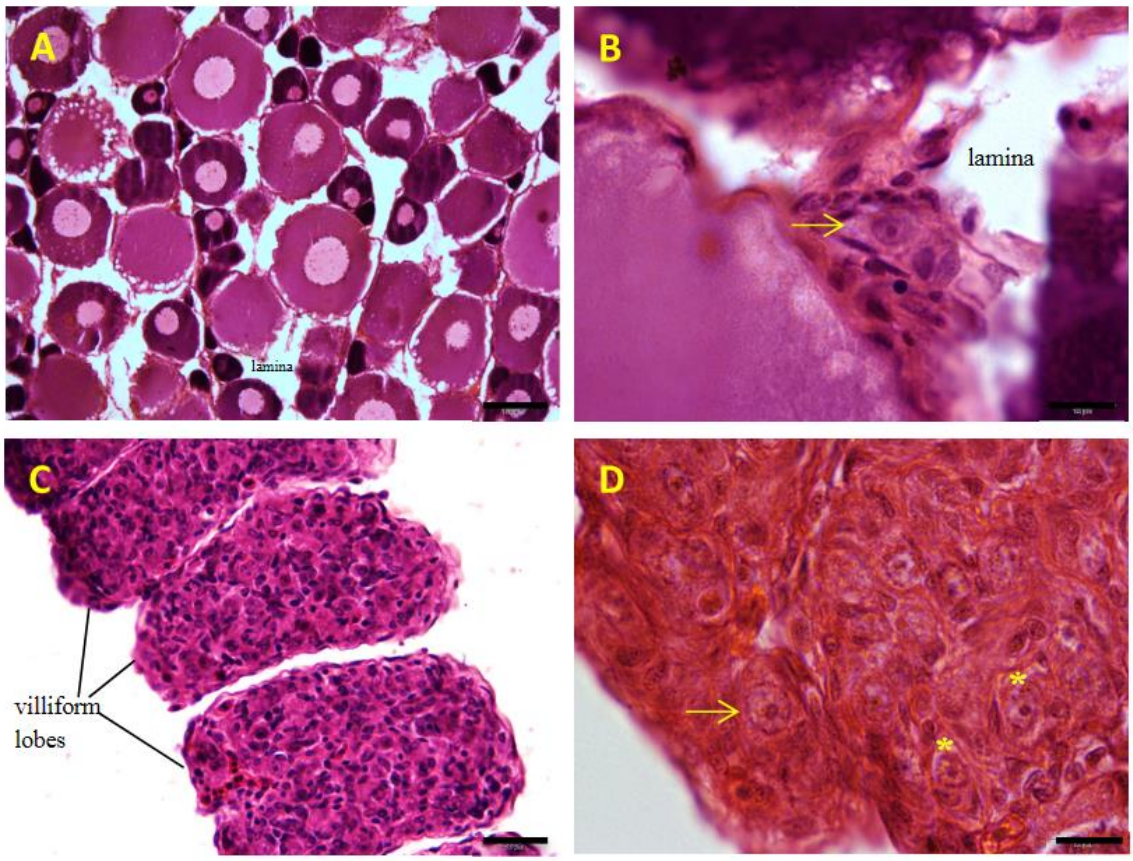

Fig. 2 Morphology of oogonia and spermatogonia in the gonads of two - year - old blue catfish (*Ictalurus furcatus*) juveniles histologically. All figures were longitudinal section. A) Sections of ovaries of two - year - old female blue catfish. B) The higher magnification of oogonia (arrow, →) in ovaries section; oogonial nest and lamina were formed in ovary. C) Sections of testes of two - year - old male blue catfish; testicular structures were elongated masses of villiform lobes. D) The higher magnification of type A spermatogonia (arrow, →) in testes section; type B spermatogonia (asterisk, *) were observed too. Bars A = 100 μm, C = 50 μm, B and D = 10 μm.

GSCs Morphology in Mature Adult Blue Catfish

In the ovaries of mature adult blue catfish, germ cells were arranged in lamellar structure, and immature eggs were formed (Fig. 3A). Oogonia were found that had the same characteristics as the ovaries of two - year- old juvenile blue catfish (Fig. 3B, arrow). Testes were still elongated masses of villiform lobes and each lobe and the base

of the lobes were white in color and full of milt (Fig. 3C). Spermatogonia were found in the basement membrane of spermatogenic cysts (Fig. 3D, arrow).

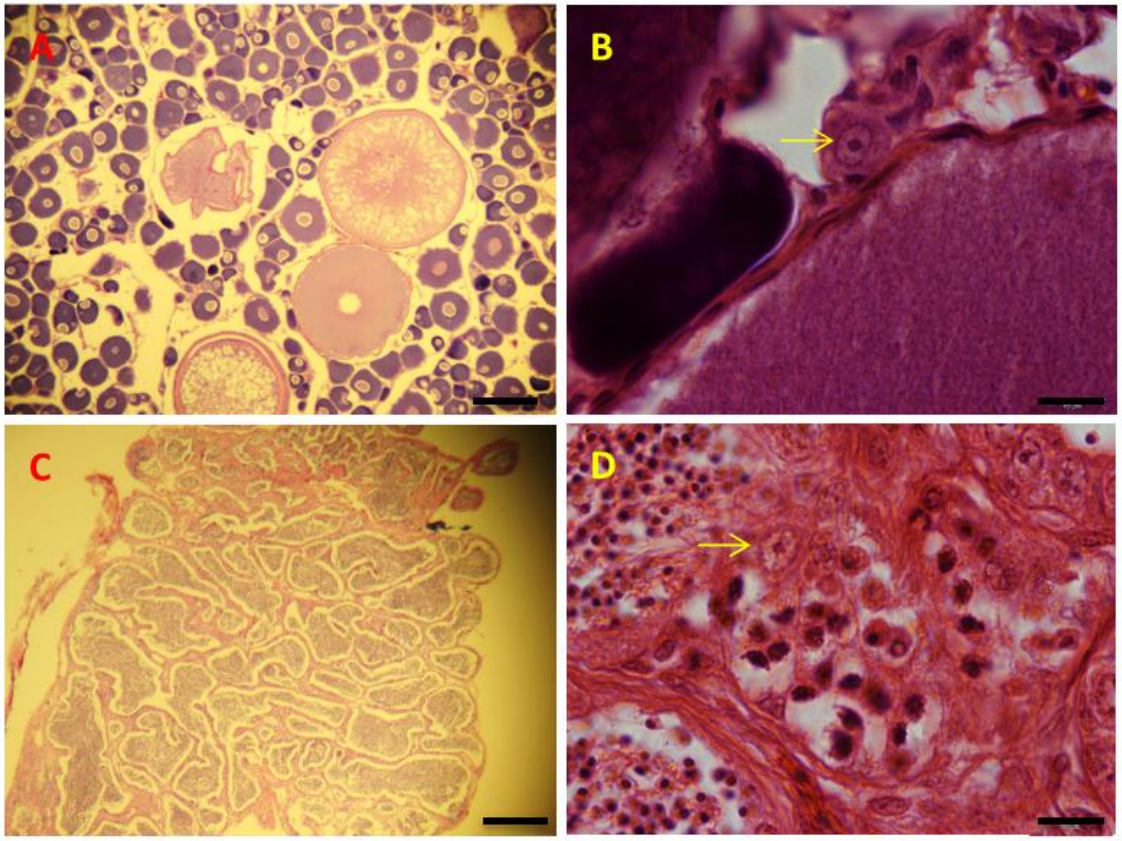

Fig. 3 Morphology of oogonia and spermatogonia in the gonads of five - year - old mature blue catfish (*Ictalurus furcatus*) adults histologically. All figures were longitudinal section. A) Sections of ovaries of five - year - old mature female blue catfish. B) The higher magnification of oogonia (arrow, →) in ovaries section. C) Sections of testes of five - year - old mature male blue catfish. D) The higher magnification of type A spermatogonia (arrow, →) in testes section. Bars A and C = 1000 μm, B and D = 10 μm.

Spermatogonial Stem Cell Enrichment

Enzymatic Efficiency by Using Different Concentrations of Trypsin

A Higher concentration of trypsin - EDTA, 0.25%, was used to digest testes from group one. A Lower concentration of trypsin - EDTA, 0.05%, was used to digest testes from group two. The testicular germ cells yield and cell viability are shown in Table 1. The result shows that we could isolate a higher number of cells (average 1.63×10^9) when testes were digested by 0.25% trypsin - EDTA, but the cell viability was low (average 85.1% cells alive), and 1.39×10^9 cells were alive. When testes were digested by 0.05% trypsin - EDTA, a lower amount of cells (average 3.89×10^5) were isolated with higher cell viability (average 98.4% cells alive), and 3.82×10^5 cells were alive. The higher concentration of trypsin - EDTA would cause more damage to the cells, but it was more efficient in testicular tissues digestion and achieve higher alive cells yield. The 0.25% Trypsin - EDTA was used in testicular tissues digestion in the following studies.

Density Gradient Centrifugation Enrichment

For cells isolated from group one fish, four distinct cell bands were generated in the tube after the Percoll density gradient centrifugation. The top band (Fig. 4B), which was named as band I, was present on the top 35% of Percoll; second band (Fig. 4C), which was usually very thin and not easy to be observed, was present in the middle of 35% Percoll and named as band II; the third band (Fig. 4D), was present in the top of 70% Percoll and named as band III; pellet on the bottom of the tube was considered as the fourth band and named as band IV. The cell types and the estimated percentage of each type of the cells are shown in Table 2. The exact percentage of each type of the cells was hard to determine because some of the spermatogonia, spermatocytes and spermatids adhered to each other and formed cell clusters. The cell band I contained a high percentage of type A spermatogonia (40%, diameter 12 ~ 15 μm, Fig. 4B, arrows), type B spermatogonia (15%, diameter 10 ~ 11 μm, asterisk) and primary spermatocytes (35%, diameter 8 ~ 9 μm, arrowhead). About 55% of the total cells were type A spermatogonia and type B spermatogonia. In cell band II, most of the cells were spermatocytes (60%, diameter 5 ~ 9 μm, Fig. 4C, arrowhead), some were type B spermatogonia (30%), a few

were spermatids (10%) and cell clusters could also be observed. The cell band III contained most of the spermatids (95%, diameter < 1 μm) and a very few secondary spermatocytes (5%, diameter 5 ~ 8 μm, fig. 4D, arrowhead). Red blood cells (95%, Fig. 4D, arrowhead) and few spermatids (5%) were the predominant cells in the cell band IV.

Cells from group two were sorted by the Percoll density gradient centrifugation too. Cell concentration and viability of different cell bands after the Percoll density gradient centrifugation were shown in Table 3. Cell viability was decreased after the density gradient centrifugation. For group one testes which were digested by 0.25% trypsin - EDTA, cell viability average was 76.0% in the cell band I and 74.6% in the cell band II. For group two testes which were digested by 0.05% trypsin - EDTA, the average cell viability was 86.3% in the cell band I and 88.1% in the cell band II.

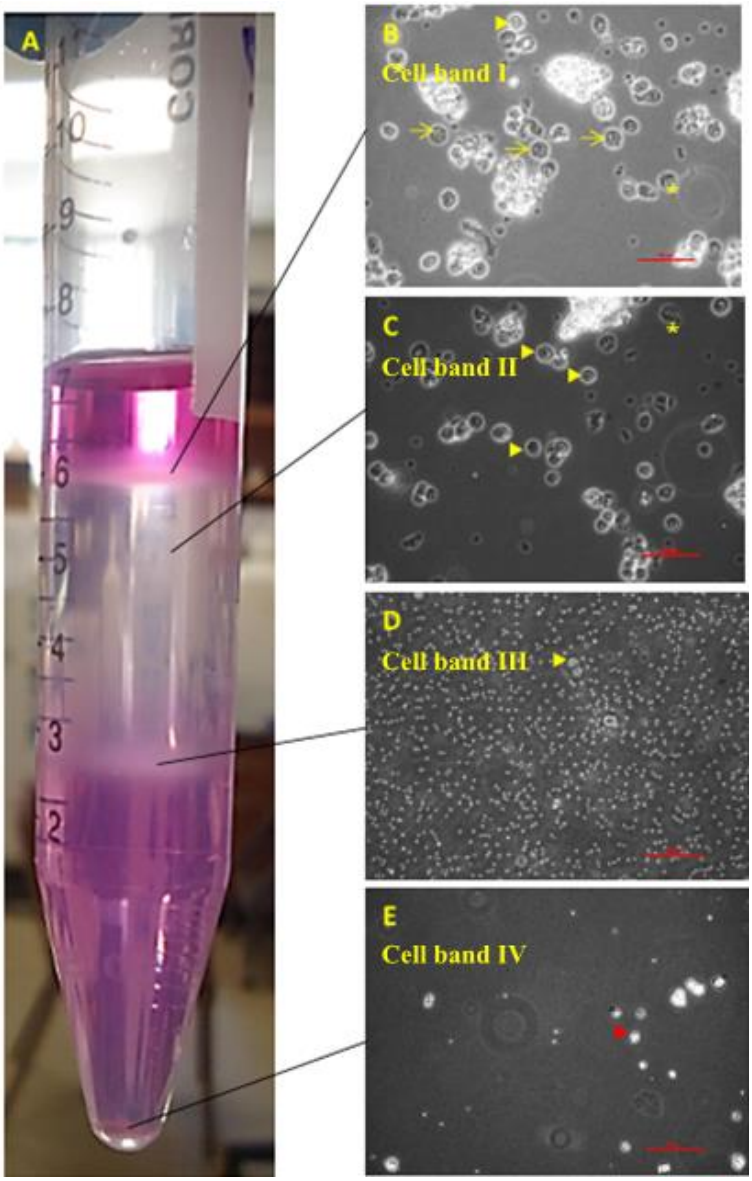

Fig. 4 Enrichment of SSCs from testes of two - year - old juvenile blue catfish (*Ictalurus furcatus*) utilizing Percoll density gradient centrifugation. A) After the Percoll density gradient centrifugation, four distinct cell bands were present in the centrifuge tube. B) The cells present in the upper - cell band I contained higher percentage (about 55%) of type A spermatogonia (arrows, →), type B spermatogonia (asterisk, *) and some primary spermatocytes (arrowhead). C) The cells present in the cell band II contained higher percentage of type B spermatogonia (asterisk, *) and some primary spermatocytes (arrowhead). D) The cells present in the cell band III contained secondary spermatocytes (arrowhead) and most of the spermatids. E) In the pellet - cell band IV, red blood cells (arrowhead) and few spermatids were the predominant cells. Bars B, C and D = 50 μm, E = 100 μm.

Table 1 Testicular germ cell yield and cell viability when testes from two - year - old male juvenile blue catfish (*Ictalurus furcatus*) were digested by different concentrations of trypsin. Each replicate contained testicular tissues from three two - year - old male juvenile blue catfish. Testicular tissues of two replicates in group one were digested by 0.25% trypsin - EDTA, and testicular tissues of two replicates in group two were digested by 0.05% trypsin - EDTA.

Blue Catfish Testes		Total Length (cm)	Standard Length (cm)	Testes Weight (g)	Concentration of Trypsin	Number of Isolated Testicular Germ Cells	Percentage of live cells	Total live Cell Number
Group 1	replicate 1	25.9 ± 1.88	20.1 ± 1.34	0.347	0.25%	1.56×10^9	83.5%	1.30×10^9
	replicate 2	25.8 ± 1.23	21.6 ± 0.36	0.396	0.25%	1.69×10^9	86.7%	1.47×10^9
Group 2	replicate 1	26.6 ± 1.12	21.7 ± 0.67	0.412	0.05%	4.05×10^5	98.2%	3.98×10^5
	replicate 2	25.8 ± 0.61	21.4 ± 0.38	0.358	0.05%	3.72×10^5	98.5%	3.66×10^5

Table 2 Four cell bands which contained different types of testicular germ cells from group one two - year - old male juvenile blue catfish (*Ictalurus furcatus*) generated after Percoll discontinuous density gradient centrifugation. Testes were digested by 0.25% trypsin - EDTA. From top of the centrifuge tube, the four cell bands were named as cell band I, cell band II, cell band III and cell band IV, successively[1].

	The Estimated Percentage of Different Cell Types after the Percoll Density Gradient Centrifugation				
Cell Band	Type A Spermatogonia	Type B Spermatogonia	Spermatocytes	Spermatids	Red Blood Cells
Initial Cell Mixture	10%	10%	60%	20%	0
Cell Band I	40%	15%	35%	10%	0
Cell Band II	0	30%	60%	10%	0
Cell Band III	0	0	5%	95%	0
Cell Band IV	0	0	0	5%	95%

[1] Discontinuous density gradient was made by 70%, 45% and 35% of Percoll. After the centrifugation, the top band, which was named as the cell band I, was present on the top 35% of Percoll; the cell band II was present in the middle of 35% Percoll; the cell band III, was present in the top of 70% Percoll; pellet on the bottom of the tube was considered as the cell band IV.

Table 3 Two - year - old male juvenile blue catfish (*Ictalurus furcatus*) testicular germ cells concentration and viability in different bands after Percoll discontinuous density gradient centrifugation. Each replicate contained testicular tissues from three two - year - old male juvenile blue catfish. Testicular tissues in group one were digested by 0.25% trypsin - EDTA, and testicular tissues in group two were digested by 0.05% trypsin - EDTA[2].

		Cell Band I		Cell Band II		Cell Band III	Cell Band IV
Blue Catfish Testes		Concentration (/mL)	Viability	Concentration (/mL)	Viability	Concentration (/mL)	Concentration (/mL)
Group 1	replicate 1	1.89×10^8	73.4%	4.77×10^7	75.4%	6.37×10^8	6.37×10^2
	replicate 2	1.64×10^8	78.6%	4.28×10^7	73.8%	6.25×10^8	7.25×10^2
Group 2	replicate 1	7.53×10^4	85.2%	1.86×10^4	87.6%	3.24×10^5	6.46×10^2
	replicate 2	7.44×10^4	87.3%	1.74×10^4	88.5%	2.98×10^5	6.88×10^2

[2]In the cell band I, 55% of the cells were type A spermatogonia and type B spermatogonia, 35% of the cells were spermatocytes, and the rest of the cells were spermatids. In the cell band II, 30% of the cells were type B spermatogonia, 60% of the cells were spermatocytes and the rest of the cells were spermatids. In the cell band III, 5% of the cells were spermatocytes and 95% of the cells were spermatids. In the cell band IV, 95% of the cells were red blood cells and 5% of the cells were spermatids

Discussion

Gonad Morphological Structure of Different Ages of Blue Catfish

This is the first report of gonad morphological structures in different ages of blue catfish fish, and the corresponding distribution of oogonia and spermatogonia in the gonads of different aged fish. Based on the ovaries and testes morphological structural differences in 90 ~ 100 day - old blue catfish fry, sex differentiation in female is earlier than in male. In ovaries, oogonia differentiated to early stage primary oocytes and vitellogenesis was already started in some of the oocytes. However in testes, type A undifferentiated spermatogonia and Sertoli cells were the only types of cells. Blue catfish early stage sex differentiation was similar to channel catfish. Female ovarian activity and ovarian meiosis were first observed at 22 day - post - fertilization and male lobule structures were observed between 90 and 102 day - post - fertilization (Patino et al. 1996).

In the ovaries of two - year - old juvenile blue catfish, the oogonial nest was surrounded by a monolayer of granulosa cells filled with lamina as was found in adult zebrafish (Selman et al. 1993). Oogonia were found in "nests" associated with the lining of the ovarian lumen. Oogonia were very few compared to other germ cells in the ovaries. Primary oocytes and follicular secondary oocytes were the predominant cell types. As found in channel catfish (*I. punctatus*) testes, blue catfish testes were villiform lobed structures. Grizzle et al. (1976) described the structure of channel catfish testes as lobate, consisting of numerous finger - like projections. Type A spermatogonia, type B spermatogonia and primary spermatocytes were easily recognized. Again, no differences were observed between blue catfish development and that observed by Grizzle et al. (1976) for channel catfish.

Similar to the findings for channel catfish (Grizzle et al. 1976), lamellar structure were seen in longitudinal sections of the ovaries of mature adult blue catfish, and immature eggs were formed. A very low amount of oogonia was also found in both species. In adult channel catfish spermatids and abundant mitotic figures were present in the seminiferous tubules of adults during all seasons (Grizzle et al. 1976). In testes of

mature adult blue catfish, spermatogenic cysts were also full of spermatids, and spermatogonia could be found in the basement membrane of spermatogenic cysts.

Based on the morphological characteristics of the testes from different ages of fish, the percentage of spermatogonia in juvenile blue catfish testes was much higher than adults. Even though testes of the 90 ~ 100 day - old fry contain only spermatogonia, the testes (about 200 μm) were too small to dissect. This makes juveniles the logical choice from which to harvest the greatest number of SSCs.

Enzymatic Efficiency

In tissue dissociation, the type of the enzyme and the concentration of the enzyme are determined by the type of the tissue. For 1 ~ 8 - cell stage zebrafish embryos digestion, 0.1% trypsin, 0.1% actinase E and 0.4% urea were used (Higaki et al. 2010). In rainbow trout PGCs isolation, genital ridges were digested by 0.5% trypsin (Takeuchi et al. 2002, Kobayashi et al. 2004). In Nile tilapia spermatogonia isolation, the testes from adult males were digested by 0.25% trypsin/1 mM EDTA and 0.03% DNase I. Collagenase, 500 units/mL, was used in carp, *Labeo rohita*, testicular tissue digestion (Panda et al. 2011).

When testes from two - year - old male juvenile blue catfish were digested with a higher concentration of trypsin - EDTA, higher germ cell yield was achieved, but the cell viability was lower. Lower concentrations of trypsin - EDTA cannot completely digest the testes and a low cell yield was harvested. The 0.25% trypsin - EDTA was much better than 0.05% for testicular tissues digestion as 0.25% trypsin - EDTA yielded almost 10^4 more live cells than the 0.25% trypsin - EDTA treatment. Digestion efficiency of different enzymes on catfish testes should be compared in future research.

Density Gradient Centrifugation for SSCs Enrichment

Three different concentration of Percoll, 70%, 45% and 35%, were used in this experiment, resulting in four distinct cell bands. However, Yoshikawa et al. (2009) reported three bands were harvested in loach (*Misgurnus anguillicaudatus*) using density

gradient of Percoll centrifugation (65%, 50%, 40%, 36%, 33%, 30%, 25%, and 20% Percoll solution in PBS).

The cell types of our study were in agreement with Yoshikawa et al. (2009). The cell band I contained high percentage of type A spermatogonia, type B spermatogonia and primary spermatocytes; the cell band II contained high percentage of spermatocytes and few spermatogonia; the cell band III contained most of the spermatids and very few secondary spermatocytes; the cell band IV contained red blood cells and few spermatids. In loach, the cell types in the three layers were described as follows: type A and early type B spermatogonia were in the 36% ~ 30% Percoll; spermatids, spermatocytes, and late type B spermatogonia were in the 40% Percoll fraction; spermatozoa and a few erythrocytes were in the 65% Percoll. In the model of Nile tilapia spermatogonia enrichment, four distinct cell layers were observed after the Percoll centrifugation (Lacerda et al. 2006). However, the gradient of Percoll used was unclear.

For blue catfish, more than 55% of the total cells in top two bands in the 35% Percoll gradient were type A spermatogonia and type B spermatogonia. This was similar to the result of Yoshikawa et al. (2009) that approximately 60% type A and early type B spermatogonia were harvested in the 36% ~ 30% Percoll. Considering the expense of Percoll used, time of isolation, target cells in the isolation; it was more cost effective and less time consuming applying this protocol. The spermatogonia and spermatocytes were easily adhering to each other and forming cell clusters. This has not been reported before.

Percoll density gradient centrifugation was a good method for SSCs enrichment, but it was not efficient. The percentage of spermatogonia was still low in comparison with that from flow cytometry sorting. When using flow cytometry to sort the GFP - positive PGCs from transgenic trout embryo which carried p*vasa* - GFP construct (the green fluorescent protein gene driven by the rainbow trout *vasa* - like gene regulatory regions) (Yoshizaki et al. 2000; Takeuchi et al. 2002). The percentage of GFP - positive cells, initially 0.5%, was increased to 99% after flow cytometry sorting. When using flow cytometry to sort the GFP - positive SSCs from 8 ~ 12 month old *vasa* - GFP transgenic rainbow trout, the percentage of GFP - positive cells was increased to 90% (Kise et al. 2012). However, flow cytometry sorting required further efforts of making transgenic

individuals or transgenic lines which need high technology and long time. Therefore, density gradient centrifugation was appropriate method for catfish SSCs enrichment.

References

Durcova - Hills, G., Tokunaga, T., Kurosaka, S., Yamaguchi, M., Takahashi, S., Imai, H. 1999. Immunomagnetic isolation of primordial germ cells and the establishment of embryonic germ cell lines in the mouse. Cloning, 1: 217 - 224

Fan, L., Moon, J., Wong, T., Crodian, J., Collodi, P. 2008. Zebrafish primordial germ cell cultures derived from *vasa* : RFP transgenic embryos. Stem Cells and Development, 17: 585 - 598

Goto - Kazeto, R., Saito, T., Takagi, M., Arai, K., Yamaha, E. 2010. Isolation of teleost primordial germ cells using flow cytometry. The International Journal of Developmental Biology, 54: 1487 - 1492.

Grizzle, J. M., Rogers, W. A. 1976. Anatomy and Histology of the Channel Catfish. U.S. Department of Commerce, National Oceanic and Atmospheric Administration, p. 66 - 70.

Higaki, S., Eto, Y., Kawakami, Y., Yamaha, E., Kagawa, N., Kuwayama, M., Nagano, M., Katagiri, S., Takahashi, Y. 2010. Reproduction. Production of fertile zebrafish (*Danio rerio*) possessing germ cells (gametes) originated from primordial germ cells recovered from vitrified embryos. 139(4): 733 - 40.

Kise, K., Yoshikawa, H., Sato, M., Tashiro, M., Yazawa, R., Nagasaka, Y., Takeuchi, Y., Yoshizaki, G. 2012. Flow - cytometric isolation and enrichment of teleost type A spermatogonia based on light - scattering properties. Biology of Reproduction, 86: 107, 1 - 12.

Kobayashi, T., Yoshizaki, G., Takeuchi, Y., Takeuchi, T. 2004. Isolation of highly pure and viable primordial germ cells from rainbow trout by GFP - dependent flow cytometry. Molecular Reproduction and Development, 67(1): 91 - 100.

Lacerda, S. M. S. N., Batlouni, S. R., Silva, S. B. G., Homem, C. S. P., França, L. R. 2006. Germ cells transplantation in fish: the Nile - tilapia model. Animal Reproduction Science, 3: 146 - 159.

Majhi, S. K., Hattori, R. S., Yokota, M., Watanabe, S., Strüssmann, C. A. 2009. Germ cell transplantation using sexually competent fish: an approach for rapid propagation of endangered and valuable germlines. PLoS ONE, 4 (7): e6132.

Mozdziak, P. E., Angerman - Stewart, J., Rushton, B. , Pardue, S. L., Petitte J. N. 2005. Isolation of chicken primordial germ cells using fluorescence - activated cell sorting. Poultry Science, 84: 594 - 600.

Okutsu,T., Yano, A., Nagasawa, K., Shikina,S., Kobayashi, T., Takeuchi, Y., Yoshizaki, G., 2006. Manipulation of fish germ cell: visualization, cryopreservation and transplantation. Journal of Reproduction and Development, 52: 685 - 693

Panda, R. P., Barman, H. K., Mohapatra, C. 2011. Isolation of enriched carp spermatogonial stem cells from *Labeo rohita* testis for in vitro propagation. Theriogenology, 76: 241 - 251

Patino, R., Davis, K. B., Schoore, J. E., Uguz, C., Struessmann, C. A., Parker, N.C., Simco, B.A., Goudie, C.A. 1996. Sex differentiation of channel catfish gonads: Normal development and effects of temperature. Journal of Experimental Zoology, 276: 209 - 218.

Pesce, M., De Felici, M., 1995. Purification of mouse primordial germ cells by MiniMACS magnetic separation system. Developmental Biology, 170: 722.

Pudney, J. 1995. Spermatogenesis in nonmammalian vertebrates. Microscopy Research and Technique, 32: 459 - 497.

Saito, T., Goto - Kazeto, R., Arai , K., Yamaha, E. 2008. Xenogenesis in teleost fish through generation of germ - line chimeras by single primordial germ cell transplantation. Biology of Reproduction, 78: 159 - 166.

Schulz, R.W., Menting, S., Bogerd, J., França, L.R., Vilela, D.A., Godinho, H.P. 2005. Sertoli cell proliferation in the adult testis - evidence from two fish species belonging to different orders. Biology of Reproduction, 73: 891 - 898.

Selman, S., Wallace, R.A., Sarka, A., Qi, X. 1993. Stages of oocyte development in the zebrafish, *Brachydanio rerio*. J. Morphology, 218: 203 - 224.

Sheehan, D. C., Hrapchack, B. B.1980. Theory and Practice of Histotechnology, 2nd ed. Mosby C, St Louis, p. 196 - 197.

Shikina, S., Ihara, S., Yoshizaki, G. 2008. Culture conditions for maintaining survival and mitotic activity of rainbow trout transplantable A - type spermatogonia. Molecular Reproduction and Development, 75: 529 - 537.

Shikina, S., Yoshizaki, G. 2010. Improved *in - vitro* culture conditions to enhance the survival, mitotic activity and transplatability of rainbow trout type A spermatogonia. Biology of Reproduction, 83: 268 - 276

Takeuchi, Y., Yoshizaki, G., Kobayashi, T., Takeuchi, T. 2002. Mass isolation of primordial germ cells from transgenic rainbow trout carrying the green fluorescent protein gene driven by the *vasa* gene promoter. Biology of Reproduction, 67: 1087 - 1092.

Yano, A., von Schalburg, K., Cooper, G., Koop, B.F., Yoshizaki ,G. 2009. Identification of a molecular marker for type A spermatogonia by microarray analysis using gonadal cells from p*vasa* - GFP transgenic rainbow trout (*Oncorhynchus mykiss*). Molecular Reproduction and Development, 76: 246 - 254.

Yoshizaki, G., Takeuchi, Y., Sakatani, S., Takeuchi, T. 2000. Germ cell - specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout vasa-like gene promoter. The International Journal of Developmental Biology, 44: 323 - 326.

Yoshizaki, G., Tago, Y., Takeuchi, Y., Sawatari, E., Kobayashi, T., Takeuchi, T. 2005. Green fluorescent protein labeling of primordial germ cells using a nontransgenic method and its application for germ cell transplantation in salmonidae. Biology of Reproduction, 73: 88 - 93.

Yoshizaki, G., Takeuchi, Y., Kobayashi, T., Takeuchi, T. 2003. Primordial germ cell: a novel tool for fish bioengineering. Fish Physiology and Biochemistry, 28: 453 - 457.

Yoshikawa, H., Morishima, K., Fujimoto, T., Saito, T., Kobayashi, T., Yamaha, E., Arai, K. 2009. Chromosome doubling in early spermatogonia produces diploid spermatozoa in a natural clonal fish. Biology of Reproduction, 80: 973 - 979.

CHAPTER THREE

Gene Expression of Blue Catfish (*Ictalurus furcatus*) and Channel Catfish (*I. punctatus*) Spermatogonial Stem Cell

Abstract

Percoll density gradient centrifugation of channel and blue catfish testicular germ cells generated distinct banding patterns containing unique cell assemblages. Unique gene expression patterns were revealed by qRT - PCR for each of the density gradient bands. Expression of seventeen genes was examined; they were as follows: pfkfb4, urod, Oct4, Plzf, sycp3, SOX2, Integrin6, neurogenin3, ID - 4, integrinV, Thy1, GFRα, CDH1, Smad5, Pum2 and Prdm14 and Kit. Relative gene expressions in putative spermatocyte cells from cell band II and putative spermatid cells from cell band III were calculated as fold changes over the putative spermatogonial cells of cell band I. Three kinds of expression patterns were observed in channel catfish. pfkfb4, urod, Plzf, Integrin6, ID - 4, integrinV, Thy1 and CDH1 gene expressions were down - regulated in putative spermatocyte cells of band II and even stronger down - regulation in putative spermatid cells of band III. SOX2 and GFRα gene expressions were up - regulated in putative spermatocyte cells of band II and even greater up - regulation in spermatid cells of band III. Oct4, Pum2 and Prdm14 gene expressions were highly up - regulated in spermatocyte cells of band II, but less strongly up - regulated in spermatid cells of band III. For the expression pattern in different blue catfish testicular cells, three expression patterns were found as well. The first two trends of gene expression showed a similar pattern as that of channel catfish. pfkfb4, urod, Oct4, Plzf, sycp3, Kit, Integrin6, integrinV, Thy1, GFRα,

CDH1 and Pum2 gene expressions were down - regulated in spermatocyte cells of band II and even stronger down - regulation in spermatid cells of band III. Neurogenin3 and SOX2 gene expressions were up - regulated in spermatocyte cells of band II and even higher up - regulation in spermatid cells of band III. The third trend of gene expression was different from channel catfish. ID - 4, Smad5 and Prdm14 gene expression were strongly down - regulated in spermatocytes cells band II, but up - regulated in spermatid cells of band III. pfkfb4, urod, Plzf, Integrin6, ID - 4, integrinV, Thy1 and CDH1 genes showed the same expression change pattern in different types of testicular germ cells of both channel and blue catfish. They were all down - regulated in spermatocytes and spermatids. Specially, Plzf and Integrin6 were strongly (more than 10 fold change) down - regulated ($p < 0.05$), and they were identified as spermatogonia markers. SOX2 gene showed the same expression change pattern in testicular germ cells of both channel and blue catfish. It was up - regulated in spermatocytes and even more highly up - regulated in spermatids, which indicated it could be the spermatids marker. Oct4, ID - 4, GFRα, Pum2 and Prdm14 genes showed different expression patterns in the testicular germ cells of channel and blue catfish.

Keywords: Spermatogonial stem cell, Gene expression, Marker gene

Introduction

Fish Germ Cells

Primordial Germ Cell (PGC)

In fishes, as in many other organisms, primordial germ cells (PGCs) are the earliest germ cell form. They are formed before gastrulation, at an earlier time and in a distinct position from where the gonad develops. During embryogenesis, the PGCs pass through various organs, and finally migrate to the genital ridge (Xu et al. 2010). Based on this characteristic, there are three stages of PGCs - premigratory, migratory and postmigratory. Once they reach the genital ridge, PGCs combine with the somatic cells to form gonads (Xu et al. 2010). PGCs differentiate into either spermatogonia or oogonia after gonadal sex differentiation (Yoshizaki et al. 2010). Eventually, PGCs give rise to gametes that can pass genetic information from generation to generation *via* the fertilization process. Fish become sterile if PGC migration was prevented during embryogenesis (Weidinger et al. 2003; Slanchev et al. 2005). Therefore, PGCs, which are primordial stem cells, have unique properties that can be exploited for fish bioengineering. PGCs have several distinct morphological characteristics, such as large size (diameter 15 ~ 20μm), high nuclear - cytoplasmic ratio (large nuclei: 6 ~ 10μm and relatively little cytoplasm), one or two prominent nucleoli, and "nuage" - a germ line - specific electron - dense structure formed by association between RNA and proteins (Schulz et al. 2010). Based on morphological characteristics, PGCs had been observed in different fish species at different stages of embryonic development by light and electron microscopy.

PGC migration study in fish was revolutionized by the cloning of the *vasa* gene (Olsen et al. 1997; Yoon et al. 1997). *Vasa* transcript was first detected by *in situ* hybridization in four strips at the edges of the first two cleavage planes of 4 - cell stage zebrafish embryos. At the 32 - cell stage, the transcript was detected in four cell clusters which subsequently developed from four cells and located close to the blastoderm margin at late blastula stages. At 24 hours of post - fertilization, two bilateral rows formed by

PGCs were found around the anterior end of the yolk extension (Yoon et al. 1997). In medaka, *vasa* RNA didn't show cell - specific expression in cells of the blastomere at early developmental stages. At the late gastrula stage, specific expression was observed only in germline cells located at the posterior shield. PGCs were visualized in transgenic zebrafish, medaka, rainbow trout and Nibe croaker (*Nibea mitsukurii*) during early transgenic embryonic development (Koprunner et al. 2001; Kurokawa et al. 2006; Yoshizaki et al. 2005).

In catfish, PGCs were characterized by their large size (approximately 12 μm diameter) and large, distinct nucleus (7 ~ 8 μm) with prominent, centrally located nucleolus and fine chromatin filament (Grizzle 1985). Patifio et al. (1996) reported that PGCs had been found in the undifferentiated gonad of 7 day post - fertilization channel catfish fry at 28 ℃ by light microscopy. Early PGCs started differentiating in ovaries of female at 22 day post - fertilization, and male lobule structures were observed between 90 and 102 day post - fertilization. Slanchev et al. (2005) determined that the mechanism of sexual fate of PGCs in channel catfish was different from that of zebrafish. In channel catfish, the undifferentiated gonads gave rise to ovaries in females and testes in males. But in zebrafish, an ovary - like structure formed in all embryos would develop into ovaries in females or into testis in males if the oocytes died.

Testicular Germ Cells

Compared to amniotic vertebrates' (reptiles, birds, mammals) non - cystic spermatogenesis, fish has the cystic type of spermatogenesis (Callard 1996), which contains two unique testicular structures. First, within the spermatogenic tubules, synchronously, each spermatogonium is enveloped by Sertoli cells to form a cyst from which germ cells are derived clonally. Second, in adult fish, the cysts retain their capacity to proliferate (Schulz et al. 2005). Hence, a spermatogenic cyst is the basic functional unit of the spermatogenesis in fish. In a typical histological micrograph of fish testes, one observes multiple different cysts in different stages of development. The different stages of development are evident by their different sized groups of germ cells.

Based on their morphology and location in teleosts, spermatogonia, which are always completely surrounded by Sertoli cells, can be classified in two different types, type A spermatogonia and type B spermatogonia. Type A spermatogonia have two types, the undifferentiated type A spermatogonia and the differentiated type A spermatogonia. Undifferentiated type A spermatogonia are the largest germ cell in the testis, have large nuclei with one to three nuclear bodies, and contain large amounts of "nuage" as PGCs (Hogan 1973; Hamaguchi 1982; Billard et al. 1986). Undifferentiated type A spermatogonia are spermatogonial stem cells (SSCs), which can self - renew and produce differentiated type A spermatogonia. Differentiated type A spermatogonia have almost the same morphological characteristics as undifferentiated type A spermatogonia, but contain no or very little nuage - like material and have less potential to self - renew. During further maturation and division, differentiated type A spermatogonia give rise to type B spermatogonia, which are irreversibly determined. Type B spermatogonia have a smaller cell size and an oval to round clearly defined nuclei surrounded by a small rim of cytoplasm (Billard 1986). Type B spermatogonia divide much more rapidly and usually have several generations in one cyst. The number of generations per cyst is different between fish species.

After the final mitosis, meiosis takes place. Through meiosis I, primary spermatocyte differentiates into secondary spermatocytes and the homologous chromosomes from one primary spermatocyte are segregated into two secondary spermatocytes. In zebrafish, the leptotene / zygotene spermatocyte had larger (5.5 μm) round nucleus with chromatin which was clear with small dots of heterochromatin outlining the nuclear membrane (Schulz et al. 2010). The pachytene spermatocyte had a larger (6.5 μm) and dense nucleus containing bold lines chromosomes. In diplotenic primary spermatocyte, the chromosomes reached maximum degree of condensation and closed to the nuclear membrane. Through meiosis II, secondary spermatocyte differentiated into spermatid and the sister chromatids from one secondary spermatocyte were segregated into two spermatids. In zebrafish, the secondary spermatocyte was small with a dense nucleus (4 μm), and it was also rarely observed because it quickly differentiated into spermatids (Schulz et al. 2010).

The spermatid is a haploid cell. The process of spermatids differentiating into spermatozoa is called spermiogenesis, which includes a series of morphological changes, such as nuclear condensation, organelle and cytoplasm elimination and rearrangement, and flagellum formation. Spermatozoa have spherical nuclei with highly condensed chromatin and a nuclear fossa, a midpiece of variable size with or without a cytoplasmic channel, and one or two long flagella (Jamieson and Leung 1991). In teleost fish, spermatozoa penetrate the membrane of the oocyte through a micropyle instead of initiating an acrosome reaction.

Sertoli cells are indispensable somatic cells in testes that play a crucial role in spermatogenesis. Sertoli cells support germ cell survival, development and physiological functioning, secrete fluid to generate the tubular lumen, and phagocytize apoptotic germ cells. The number of Sertoli cells per cyst is different in different fish species at a certain stage of germ cell development (Billard.1969; Matta et al. 2002). In seasonal species, the number of Sertoli cells increases during the reproductive season when spermatogonia proliferation is resumed, but the number drops dramatically during the post - spawning.

Unlike the typical smooth cylindrical testes in most fish species, channel and blue catfish testes are villiform (Grizzle 1985). Spermatogenesis occurs along the anterior region of the testis, which contains the seminiferous tubules. The posterior region is called the glandular testis, which is formed by a different type of progenitor cell, and no spermatogenesis occurs in this region. Before spawning season, the anterior region of the testis white because of the increasing number of spermatozoa.

Ovarian Germ Cells

In female fish, when the PGCs reach the genital ridge, they start differentiating into oogonia. Each oogonium multiplies by mitosis and forms an oogonial nest surrounded by a monolayer of granulosa cells that secrete a basement lamina. Subsequently, in the oogonial nest, oogonia are transformed into primary oocytes followed by a massive growth - vitellogenesis, which is also conceded as meiotic arrest (Lubzens et al. 2010). During vitellogenesis, oocytes store nutrition needed for the future embryo development and accumulate RNA. Oocytes are considered mature when they

show reduced or no endocytosis, resumption of meiosis, germinal vesicle breakdown (GVBD), the formation of a monolayer of cortical alveoli under the oolemma, yolk platelet dissolution and pelagophil oocytes undergo hydration. Through meiosis I, oocytes divide into two cells with different size. The small cell degenerates with the first polar body and the large cell forms a secondary oocyte. The secondary oocyte is extruded through the follicular cell layers, moves into the ovarian lumen or abdominal cavity and forms the ovum. , The ovum becomes haploid at meiosis II, whereupon the second polar body is formed and later degenerates.

Germline Stem Cell Genes

PGCs Genes

The *vasa* gene is the first germline - specific marker identified in zebrafish PGCs. *Vasa*, which encodes an ATP - dependent RNA helicase of the DEAD BOX family, is expressed in germplasm, PGCs and germ cells during gametogenesis of many species (Gustafson et al. 2010). In the early embryonic development, *vasa* is expressed equally in all cells, but becomes restricted to the PGCs during gastrulation. As previously mentioned, PGC formation and migration has been tracked by identifying the expression of *vasa* in zebrafish embryos (Olsen et al. 1997; Yoon et al. 1997). Artificial 3'UTR mRNAs constructs: *vasa* promoter fused with green fluorescent protein (GFP) and red fluorescent protein (RFP) genes, have been used to generate the transgenic lines in several transgenic fish species including medaka, zebrafish (Fan et al. 2008) and rainbow trout (Takeuchi et al. 2002). PGCs were visualized in developing embryos during migration toward genital ridge, and were also sorted by flow cytometry based on their fluorescence.

Nanos - 1 encodes an RNA - binding zinc - finger protein. Zebrafish *nanos* - 1 gene is essential for PGC formation, proper migration and survival (Koprunner. et al 2001). *Nanos* - 1 gene was expressed in the germplasm, and the expression could be detected until 5 days post - fertilization. *Nanos* deficient PGCs migrate, but they exhibit abnormal morphology, and express mRNA in the soma. When *nanos* - 1 was knocked down, the PGC migration stopped and the PGCs eventually died.

Dead end (*dnd*) is a newly discovered protein that contains a single stranded RNA - binding domain, and it is also an important gene in PGCs' migration and survival (Liu et al. 2010). In *dnd* knockdown zebrafish, PGCs were confined to the deep blastoderm, and PGC migration was prevented and eventually died. But during further embryo development, PGC specification was not influenced because *nanos* - 1 and *vasa* were normally expressed till early gastrulation stages. If PGCs die, *dnd* RNA expression persisted, but the expression of both *nanos* - 1 and *vasa* markers gradually disappeared (Weidinger et al. 2003). *Dnd* was vital for female development (Slanchev et al. 2005). After PGCs were ablated by using a *dnd* antisense morpholino oligonucleotide (MO), only male zabrafish survived (Slanchev et al. 2005).

The *dazl* gene is a member of the DAZ gene family which encodes RNA - binding proteins and plays a pivotal role in gametogenesis (Maegawa et al. 1999). In zebrafish, *dazl* mRNA was expressed in the vegetal pole of the egg, and transported to the blastomeres during cleavage stages and ceases expression before gastrulation. *Dazl* is expressed in gonads of both sexes. In the ovary, it is localized in the cortex of oocytes (Maegawa et al. 2002).

The orthologues of *vasa*, *nanos* - 1, *dnd* and *dazl* are also found in mammalian PGCs and play a very important role in migration and survival of PGCs. But they were expressed in all stages of differentiated germ cells, and therefore cannot be used as PGC specific markers.

A substantial amount of research has focused on PGC - specific marker identification, both in mammals and fish. The *Oct4* gene encodes a class V POU domain transcription factor expressed in pluripotent embryonic and germ line cells (Khan et al. 2012). In mammals, Oct4 protein has the function of maintaining the pluripotency and proliferation characteristics of embryonic stem cell when combined with transcription factors Nanog and Sox2 (Boyer et al. 2005; Loh et al. 2006; Wang et al. 2006). In zebrafish, the *Oct4* ortholog gene - *spg/pou2* was not expressed in the developing PGCs or the adult gonads, but was necessary for pregastrula morphogenesis, mid - hindbrain boundary (MHB) formation (Belting et al. 2001; Burgess et al. 2002), specification of the endoderm (Lunde et al. 2004; Reim et al. 2004), and participates in establishing the dorso

- ventral axis (Reim and Brand, 2006). Further study on medaka, by using Oct4 protein specific antibodies, showed that *Oct4* was expressed in primordial germ cells, spermatogonia (male germline stem cells), and multiple stages of oocyte development (Sanchez - Sanchez et al. 2010). Fish *Oct4* mRNA and protein expression patterns are similar to those of mammalian *Oct4*.

SSC Genes

Several cell surface markers have been reported as SSCs specific markers. After selection by anti - β1- or anti - α6 - integrin antibody, but not anti - c - kit antibody, mouse SSCs with a significantly enhanced ability to colonize recipient testes and generate donor cell - derived spermatogenesis were isolated from the testicular cells (Shinohara et al. 1999). Shinohara et al. (2000) proved that SSCs were also negative for αV - integrin. Kubota et al. (2003) characterized the SSC surface phenotype using flow cytometric cell sorting in conjunction with a transplantation functional assay. Mouse SSCs were positive for Thy1, a glycosyl phosphatidylinositol anchored glycoprotein of the Ig superfamily. Later, Oct4 (Ohmura et al. 2004), Stra8 (a novel gene inducible by retinoic acid in P19 embryonal carcinoma cells; Giuili et al. 2002), GFRα (a glial cell line derived neurotrophic factor family receptor - α; Meng et al. 2000; von Schonfeldt et al. 2004; Hofmann et al. 2005), Plzf, (a promyelocytic leukemia zinc finger protein transcription factor; Buaas et al. 2004; Costoya et al. 2004), Neurogenin3 (a class B basic helix - loop - helix transcription factor; Yoshida et al. 2004), and CDH1 (a homophilic cell - cell adhesion molecule spanning the cell membrane; Tokuda et al. 2007) all proved to be type A spermatogonia specific markers.

In fish, several markers have been shown to be type A spermatogonia specific markers. Notch1 (Notch homolog protein 1) homologue showed high expression in the type A spermatogonia in rainbow trout. Lymphocyte antigen 75 (Ly75, also called CD205) mRNA was predominantly localized in the PGCs, type A spermatogonia, oogonia, and chromatin nucleolus - stage oocytes of rainbow trout, but not in spermatocytes, spermatids, spermatozoa, vitellogenic oocytes, or gonadal somatic cells (Nagasawa et al. 2010). Ozaki et al (2011) tested the suitability of three proteins, Sycp3, Plzf and Cyclin B3, as germ cell markers in zebrafish by immunohistochemistry. Plzf and

Cyclin B3 were proved as markers of the early stages of spermatogonia, and Sycp3 was a spermatocyte marker.

The objective of this research was to determine gene expression of different types of testicular germ cells of both channel and blue catfish, and to identify specific markers for spermatogonium, spermatocyte and spermatids, respectively. Identification of specific markers might be useful in verifying spermatogonia sorting and the results of transplantation studies.

.

Materials and Methods

Primer Design

The genes, pfkfb4, urod, Oct4, Plzf, sycp3, sox2, Integrin6, neurogenin3, ID - 4, integrinV, Thy1, GFRα, CDH1, Smad5, Pum2 and Prdm14 and Kit, were SSCs markers in zebrafish, medaka, rainbow trout, mouse and human. These genes were evaluated as potential catfish SSCs specific markers, and accession information is shown in Table 4, and gene functions are found in Table 5.

The corresponding bony fish protein sequences of the seventeen genes were found in NCBI protein database. To identify the seventeen genes from catfish, the channel catfish testes transcriptome database and blue catfish transcriptome database were searched using all available bony fish protein sequences as queries. TBLASTN was used to obtain the initial pool of seventeen gene sequences, with a cutoff E - value (Expect value, E = the likelihood that the matching sequence is obtained by chance) of 1e - 5. Upon identification of the initial pool of catfish seventeen sequences, sequences were aligned to delete the repeated entries and a unique set of sequences were subjected to further analysis. The cDNA sequences (contigs) obtained from RNA - seq database was confirmed by using BLASTX against NCBI non - redundant protein sequence database. If more than one contigs corresponded to one gene, all the contigs for that gene were aligned with Multiple Sequence Alignment by ClustalW2 program (EMBL - EBI) and the conserved region was isolated for primer design. Primers were designed using Primer3 (Online primer design tool). The accession number of the contigs whose sequences were used in primer design and primer information was listed in Table 6.

RNA Extraction

Six two - year - old male juvenile channel catfish (TL: 21 ~ 25 cm) and six two - year - old male juvenile blue catfish (TL: 25 ~ 30 cm) were collected from the Fish Genetics Research Unit, Auburn University, AL. By the same method described in Chapter Two, testes from six fish of each species were pooled together and trypsinized,

testicular cells were isolated. One - tenth of the isolated testicular cells were removed for RNA extraction using TRIzol Reagent (Ambio) directly. The remaining testicular cells were separated by Percoll density gradient centrifugation. RNAs from cells of four bands (the cell band I was on the top of 35% Percoll; the cell band II was in the middle of 35% Percoll; the cell band III was on the top of 70% Percoll; the cell band IV was the pellet on the bottom of the tube) were extracted using TRIzol Reagent separately and the extraction procedure followed the manufacturer's recommendation. The quality and concentration of all the samples were checked by gel electrophoresis and a ND - 1000 spectrophotometer (NanoDrop Technologies). All extracted samples must have an A260/280 ratio greater than 1.8.

Primer Test

RNAs from one - tenth of the testicular cells of both channel and blue catfish were reverse transcribed into cDNAs by iScript Synthesis Kit (Bio - Rad). Each reaction consisted of a total volume of 10 μL containing 4.0 μL iScript reaction mix, 1.0 μL iScript reverse transcriptase, 500 ng RNA template, and RNase / DNase - free water to reach the 10 μL volume. The reaction followed the protocol: 5 minutes at 25 ℃, 30 min at 42 ℃, 5 min at 85 ℃.

The PCR reactions were prepared in 10.0 μL volume containing 200 ng cDNA, 1.0 μL of each primer (5 μM/L), 0.8 μL2.5 mM of each dNTP, 1.0 μL 10 × PCR buffer, 0.4 μL 50 mM $MgCl_2$, RNase / DNase - free water. The PCR procedures were performed using the following thermo profile: after an initial denaturation at 95 ℃ for 5 min; PCR amplifications were carried out at 95 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min for 35 cycles. A final extension at 72 ℃ for 10 min was included. The seventeen amplification products were analyzed on an ethidium - bromide - stained 1.0 % agarose gel. A Tracklt 100bp DNA ladder (Invitrogen) was used to check the band size after electrophoresis.

Table 4 GenBank protein sequence accession number for seventeen putative germ cell genes.

Gene Name	Accession No.	Species
pfkfb4	CBN81048.1	European seabass (*Dicentrarchus labrax*)
urod	ADO27991.1	blue catfish (*Ictalurus furcatus*)
Oct4	NP_571513.1	zebrafish (*Danio rerio*)
Plzf	AAI65228.1	zebrafish (*D. rerio*)
sycp3	AAI15344.1	zebrafish (*D. rerio*)
SOX2	AAH65656.1	zebrafish (*D. rerio*)
Integrin6	NP_001013466.1	zebrafish (*D. rerio*)
neurogenin3	AAI62120.1	zebrafish (*D. rerio*)
ID - 4	NP_001035079.1	zebrafish (*D. rerio*)
IntegrinV	AAI63566.1	zebrafish (*D. rerio*)
Thy1	AAI52207.1	zebrafish (*D. rerio*)
GFRα	AAR99464.1	zebrafish (*D. rerio*)
CDH1	NP_571895.1	zebrafish (*D. rerio*)
Smad5	AAF06361.1	zebrafish (*D. rerio*)
Pum2	BAI68041.1	zebrafish (*D. rerio*)
Prdm14	NP_001157303.1	zebrafish (*D. rerio*)
Kit	NP_571128.1	zebrafish (*D. rerio*)

Table 5 Germ cell gene expression in fish and mice

Gene	Specification	Species investigated	Expression by cell type	Reference
pfkfb4	6 - phosphofructo - 2 - kinase/fructose - 2, 6 - biphosphatase 4	rainbow trout	type A and type B spermatogonia	Yano et al. 2009
urod	uroporphyrinogen decarboxylase	rainbow trout	type A and type B spermatogonia	Yano, et al. 2009
Oct4	POU domain, class 5, transcription factor 1	medaka	type A spermatogonia	Sanchez et al. 2010
Plzf	promyelocytic leukemia zinc finger	zebrafish	type A and type B spermatogonia	Ozaki et al. 2011
sycp3	synaptonemal complex protein 3 like	zebrafish	spermatocytes	Ozaki et al. 2011
SOX2	SRY - box containing gene 2	mouse	spermatogonial stem cells	Western et al. 1999
Integrin6	A6 - integrin	mouse	spermatogonial stem cells	Shinohara et al. 1999
neurogenin3	a class B basic helix - loop - helix transcription factor	mouse	spermatogonial stem cells	Yoshida et al. 2004
ID - 4	DNA - binding protein inhibitor ID - 4	mouse	spermatogonial stem cells	Oatley et al. 2011
IntegrinV	αV - integrin	mouse	type A spermatogonia	Shinohara et al. 2000
Thy1	a glycosyl phosphatidylinositol anchored glycoprotein of the Ig superfamily	mouse	type A spermatogonia	Kubota et al. 2003
GFRα	a glial cell line derived neurotrophic factor family receptor - α	mouse	type A spermatogonia	Hofmann et al. 2005
CDH1	E - cadherin	mouse	type A spermatogonia	Tokuda et al. 2007
Smad5	downstream signal mediators	mouse	PGCs	Chang et al. 2001
Pum2	a human homolog of Pumilio	human	PGCs	Moore et al. 2003
Prdm14	a PR domain - containing transcriptional regulator	mouse	PGCs	Yamaji et al. 2008
Kit	Mast/stem cell growth factor receptor Kit	mouse	stem cells	Zayas et al. 2008

Table 6 Primers used for amplification of seventeen germ cell genes in blue catfish (*Ictalurus furcatus*) and channel catfish (*I. punctatus*)

Gene	sequence source contig number	forward primer	reverse primer	product size	Testicular germ cells
pfkfb4	Contig22237	CTCGCTTCTTGGGACAGTTC	CGATCTCGTTGAGAGCCTTC	135	channel and blue catfish
urod	Contig16176	TGGATAACGTCCCCATGATT	TGACTTTCCCACCAGTCCTC	141	channel and blue catfish
OCT4	Contig3283	GGCGGTTACAGAACCACACT	CGACGCCGTAAGAAAAGAAC	163	channel and blue catfish
Plzf	k61:1685651	GCCACTGTCCTCAATGGAAT	TTCAGACGAGAACGATGCAG	124	channel catfish
	comp410141_c0_seq1	TGCAGAGCAACACAGGAAAC	ACCAGTGCTTTTGGTCCATC	132	blue catfish
sycp3	Contig29397	AGCAGAAGCTCTTCCAGCAG	GCAGAGCCATGTCCTTTTTC	162	blue catfish
SOX2	k50:2879500	GCGGTCGGTACTTGTAGTCC	AACCCGAAGATGCACAACTC	151	channel and blue catfish
Integrin6	k78:575431	TGAGGTTGGGGATGAAAGTC	GCTCCAGACACCACAATCAG	129	channel and blue catfish
neurogenin3	k60:1739495	TCGCGCACAATTATATCTGG	AGTGGAAAGTGACGGAGCTG	123	blue catfish
ID - 4	k50:1705039	GGCAGGTCCAGGATGTAGTC	CCTACAGCACGACATGAACG	126	channel and blue catfish
IntegrinV	Contig16130	ACCAGTCCAAACCAGCAAAC	ACGATTCTCTGCCGTCACTT	167	channel and blue catfish
Thy1	k53:2338234	TTCAACATCCGACACAGGAA	GGTGAACCTGCATTCCATCT	168	channel and blue catfish
GFRα	k51:2495180	CCGACTGTTTACCCCAACAC	GTTCCACACAGGGTTCTCGT	129	channel and blue catfish
CDH1	Contig30216	CACCAGAGCTGGAGGAGTTC	CAGAGGAGATTGGCACCTTC	153	channel and blue catfish
Smad5	Contig490	ACACGTCCTTCTGCTTGGAT	GACTGCAGGTGTCACATCGT	141	blue catfish
Pum2	Contig24823	GGCTGATGATGACAGAAGCA	CTTTGGCATTTGGACAAGGT	156	channel and blue catfish
Prdm14	k74:1098069	ATGTAGCCAAATGCGATGCT	ACAGAACCTTGTGGCGTTTC	135	channel and blue catfish
Kit	comp70946_c0_seq7	CCGAAGGCGAGATTGTCTAC	ACAGGATGAGGCAAAGGATG	120	blue catfish

Real - time PCR

RNAs were reverse transcribed into cDNAs by iScript Synthesis Kit (Bio - Rad). Each reaction consisted of a total volume of 10 μL containing 4.0 μL iScript reaction mix, 1.0 μL iScript reverse transcriptase, 500 ng RNA template, and RNase / DNase - free water to reach the 10 μL volume. The reaction followed the protocol: 5 minutes at 25 ℃, 30 min at 42 ℃, and 5 min at 85 ℃.

qRT - PCR was carried out on a C1000 Thermal Cycler (Bio - Rad) by using SsoFast EvaGreen supermix kit (Bio - Rad) following the manufacturer's instructions with modification. The reaction systems for all qRT - PCR were as follows: 1.0 μL of each primer (5 μM/L), 5.0 μL SsoFast EvaGreen supermix, 2.0 μL RNase / DNase - free water, and 1.0 μL 200 ng/μL cDNA. The PCR procedure was as follows: denaturation, 95 ℃ / 30 s, 40 cycles of 95 ℃ / 5 s, and 57 ℃ / 5 s followed by melting curve analysis, 5 s at 65 ℃, then up to 95 ℃ at a rate of 0.1 ℃ / s increasement. Ribosome 18S mRNA was used as reference gene. Each sample was repeated three times for the expression analysis. A Negative control was included on all the plates. Ct (threshold crossing) values were exported into Excel sheet from Bio - Rad CRX Manager (Version 1.6.541.1028, 2008). The relative expression ratios of target genes were analyzed for significance using a randomization test in the REST (Pfaffl et al. 2002) software assuming 100% efficiencies. The results were graphed with corresponding standard errors.

Results

Primer Test

Thirteen genes expression, pfkfb4, urod, OCT4, Plzf, SOX2, Integrin6, ID - 4, IntegrinV, Thy1, GFRα, CDH1, Pum2 and Prdm14, were detected in testicular cells of the channel catfish. Sycp3, neurogenin3, Smad5 and Kit gene expression weren't detected in channel catfish testicular cells (Fig. 5 A). Seventeen genes expression, pfkfb4, urod, OCT4, Plzf, sycp3, SOX2, Integrin6, neurogenin3, ID - 4, IntegrinV, Thy1, GFRα, CDH1, Smad5, Pum2, Prdm14 and Kit, were all detected in blue catfish testicular cells (Fig. 5 B).

Melting Curve

After the qRT - PCR process, melting curves from each run were checked for each cell band before data analysis. Amplification products of each gene had the correct size and showed a single specific peak in the melting curve, which meant that these genes were expressed in both channel catfish and blue catfish cells of the upper three bands (cell band I, cell band II and cell band III). For the cells in band IV, some melting cures contained non - specific peaks, which were because that the cell band IV only contained red blood cells and very few spermatids, and some genes were not expressed in those types of cell. The expression of control gene - 18S mRNA was very low in the cells of band IV, which meaned that the quantity of the extracted RNAs was very low and some of the marker gene expressions were undetectable. The relative gene expressions in cells of band IV were not included during the data analysis.

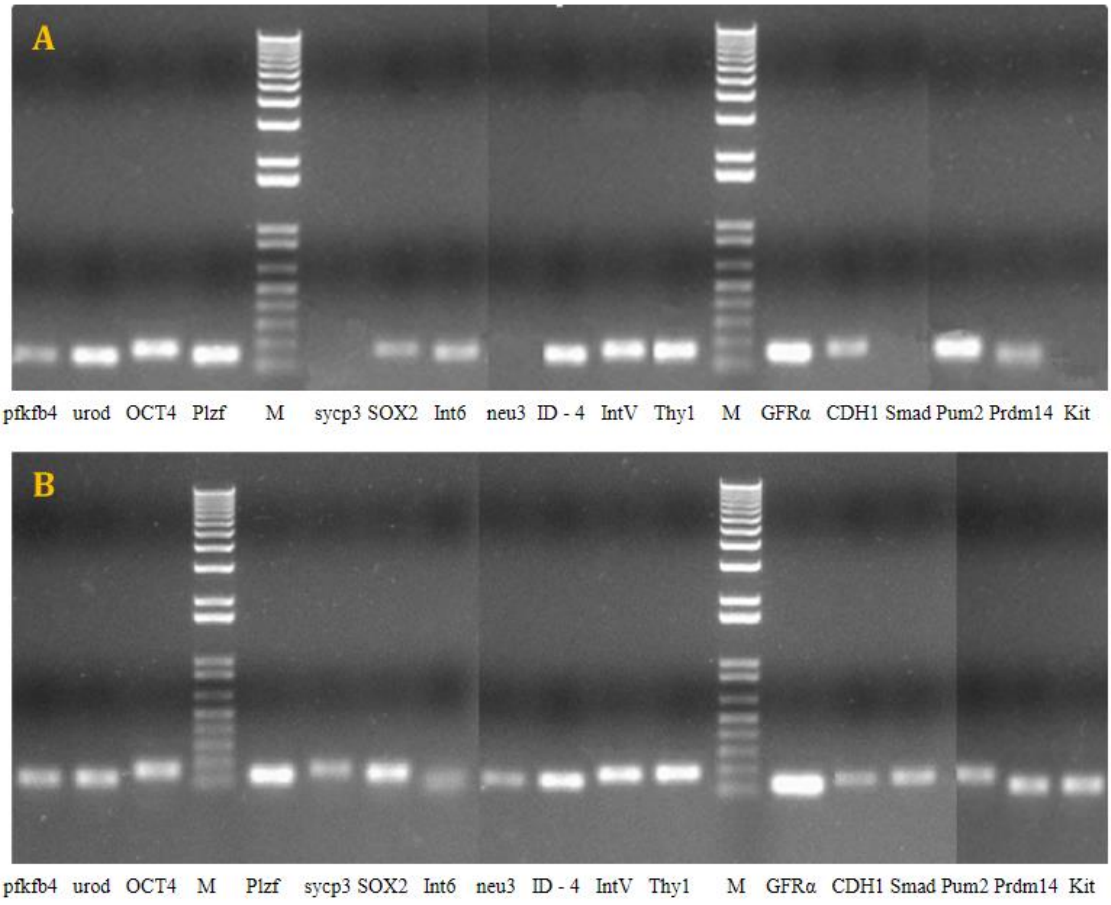

Fig. 5 Seventeen gene, pfkfb4, urod, OCT4, Plzf, sycp3, SOX2, Integrin6, neurogenin3, ID - 4, IntegrinV, Thy1, GFRα, CDH1, Smad5, Pum2, Prdm14 and Kit, amplification products for testicular cells of channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). A) Genes, sycp3, neurogenin3, Smad5 and Kit, were not detected in channel catfish testicular cells. B) All Seventeen genes were detected in blue catfish testicular cells.

Relative Gene Expression in Different Types of Channel Catfish Testicular Germ Cells

After Percoll density gradient centrifugation, channel catfish testicular germ cells showed the same characteristics as blue catfish. Four distinct cell bands were generated and the percentage of each cell type in each band was the same as blue catfish. Expression of thirteen genes, pfkfb4, urod, Oct4, Plzf, SOX2, Integrin6, ID - 4, IntegrinV, Thy1, GFRα, CDH1, Pum2 and Prdm14, were detected in cells from each band.

Three expression trends were observed: pfkfb4, urod, Plzf, Integrin6, ID - 4, IntegrinV, Thy1 and CDH1 gene expressions were down - regulated in cells of band II (60% of the cells were spermatocytes, 30% were type B spermatogonia, and 10% were spermatids) and even more strongly down - regulated in cells of band III (95% of the cells were the spermatids, and 5% were spermatocytes) compared to cells of band I (55% of the cells were type A spermatogonia and type B spermatogonia, 35% were spermatocytes, 10% were spermatids) (Fig. 6); SOX2 and GFRα gene expressions were up - regulated in cells of band II and even more strongly up - regulated in cells of band III compared to cells of band I (Fig. 7); Oct4, Pum2 and Prdm14 gene expressions were highly up - regulated in cells of band II, but not as strongly up - regulated in cells of band III compared to cells of band I (Fig. 8).

Relative Gene Expression in Different Types of Blue Catfish Testicular Germ Cells

Seventeen genes', pfkfb4, urod, Oct4, Plzf , sycp3, SOX2, Integrin6, neurogenin3, ID - 4, IntegrinV, Thy1, GFRα, CDH1, Smad5, Pum2, Prdm14, Kit, expression were detected for blue catfish testicular germ cells from each band.

Three expression trends were observed: pfkfb4, urod, Oct4, Plzf, sycp3, Kit, Integrin6, IntegrinV, Thy1, GFRα, CDH1 and Pum2 gene expressions were down - regulated in cells of band II and even more strongly down - regulated in cells of band III compared to cells of band I (Fig. 9); neurogenin3 and SOX2 gene expressions were up - regulated in cells of band II and even more strongly up - regulated in cells of band III compared to cells of band I (Fig. 10); ID - 4, Smad5 and Prdm14 gene expressions were highly down - regulated in cells of band II, but up - regulated in cells of band III compared to cells of band I (Fig. 11).

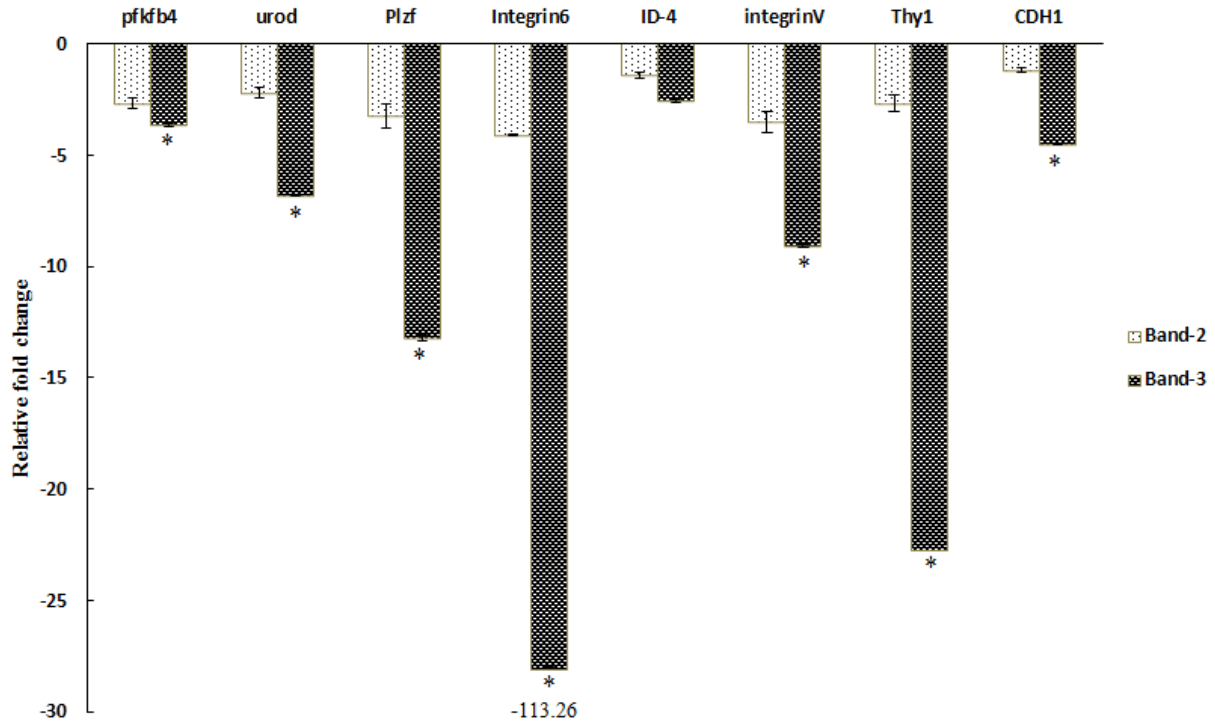

Fig. 6 Analysis of relative expression of pfkfb4, urod, Plzf, Integrin6, ID - 4, IntegrinV, Thy1 and CDH1 genes, which were down - regulated in channel catfish (*Ictalurus punctatus*) testicular germ cells of band II (60% of the cells were spermatocytes, 30% were type B spermatogonia, and 10% were spermatids) and even more strongly down - regulated in cells of band III (95% of the cells were the spermatids, and 5% were spermatocytes). Gene expression in the cells of band II and band III was expressed as fold - change over that of the cell band I (55% of the cells were type A spermatogonia and type B spermatogonia, 35% were spermatocytes, 10% were spermatids) as normalized to fold change in the expression of ribosomal 18S RNA. Relative fold changes were shown as mean ± SE. All the values were significant at the level of $P < 0.05$ and marked with asterisk (*).

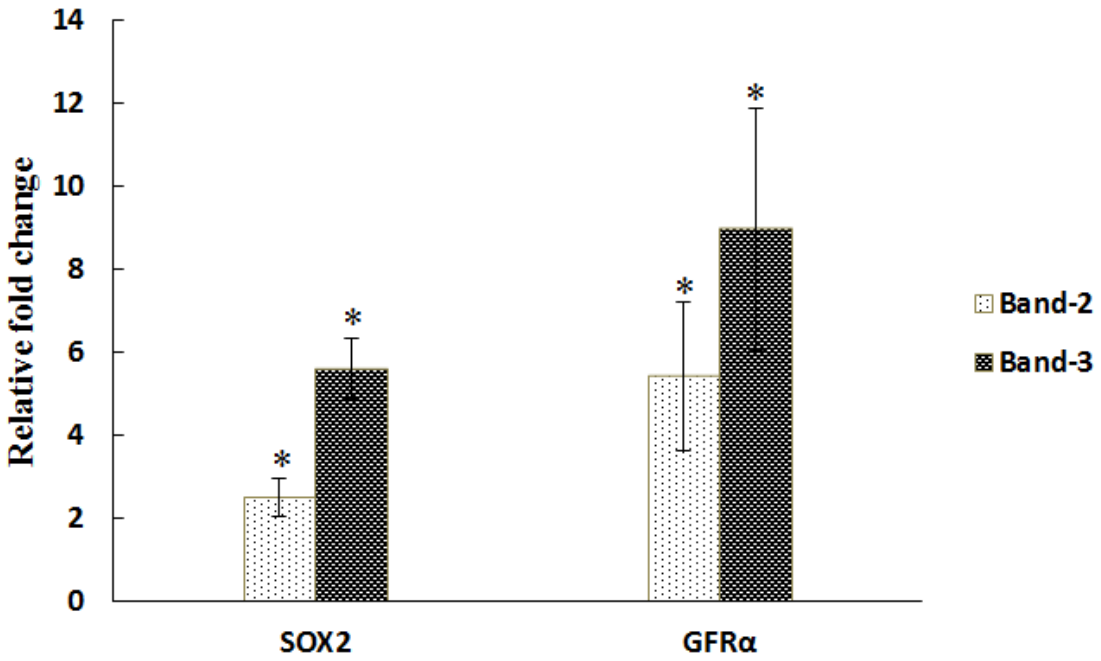

Fig. 7 Analysis of relative expression of SOX2 and GFRα genes, which were up - regulated in channel catfish (*Ictalurus punctatus*) testicular germ cells of band II (60% of the cells were spermatocytes, 30% were type B spermatogonia, and 10% were spermatids) and and even more strongly up - regulated in cells of band III (95% of the cells were the spermatids, and 5% were spermatocytes). Gene expression in the cells of band II and band III was expressed as fold - change over that of the cell band I (55% of the cells were type A spermatogonia and type B spermatogonia, 35% were spermatocytes, 10% were spermatids) as normalized to fold change in the expression of ribosomal 18S RNA. Relative fold changes were shown as mean ± SE. All the values were significant at the level of $P < 0.05$ and marked with asterisk (*).

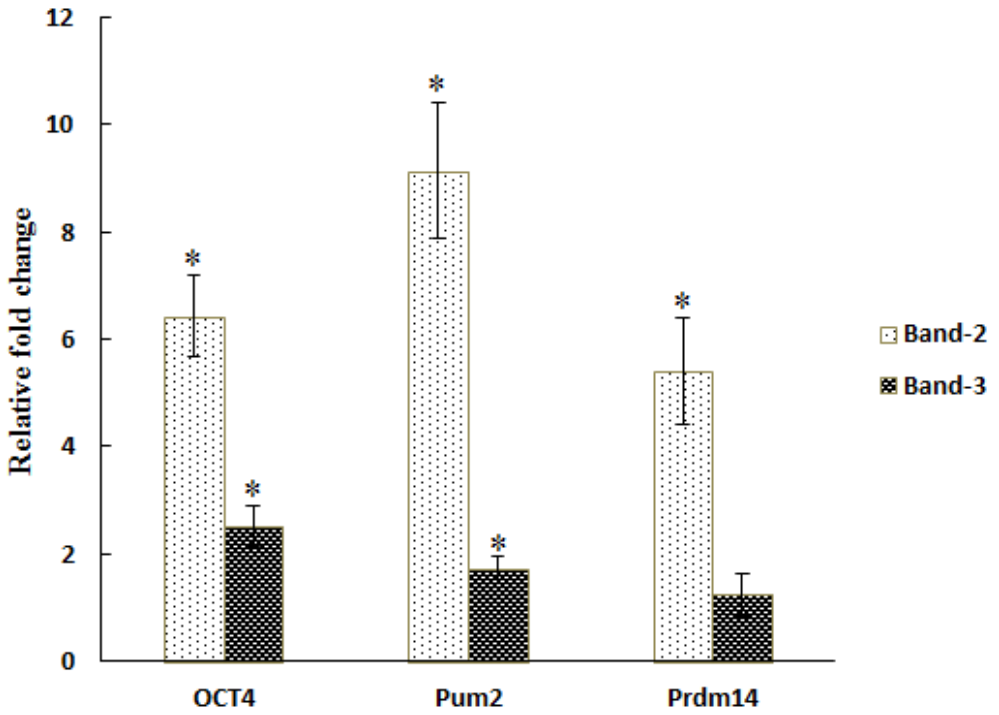

Fig. 8 Analysis of relative expression of Oct4, Pum2, and Prdm14 genes, which were highly up - regulated in channel catfish (*Ictalurus punctatus*) testicular germ cells of band II (60% of the cells were spermatocytes, 30% were type B spermatogonia, and 10% were spermatids), but not as strongly up - regulated in cells of band III (95% of the cells were the spermatids, and 5% were spermatocytes). Gene expression in the cells of band II and band III was expressed as fold - change over that of the cell band I (55% of the cells were type A spermatogonia and type B spermatogonia, 35% were spermatocytes, 10% were spermatids) as normalized to fold change in the expression of ribosomal 18S RNA. Relative fold changes were shown as mean ± SE. All the values were significant at the level of $P < 0.05$ and marked with asterisk (*).

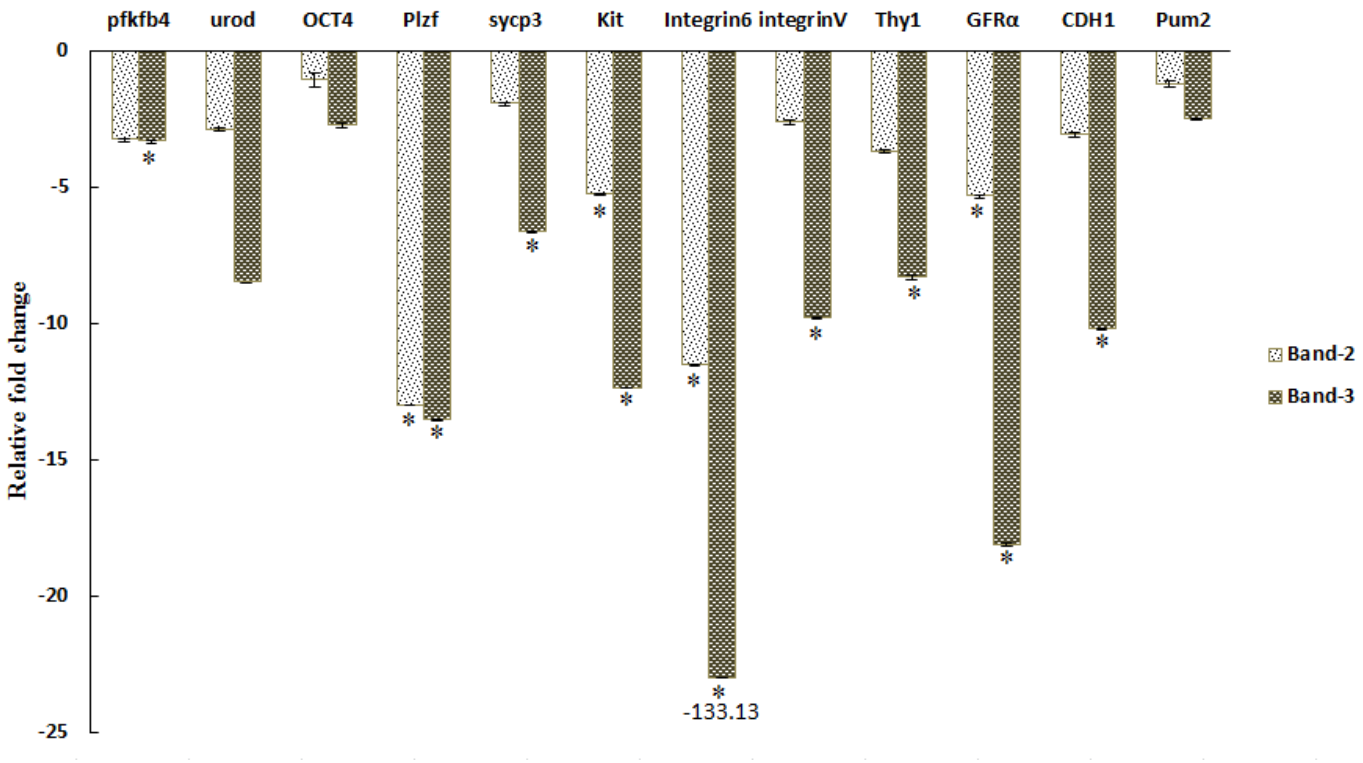

Fig. 9 Analysis of relative expression of pfkfb4, urod, Oct4, Plf, sycp3, kit, Integrin6, IntegrinV, Thy1, GFRα, CDH1 and Pum2 genes, which were down - regulated in blue catfish (*Ictalurus furcatus*) testicular germ cells of band II (60% of the cells were spermatocytes, 30% were type B spermatogonia, and 10% were spermatids) and even more strongly down - regulated in cells of band III (95% of the cells were the spermatids, and 5% were spermatocytes). Gene expression in the cells of band II and band III was expressed as fold - change over that of the cell band I (55% of the cells were type A spermatogonia and type B spermatogonia, 35% were spermatocytes, 10% were spermatids) as normalized to fold change in the expression of ribosomal 18S RNA. Relative fold changes were shown as mean ± SE. All the values were significant at the level of $P < 0.05$ and marked with asterisk (*).

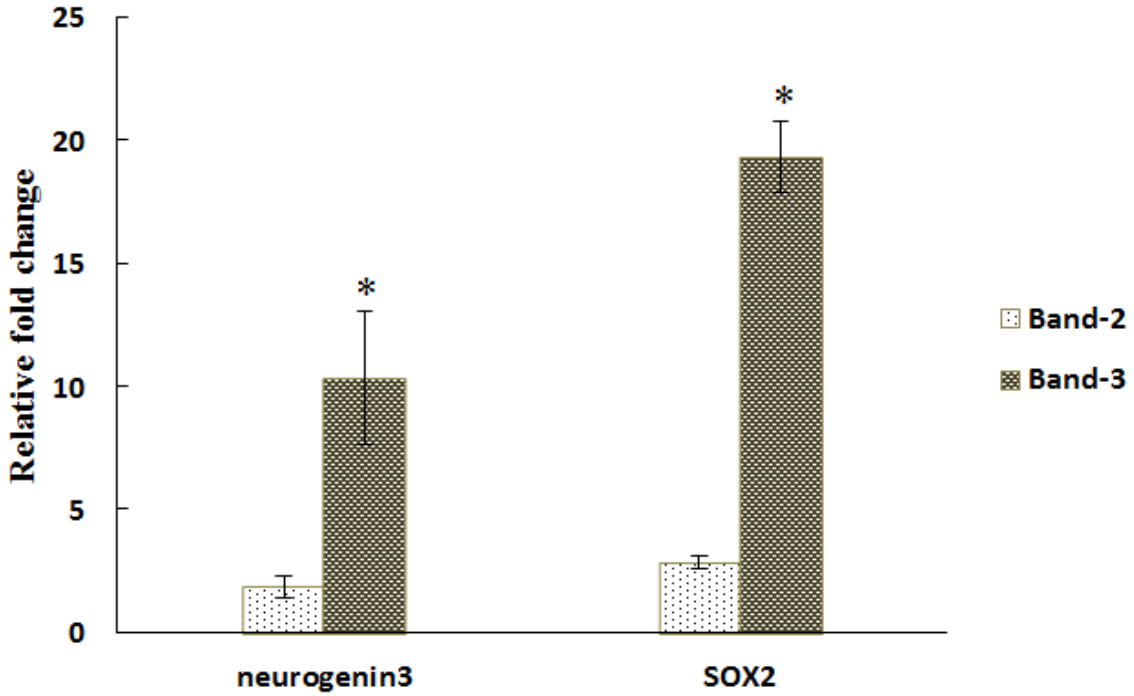

Fig. 10 Analysis of relative expression of neurogenin3 and SOX2 genes, which were up - regulated in blue catfish (*Ictalurus furcatus*) testicular germ cells of band II (60% of the cells were spermatocytes, 30% were type B spermatogonia, and 10% were spermatids) and even more strongly up - regulated in cells of band III (95% of the cells were the spermatids, and 5% were spermatocytes). Gene expression in the cells of band II and band III was expressed as fold - change over that of the cell band I (55% of the cells were type A spermatogonia and type B spermatogonia, 35% were spermatocytes, 10% were spermatids) as normalized to fold change in the expression of ribosomal 18S RNA. Relative fold changes were shown as mean ± SE. All the values were significant at the level of $P < 0.05$ and marked with asterisk (*).

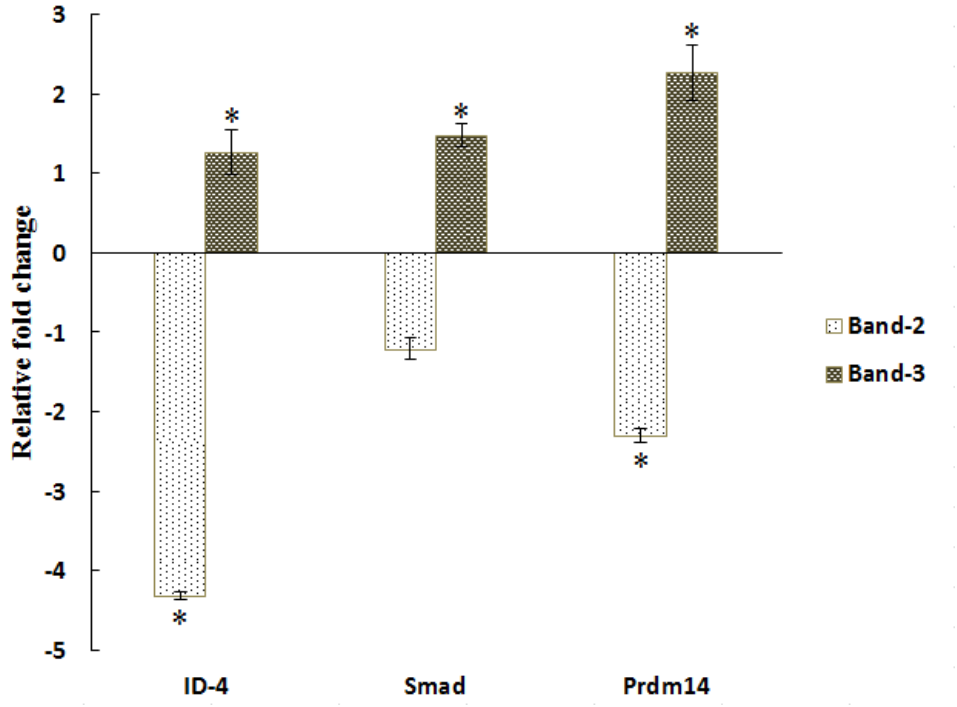

Fig. 11 Analysis of relative expression of ID - 4, Smad5 and Prdm14 genes, which were highly down - regulated in blue catfish (*Ictalurus furcatus*) testicular germ cells of band II (60% of the cells were spermatocytes, 30% were type B spermatogonia, and 10% were spermatids), but up - regulated in cells of band III (95% of the cells were the spermatids, and 5% were spermatocytes). Gene expression in the cells of band II and band III was expressed as fold - change over that of the cell band I (55% of the cells were type A spermatogonia and type B spermatogonia, 35% were spermatocytes, 10% were spermatids) as normalized to fold change in the expression of ribosomal 18S RNA. Relative fold changes were shown as mean ± SE. All the values were significant at the level of $P < 0.05$ and marked with asterisk (*).

Discussion

Expression of sycp3, neurogenin3, Smad5 and Kit, were not detected in channel catfish testicular cells. The fundamental expression of channel catfish testicular cells may differ from that of blue catfish or a more likely explanation is that the primers were not able to amplify these genes from channel catfish as these primers were based on blue catfish transcriptome database. If the latter is true, significant sequence differences exist between these two species for sycp3, neurogenin3, Smad5 and Kit.

Sycp3 gene coding for synaptonemal complex protein 3, which was formed between homologous chromosomes during meiotic prophase, has an essential meiotic function in spermatogenesis (Yuan et al. 2000). In zebrafish, sycp3 was specifically localized in spermatocytes in typical nuclear patterns at each meiotic stage (Ozaki et al 2011). The class B basic helix - loop - helix (bHLH) transcription factor neurogenin3 was first expressed at the prepubertal stage in c - Kit negative prespermatogonia in mouse (Yoshida et al. 2004). Smad5 protein was found to be a downstream signaling mediator for TGF - β superfamily members and expressed ubiquitously in mouse adult tissues and during embryogenesis (Chang et al. 2001). Kit gene coded protein - Mast / stem cell growth factor receptor was an important cell surface marker used to identify certain types of hematopoietic (blood) progenitors in the bone marrow (Leong et al. 2008). It was s also a marker for mouse prostate stem cells. However, mouse SSCs did not exhibit Kit gene expression (Shinohara et al. 1999).

pfkfb4, urod, Plzf, Integrin6, ID - 4, IntegrinV, Thy1 and CDH1 genes showed the same expression pattern in different types of testicular germ cells of both channel catfish and blue catfish. In the case of channel catfish, they were all down - regulated in cells from the second band, which contained type B spermatogonia, spermatocytes and spermatids, but not significantly despite a consistent trend of a 1 ~ 4 × decrease in expression. All of these genes except ID - 4 were more strongly and significantly down - regulated in the third band, which contained spermatocytes and spermatids. These genes were highly expressed in cells of the first band, which contained higher percentage of

spermatogonia and primary spermatocytes, and this was the only band containing type A spermatogonia. The implication is that these genes are most strongly expressed in type A spermatogonia. Among those genes, Plzf, Thy1 and Integrin6 were the most strongly down - regulated genes, especially in cells from the third band (> 12 -, 22 - and 113 - fold change), implying that it was down - regulated in type B spermatogonia and further down - regulated in spermatocytes and spermatids.

Down - regulation of these genes was stronger in the second band, type B spermatogonia, spermatocytes and spermatids, for blue catfish than channel catfish. Plzf, integrin6 and GFRα were significantly down - regulated (5 ~ 12 times).

Plzf protein, zinc finger and BTB domain - containing protein 16, have been studied in zebrafish using immunohistochemistry, and it could be used as both type A and type B spermatogonia markers (Ozaki et al. 2011). Thy1 coded thymocyte antigen, which can be used as a marker for a variety of stem cells (Nakamura et al. 2006), and it had been used for SSC enrichment. Integrin6 protein was used as a marker for mouse SSCs (Shinohara et al. 1999). In this study, Plzf, Thy1 and Integrin6 protein coding gene were highly expressed in catfish spermatogonia.

The SOX2 gene showed the same expression trend in different types of testicular germ cells of both channel catfish and blue catfish. It was up - regulated in cells from the second band and even more strongly up - regulated in cells from the third band, which indicated that SOX gene was highly expressed in catfish spermatids. The up - regulation in the spermatids compared to the spermatogonia heavy band was observed to be 20 - fold higher in blue catfish than in channel catfish, which showed only a 5.7 - fold up - regulation. SOX2 has been reported to be a spermatogonia marker in mouse (Loh et al. 2006; Wang et al. 2006). But in our case, SOX2 gene was not a spermatogonia marker, but instead, a spermatid marker. This gene appears to have evolved to have different functions in mammals and fish.

GFRα was strongly up - regulated in bands 2 and 3 containing spermatogonia B, spermatocytes and spermatids in channel catfish, but strongly down - regulated in blue catfish. This gene expression could also help identify spermatogonia A in both species,

but the response of the two species was opposite. Thus, the identification procedure of the cells for channel catfish and blue catfish differs. GFRα protein, the receptor for glial cell line - derived neurotrophic factor (GDNF) plays a crucial role in maintaining germline stem cell proliferation and/or renewal in mouse.

Oct4, Pum2, and Prdm14 genes in channel catfish testicular germ cells exhibited an interesting expression pattern. As type A spermatogonia were eliminated from the cell population, expression greatly increased, 5 ~ 9 fold, and type B spermatogonia were eliminated, expression levels decreased, but were still 1.5 ~ 2.5 fold higher than when type A spermatogonia were present. Expression levels must have been highest in type B spermatogonia, lower in spermatocytes and spermatids and lowest in type A spermatogonia. The expression pattern for these 3 genes was different for blue catfish compared to channel catfish. The observed expression slightly (1 ~ 2.5 ×) and progressively decreased for Oct4 and Pum2 as first type A spermatogonia and then type B spermatogonia were removed from the cell population. Expression level of Prdm14 decreased 2.3 × upon removal of type A spermatogonia and increased 2.2 × upon removal of type B spermatogonia suggesting that spermatids had the highest expression level, followed by the spermatocytes and spermatids with type B spermatogonia having the lowest expression level.

Oct4 was expressed in primordial germ cells, in the spermatogonia (male germline stem cells), and during different stages of oocyte development in medaka (Sanchez - Sanchez et al. 2010). Pum2 and Prdm14 genes coding proteins had been studied only in mammals. Pum2 is a human homolog of fly Pumilio, a translation repressor that was required for embryonic axis determination and germ cell development, especially maintenance of germline stem cell (Moore et al. 2003). Prdm14 is a PR domain - containing transcriptional regulator with exclusive expression in the mouse PGCs.

Smad5 and ID - 4 exhibited a similar expression pattern as Prdm14 in blue catfish. In the case of ID - 4, this expression pattern differed from that of channel catfish. Transcriptional repressor ID - 4 (inhibitor of DNA binding ID proteins 4) is a class of helix - loop - helix molecules, expressed by undifferentiated spermatogonia in mouse

playing an important role in the regulation of spermatogonia self - renewal (Oatley et al. 2011).

The testicular germline cells of blue catfish and channel catfish could not be distinguished by presence or absence of expression for putative, germline marker genes. However, they can be identified by examining the relative levels of expression at single loci or over several loci. The variable levels of expression confirm that the morphologically different cell types are different cell types in various stages of development.

The sexual development, age of maturity, variability in gravidness, spawning and responsiveness to hormone induced gamete maturation is different between blue catfish and channel catfish. This may functionally related to the differential gene expression observed between blue catfish and channel catfish in this study for genes, such as Oct4, ID - 4, GFRα, Pum2 and Prdm14, showed different expression trend in both channel and blue catfish. Gene expression for those genes should be examined more closely in the future, and those genes can help us explain that why channel catfish and blue catfish respond differently in hormone induced spawning.

References:

Amemiya, C. T., Aizawa, S. 2006. Evolutionary constraint on Otx2 neuroectoderm enhancers - deep conservation from skate to mouse and unique divergence in teleost. Proceedings of the National Academy of Sciences USA, 103, 19350 - 19355.

Belting, H. G., Hauptmann, G., Meyer, D., Abdelilah - Seyfried, S., Chitnis, A., Eschbach, C., Soll, I., Thisse, C., Thisse, B., Artinger, K., Lunde, K., Driever, W. 2001. Spiel ohne grenzen/pou2 is required during establishment of the zebrafish midbrain - hindbrain boundary organizer. Development, 128: 4165 - 4176.

Billard, R., 1969. La spermatogenese de Poecilia reticulata I. Estimation du nombre de generations goniales et rendement de la spermatogenese. Ann Biol Anim Biochim Biophys, 9, 251 - 271.

Billard, R. 1986. Spermatogenesis and spermatology of some teleost fish species. Reproduction Nutrition Development, 26: 877 - 920.

Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., Young, R. A. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell, 122: 947 - 956.

Buaas, F., Kirsh, A., Sharma, M., McLean, D. J., Morris, J. L., Griswold, M. D., de Rooij, D. G., Braun, R. E. 2004. Plzf is required in adult male germ cells for stem cell self - renewal. Nature Genetics, 36:647–652.

Burgess, S., Reim, G., Chen, W., Hopkins, N., Brand, M. 2002. The zebrafish spiel - ohne - grenzen (spg) gene encodes the POU domain protein Pou2 related to mammalian Oct4 and is essential for formation of the midbrain and hindbrain, and for pre - gastrula morphogenesis. Development 129(4): 905 - 916.

Callard, GV. 1996. Endocrinology of Leydig cells in normammalian vertebrates. In: The Leydig Cell. Cache River Press, Clearwater, FL. pp.307 - 332.

Chang, H., Matzuk, M.M. 2001. Smad5 is required for mouse primordial germ cell development. Mechanisms of Development, 104: 61 - 67.

Costoya, J., Hobbs, R., Barna, M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K. E., Wolgemuth, D. J., Pandolfi, P. P. 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. Nature Genetics, 36: 653 - 659.

Fan, L., Moon, J., Wong, T., Crodian, J., and Collodi, P. 2008. Zebrafish primordial germ cell cultures derived from vasa::RFP transgenic embryos. Stem Cells and Development, 17: 585 - 598

Giuili, G., Tomljenovic, A., Labrecque, N., Oulad - Abdelghani, M., Rassoulzadegan, M., Cuzin, F. 2002. Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. EMBO reports, 3:753 - 759.

Grizzle, J. M. 1985. Channel Catfish Culture. Elsevier, Amsterdam, Netherlands. Reproductive biology, C. S. Tucker (editor). Pages 229 - 282.

Gustafson, E. A., Wessel, G. M. 2010. *Vasa* genes: Emerging roles in the germ line and in multipotent cells. Bioessays, 32(7): 626 - 637.

Hamaguchi, S. 1982. A light - and electron - microscopic study on the migration of primordial germ cells in the teleost, *Oryzias latipes*. Cell and Tissue Research, 227: 139 - 151.

Hofmann, M., Braydich - Stolle, L., Dym, M. 2005. Isolation of male germ - line stem cells; influence of GDNF. Developmental Biology, 279: 114 - 124.

Hogan, J.C., 1973. The fate and fine structure of primordial germ cells in the teleost, *Oryzias latipes*. The Journal of Cell Biology, 59, 146.

Jamieson, B. G. M., and Leung, L.K.P. 1991. Chapter 5: Introduction to fish spermatozoa and the micropyle. In: Fish Evolution and Systematics: Evidence from Spermatozoa. With a survey of lophophorate, echinoderm and protochordate sperm and an account of gamete cryopreservation, edited by Jamieson, B. G. M., Cambridge University Press, Cambridge, UK. pp 56 - 72.

Khan, A., Nakamoto, A., Tai, M., Saito, S., Nakayama, Y., Kawamura, A., Takeda, H., Yamasu, K. 2012. Mesendoderm specification depends on the function of Pou2, the class V POU - type transcription factor, during zebrafish embryogenesis. Development, Growth & Differentiation, 54(7): 686 - 701.

Koprunner, M., Thisse, C., Thisse, B., and Raz, E. 2001. A zebrafish *nanos* related gene is essential for the development of primordial germ cells. Genes & Development, 15: 2877 - 2885.

Kubota, H., Avarbock, M. R. Brinster, R. L. 2003. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proceedings of the National Academy of Sciences USA, 100: 6487 - 6492.

Kurokawa, D., Sakurai, Y., Inoue, A., Nakayama, R., Takasaki, N., Suda, Y., Miyake, T., Amemiya, C.T., and Aizawa, S. 2006.Evolutionary constraint on Otx2 neuroectoderm enhancers - deep conservation from skate to mouse and unique divergence in teleost. Proceedings of the National Academy of Sciences USA, 103(51): 19350 - 19355.

Liu, W., Collodi, P. 2010. Zebrafish dead end possesses ATPase activity that is required for primordial germ cell development. The FASEB Journal, 24(8): 2641 - 50.

Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., Wong, K. Y., Sung, K. W., Lee, C. W., Zhao, X. D., Chiu, K. P., Lipovich, L., Kuznetsov, V. A., Robson, P., Stanton, L. W., Wei, C. L., Ruan, Y., Lim, B., Ng, H. H. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet. 8: 431 - 40.

Leong, K. G., Wang, B. E., Johnson, L., Gao, W. Q. 2008. Generation of a prostate from a single adult stem cell. Nature, 456 (7223): 804 - 8.

Lubzens, E., Young, G., Bobe, J., Cerda, J. 2010. Oogenesis in teleosts: how eggs are formed. General and Comparative Endocrinology, 1, 165(3): 367 - 89

Lunde, K., Belting, H. G., Driever, W. (2004) Zebrafish pou5f1/pou2, homolog of mammalian Oct4, functions in the endoderm specification cascade. Current Biology, 14(1): 48 - 55.

Reim, G., Mizoguchi, T., Stainier, D. Y., Kikuchi, Y., and Brand, M. 2004. The POU domain protein spg (pou2/Oct4) is essential for endoderm formation in cooperation with the HMG domain protein casanova. Developmental Cell, 6(1): 91 - 101.

Matta, S. L. P., Vilela, D. A. R., Godinho, H. P., França, L. R. 2002. The goitrogen 6 - n - propyl - 2 - thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromisniloticus*) model. Endocrinology 143: 970 - 978

Maegawa, S., Yasuda, K., Inoue, K. 1999. Maternal mRNA localization of zebrafish DAZ - like gene. Mechanisms of Development, 81(1 - 2): 223 - 6.

Maegawa, S., Yamashita, M., Yasuda, K., Inoue, K. 2002. Zebrafish DAZ - like protein controls translation via the sequence 'GUUC'. Genes Cells. 7: 971 - 984.

Meng, X., Lindahl, M., HyvOnen, M., 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.

Meng, K., Rodriguez - Pena, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., Deuel, T. F. 2000. Pleiotrophin signals increased tyrosine phosphorylation of beta beta - catenin through inactivation of the intrinsic catalytic activity of the receptor - type protein tyrosine phosphatase beta / zeta. Proceedings of the National Academy of Sciences USA, 97: 2603 - 2608.

Moore, F. L., Jaruzelska, J., Fox M. S., Urano, J., Firpo, M. T., Turek, P. J., Dorfman, D. M., Pera, R. A. 2003. Human Pumilio - 2 is expressed in embryonic stem cells and germ cells and interacts with DAZ (Deleted in AZoospermia) and DAZ - like

proteins. Proceedings of the National Academy of Sciences USA, 100 (2): 538 - 43.

Morinaga, C., Tomonaga, T., Sasado, T., Suwa, H., Niwa, K., Yasuoka, A., Henrich, T., Watanabe, T., Deguchi, T., Yoda, H., Hirose, Y., Iwanami, N., Kunimatsu, S., Okamoto, Y., Yamanaka, T., Shinomiya, A., Tanaka, M., Kondoh, H., Furutani - Seiki, M., 2004. Mutations affecting gonadal development in medaka, *Oryzias latipes*. Mechanical Development. 121, 829 - 839.

Nagasawa, K., Shikina, S., Takeuchi, Y., Yoshizaki, G.2010. Lymphocyte antigen 75 (Ly75/CD205) is a surface marker on mitotic germ cells in rainbow trout. Biology of Reproduction, 83:597 - 606.

Nakamura, Y., Muguruma, Y., Yahata, T., Miyatake, H., Sakai, D., Mochida, J., Hotta, T., Ando, K. 2006. "Expression of CD90 on keratinocyte stem/progenitor cells". British Journal of Dermatology, 154 (6): 1062 - 70.

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. Biology of Reproduction, 85(2): 347 - 56.

Ohmura, M., Yoshida, S., Ide, Y., Nagamatsu, G., Suda, T., Ohbo, K. 2004. Spatial analysis of germline stem cell development in Oct - 4/ EGFP transgenic mice. Archives of Histology and Cytology, 67(4): 285 - 296.

Olsen, L.C., Aasland, R., Fjose, A. 1997. A *vasa* - like gene in zebrafish identifies putative primordial germ cells. Mechanisms of Development, 66:95 - 105.

Ozaki, Y., Saito, K., Shinya, M., Kawasaki, T., Sakai, N. 2011. Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. Gene Expression Patterns, 11: 309 - 315.

Patino, R., Davis, K. B., Schoore, J. E., Uguz, C., Struessmann, C. A., Parker, N. C. , Simco, B. A., Goudie, C. A. 1996. Sex differentiation of channel catfish gonads:

Normal development and effects of temperature. Journal of Experimental Zoology, 276: 209 - 218.

Pfaffl, M. W., Horgan, G. W., Dempfle, L., 2002. Relative expression software tool (REST©) for group - wise comparison and statistical analysis of relative expression results in real - time PCR. Nucleic Acids Research, 30, e36.

Reim, G., Brand, M. 2006. Maternal control of vertebrate dorsoventral axis formation and epiboly by the POU domain protein Spg/Pou2/Oct4. Development, 133: 2757 - 2770.

Sanchez - Sanchez, AV., Camp, E., García - Espana, A., Leal - Tassias, A., Mullor, JL. 2010. Medaka Oct4 is expressed during early embryo development, and in primordial germ cells and adult gonads. Developmental Dynamics, 239(2): 672 - 9.

Shinohara, T., Avarbock, M. R., Brinster, R. L. 1999. beta1 - and alpha6 - integrin are surface markers on mouse spermatogonial stem cells. Proceedings of the National Academy of Sciences USA, 96, 5504 - 5509.

Schulz, R. W., Menting, S., Bogerd, J., França, L. R., Vilela, D. A., Godinho, H. P. 2005. Sertoli cell proliferation in the adult testis - evidence from two fish species belonging to different orders. Biology of Reproduction, 73: 891 - 898.

Schulz, R. W., de Franca, L. R., Lareyre, J. J., Le Gac, F., Chiarini - Garcia, H., Nobrega, R. H., Miura, T. 2010. Spermatogenesis in fish. General and Comparative Endocrinology, 1: 165(3): 390 - 411.

Shinohara, T., Orwig, K. E., Avarbock, M. R., Brinster, R. L. 2000. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proceedings of the National Academy of Sciences USA, 97, 8346 - 8351.

Slanchev, K., Stebler, J., Cueva - Mendez, G., Raz, E., 2005. Development without germ cells: the role of the germ line in zebrafish sex differentiation. Proceedings of the National Academy of Sciences USA, 102, 4074 - 4079.

Takeuchi, Y., Yoshizaki, G., Kobayashi, T. and Takeuchi, T. 2002. Mass isolation of primordial germ cells from transgenic rainbow trout carrying the green fluorescent protein gene driven by the *vasa* gene promoter. Biology of Reproduction, 67: 1087 - 1092.

Tokuda, M., Kadokawa, Y., Kurahashi, H., Marunouchi, T.2007. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biology of Reproduction, 76: 130 - 141.

SchÖnfeldt, V, Wistuba, J., Schlatt, S.2004. Notch -1, c - kit and GFR alpha - 1 are developmentally regulated markers for premeiotic germ cells. Cytogenetic and Genome Research, 105: 235 - 239.

Wang, W., Lin, C., Lu, D., Ning, Z., Cox, T., Melvin, D., Wang, X., Bradley, A., Liu, P. 2008. Chromosomal transposition of PiggyBac in mouse embryonic stem cells. Proceedings of the National Academy of Sciences USA, 105: 9290 - 9295.

Weidinger, G., Stebler, J., Slanchev, K., Dumstrei, K., Wise, C., Lovell - Badge, R., Thisse, C., Thisse, B., Raz, E., 2003. Dead end, a novel vertebrate germ plasma component, is required for zebrafish primordial germ cell migration and survival. Current Biology, 13, 1429 - 1434.

Xu, H., Li, M., Gui, J., Hong, Y. 2010. Fish germ cells. Science China Life Sciences, 53(4): 435 - 46.

Yamaji, M., Seki, Y., Kurimoto, K., Yabuta, Y., Yuasa, M., Shigeta, M., Yamanaka, K., Ohinata, Y., Saitou, M. 2008. Critical function of Prdm14 for the establishment of the germ cell lineage in mice. Nature Genetics, 40(8): 1016 - 22.

Yoon, C., Kawakami, K., Hopkins, N. 1997. Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2 - and 4 - cell stage embryos and is expressed in the primordial germ cells. Development, 124: 3157 - 3165.

Yoshizaki, G., Tago, Y., Takeuchi, Y., Sawatari, E., Kobayashi, T., Takeuchi, T. 2005. Green fluorescent protein labeling of primordial germ cells using a nontransgenic

method and its application for germ cell transplantation in salmonidae. Biology of Reproduction, 73: 88 - 93.

Yoshizaki, G., Ichikawa, M., Hayashi, M., Iwasaki, Y., Miwa, M., Shikina, S., Okutsu, T. 2010. Sexual plasticity of ovarian germ cells in rainbow trout. Development. 137(8): 1227 - 30.

Yoshida, S., Takakura, A., Ohbo, K., Abe, K., Wakabayashi, J., Yamamoto, M., Suda, T., Nabeshima, Y. 2004. Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Developmental Biology, 269: 447 - 458.

Yuan, L., Liu, J. G., Zhao, J., Brundell, E., Daneholt, B., and Hoog, C. 2000. The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. Molecular Cell, 5, 73 - 83.

CHAPTER FOUR

Blue Catfish (*Ictalurus furcatus*) Germ Cell Culture

Abstract

Both blue catfish (*Ictalurus furcatus*) testicular and ovarian germ cell culture were studied *in vitro*. To achieve better culture conditions, the buffering ability of L - 15 and DMEM define based culture mediums were compared. L - 15 based medium had better buffering ability than DMEM based medium in air. L - 15 medium could be used as base medium in catfish germ cell culture without a CO_2 incubator. Spermatogonial stem cells (SSCs) enriched by Percoll density gradient centrifugation, testicular germ cells isolated after trypsin digestion and testicular tissues produced by physical dispersion, were used as initial culture materials. Cells from SSCs enrichment and testicular germ cell isolation did not proliferate or adhere to the plate during culture; but, they could stay alive for 16 days. When minced testicular tissues were used as the initial culture materials, cells migrated outward to the edges of the tissues and formed monolayers during the first two weeks culture; cultured cells were fibroblast like and grew quickly after the third passage; testicular germ cell growth curves were studied based on the growth of the cells in passage six. *In vitro* ovarian germ cell culture was studied using the same culture conditions. Ovarian germ cell isolation was used as the initial culture material, and the cells attached to the plate during the first week of culture. Cultured cells from ovaries were also fibroblast - like based on morphology, as seen in cells developed from testis material. Passage six testicular germ cells were also used for evaluation of

cryopreservation in liquid nitrogen. After thawing, 77.4% of the cells were alive. After seven days of culture, a monolayer had formed and cell growth was stable.

Keywords: blue catfish (*Ictalurus furcatus*), spermatogonial stem cells, testicular germ cell culture, ovarian germ cell culture

Introduction

Fish Cell Culture

Cell culture refers to a culture derived from dispersed cells taken from original tissue - a primary culture, and from cell line - a subculture. *In vitro* cell culture systems for animals have been established since the1950s (Chaudry, 2004). Cultured cells provide important models for basic biological and physiological studies ranging from identification of cellular function and cell - environment interaction to determination of quantitative molecular and genetic manipulation. Cultured cells are routinely used in the biomedical industry to produce enzymes, vaccines, growth factors, monoclonal antibodies and hormones (Evans et al. 2006).

Since the first fish cell line, RTG - 2 from rainbow trout gonad, was established (Wolf and Quimby, 1962), fish cell culture has steadily progressed. More than 280 fish cell lines have been established from different tissues and organs of many fish species (Wolf and Mann, 1980; Fryer & Lannan, 1994; Lakra et al. 2011).

Fish used in cell culture need to be fresh and healthy with no surface wounds. Cells from juvenile fish are more commonly used as the initial material in fish cell culture than the cells from mature fish, because they have much more viability (Ostrander, 2000). For example, in gonadal cell culture, the gonads from early development stages of immature fish are used as the initial material. For higher vertebrates, most of the cell lines are derived from tumor cells. But for fish, primary cultured cells and tissues are mostly derived from normal tissue, such as embryo, gill, liver, kidney, gonad, swimming bladder and fin, with only a few from tumor cells (Evans et al. 2006).

The same terminology is used for cells cultured from either mammalian or teleost sources. Cells cultured directly from an organ or tissue and before the first passage are referred to as in primary culture. Few tissue types can be cultured directly without tissue dissociation (Kruse et al. 1973). Briefly, the tissues are cut into $1mm^3$ blocks and placed in the culture plates; during culture, cells will migrate outward from the edge of the

tissues. However, for most of the tissues, tissue dissociation is needed before culturing. Physical dispersion with a chelating agent and enzymatic digestion is one of the most common methods used in tissue dissociation (Alberts et al. 2002; Sen et al. 2004; Prater et al. 2013). Physical dispersion is applied to organs with loose and / or delicate cellular extracellular matrix, such as embryos and larvae. Repeated shearing and extruding can disperse the tissues and release the cells for culture. Chelating agents, such as ethylenediamine tetraacetic acid (EDTA), are used in blastula cell dissociation and cell line passage (Lanza et al. 2004). By binding (chelating) the cations (Ca^{2+} and Mg^{2+}) of the intercellular substance, chelating agents the cell connection and separate cells, and usually this is combined with enzymatic digestion.

Tissue dissociation is a very important step in primary cell culture. The appropriate tissue dissociation method is chosen based on the characteristics of the organs and tissue, and it can be accomplished with one method or a combination of several methods. Enzymatic digestion is the most common method used in tissue and organ dissociation. Depending upon the level of dissociation, enzymatic digestion can be divided into two categories: complete digestion and incomplete digestion. Complete digestion achieves the maximum tissue dissociation - single - cells. Incomplete digestion indicates a shorten time of digestion, which produces a mix of smaller tissue blocks and free cells. Compared to complete digestion, incomplete digestions have lower mortality, and also maintains the interactions between cells (Lanza et al. 2004). Trypsin (0.25% ~ 0.5%), collagenase and hyaluronidase are the most commonly used enzymes as well as other enzymes for tissue disaggregation, such as DNase, pronase alone or in combinations (Freshney, 2005). Trypsin digestion may be performed at high or low temperatures (‘cold digestion’). In cold digestion, $2mm^3$ tissue blocks are digested at 4 ℃ in 5 ~ 20 times volume of trypsin - containing buffer or medium overnight; but for hot digestion, the temperature is usually 15 ~ 20 ℃ for 30 ~ 60min.

Most of the culture media used in fish cell culture come directly from mammalian cell culture media. However, the culture temperature is based on the donor fish’s survival temperature. 25 ℃ is the most commonly used temperature in fish cell culture. The suitable pH for most fish cells is 7.0 ~ 7.4 (Sashikumar and Desai, 2008). A pH above 7.6

or below 6.8 is toxic to cells, causing cell degeneration and death. Cultured fish cells are different from mammalian cells in that the time between passages is more flexible, about 7 ~ 14 days or even longer. In marine fish cell culture, osmotic pressure of the culture medium is regulated by adjusting the NaCl concentration (Bejar et al. 1997).

Successful fish cell culture is critically dependent upon optimizing the culture medium. Serum, which promotes cell growth and adherence, is an integral component in the culture medium. Serum comprises 10 ~ 20% of the total volume in the primary culture medium, and 5 ~ 10% in the cell line culture medium (Bradford et al. 1994; Detrich, et al. 1999). Fetal bovine serum (FBS) is the most commonly used serum, which not only provides nutrition and promotes cell attachment, but also protects cells from damage from fatty acids and other substances such as protease (Ghosh et al. 1994). Much like mammalian cells, phosphate buffered saline (PBS) and Hank's buffered salt solution (HBSS) is suitable salt solutions used in fish cell culture. To promote cell division and proliferation, one or more growth factors are often added into the culture medium common, such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). The bFGF can strongly promote cell proliferation *in vitro*, and it was a key component in the successful isolation and culture of human ESCs for the first time in 1998 (Thomson et al. 1998), which then opened a new era for the study and application of human embryonic stem cells, and other mammalian and non - mammalian cell culture studies. Generally the most commonly used concentration is 2 ~ 20 ng/mL. EGF can promote epidermal cells and fibroblasts proliferation and division with a concentration of 0.5 ~ 25 ng/mL. Antibiotics and antifungal, such as penicillin (100 unit/mL) and streptomycin (100 ug/mL) with an antifungal amphotericin B (10 μg/mL), are added to the medium to prevent contamination (Detrich et al. 1999; Kumar, 2012).

Application of Fish Cell Culture

Cultured fish cells were firstly used for identifying and diagnosing fish viral diseases (Wolf and Ahne, 1982). Because fish viruses have variable latency periods, are highly contagious, and can cause complex symptoms and high mortality, fish cell lines have provided valuable models for isolation and identification of fish viruses, study of the molecular mechanism of virus infection, viral induction of apoptosis and viruses-

antibody interaction (Evans et al. 2006). Channel catfish ovary (CCO), chinook salmon embryo (CHSE214), carp epithelioma papulosum (EPC), bluegill fry (BF2), fathead minnow (FHM), rainbow trout gonad (RTG), and rainbow trout hepatoma (RTH) are the commonly used cell lines for identifying and diagnosing fish viral diseases (Peters 2009). With the application of other technologies to the fish cell culture such as cytopathic effect examination, electron microscopy, *in situ* hybridization, immunostaining and plaque formation assays, more than 60 fish viruses have been propagated and studied in cell culture. Fish cell cultures have also been used to study pathogenic bacteria and protozoa (Garces et al. 1991; Wongtavatchai et al. 1994).

Fish cell culture has become more important for vaccine production. The traditional way of treating and preventing fish diseases is by using chemical and antibiotics. However, long - term use of antibiotics leads to antibiotic resistant strains of bacteria. Moreover, the large amount of antibiotic residues have caused serious environment damage and aquatic bacterial disorders, and also affected food fish safety (Chalmers, 2004). Fish vaccines leave no residue in the product and environment, and have become an established, proven, and cost - effective method of controlling certain infectious diseases in aquaculture worldwide during the past 20 years (Evans et al. 2006). Commercial vaccines have been widely used in several aquaculture species, such salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), European seabass (*Dicentrarchus labrax*), Japanese amberjack (*Seriola quinqueradiata*), tilapia (*Oreochromis niloticus*) and Atlantic cod (*Gadus morhua*) (Sommerset et al. 2005). Fish cell lines are a useful tool for isolation and propagation of viruses and microorganisms, which can be used in vaccine development and commercial production.

Cultured fish cells are also an important tool in toxicology and ecotoxicology studies. Toxicants are often released first into aquatic environments, and effects on the fish can serve as a warning of possible impacts on human health. Understanding the actions and effects of toxicants on fish assists in evaluating the health of the aquatic environment and the potential impacts on human health. Recently, fish cell lines have been widely applied as a replacement for *in vivo* animal tests in aquatic toxicology and animal health to understand mechanisms of toxicity and evaluate the toxicity of

environmental samples (Segner 1998; Lee et al. 2008; Dayeh et al. 2013). Fish cell lines such as WF - 2 (walleye fry), GFSk - S1 (goldfish skin), RTL - W1 (rainbow trout liver), RTgill - W1(rainbow trout gill), FHML (fathead minnow liver) and FHMT (fathead minnow testes) were employed to evaluate oil sands process - affected waters (OSPW), and were developed for biomonitoring to identify toxicants in contaminated areas (Sansom, 2010). It is easier, faster, more reproducible, and less costly using fish cells to study toxicology and ecotoxicology. Furthermore, by using multiple fish species cell cultures, the relative sensitivity to environmental contaminants at the cellular level and molecular level can be compared (Moon et al. 2005).

Fish cell lines can be used to model the immune response of bacterial infection, mechanisms of immune functions in mammalian cells, and for studying the interaction and function of molecules, such as interleukins, leukotrienes and other cytokines (Anderson, 1992; Hasegawa et al. 1998). Both primary cell culture and cell lines are also valuable models to study immunology. Although fish are lower vertebrates, their immune systems possess distinct immune cells (B - cell, T - cell and monocytes) that are functionally similar to those of higher vertebrates. Fish kidney, spleen, thymus and lymphoid tissues near the digestive tract, as well as blood and lymph, are the immune response organs (Uribe et al. 2011). The pioneering *in vitro* studies of piscine immune function were conducted using cultured catfish peripheral blood lymphocytes (Evans et al. 2006). Proliferative responses to mitogenic factor, molecular events occurring during T - cell and macrophage activation, and cytokine induced changes in gene expression have been studied by using fish immune cell cultures.

Cultured fish cells are also an ideal material for gene function analysis, especially in immune gene function studies (Zhu et al. 2013; Uribe et al. 2011). Similar to higher vertebrates, fish have both specific and non - specific immune systems. The non - specific immunity plays an important role in defensing bacteria and pathogen infection, and the function and mechanism of non - specific immunity related genes have been studied. The non - specific immune factors, including antimicrobial peptide, interleukins, interferon, interferon regulatory factors (IRF), transforming growth factor β (TGF - β), tumor necrosis factor α (TNF - α), NK cell enhancement factor (NKEF) and major

histocompatibility complex (MHC, including MHC I and II) has been reported in zebrafish (*Brachydanio rerio*), carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), white bass (*Morone chrysops*), rainbow trout (*Oncorhynchus mykiss*), flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*) (Uribe et al. 2011).

Stem cells have the ability of self - renewal by mitotic cell division and differentiation into a number of different cell types. Stem cell culture provides a valuable model not only for *in vitro* cell growth and differentiation studies but also for gene function study during individual development and growth. In mammals, genetically modified embryonic stem cells were selected and cultured for transplantation to recipient embryos where they contribute to the germ cell lineage (Doetschman et al. 1987; Capecchi, 1989). Additionally, cultured embryonic germ cell - PGCs were used to produce transgenic mice (Piedrahita et al. 1998) and chickens (van de Laoir et al. 2006).

Stem cell culture technology has been applied in the field of aquaculture. Embryonic stem cell (ESC) and germline stem cell (GSC) culture have been established in several laboratories (Bejar et al. 2002; Chen et al. 2003 a and b; Shikina et al. 2008). Undifferentiated cells from blastula - stage embryos were used for the initiation of culture. In zebrafish ESC cells were cultured on a growth - arrested feeder layer which was formed by rainbow trout spleen cell line RTS34st (Fan et al. 2004; Ma et al. 2001). LDF medium as the basal medium supplemented with human EGF, human bFGF, bovine insulin, FBS and trout serum was used for ESC culture. The pluripotency of the cultured ESCs was evaluated by transplanting cultured ESCs which were initiated from a transgenic line of zebrofish into recipient blastula - stage embryo (Fan et al. 2004). Approximately, 50 - 100 cultured ESCs were injected into the cell mass of each recipient blastula. Three days after the transplantation, the germ - line chimeras developed from the cultured ESCs were identified in the region of gonad. Germ - line chimeras developed in the gonad and formed functional gametes. Cultured cells from multiple passages were also evaluated. When cultured ESCs at the first passage were injected into embryos, 5% embryos survived to sexual maturity. While, when cultured ESCs at the fifth passage were used as donor cells, less than 1% embryos survived to sexual maturity.

The ESC culture has also been established in medaka without feeder cells (Wakamatsu et al. 1994; Hong et al. 1996). ES - like cells in a feeder - free culture system were also established in several marine fish species, including the gilthead sea bream (*Sparus aurata*) (Bejar et al. 2007), red sea bream (*Pagrus major*) (Chen et al. 2003 b), sea perch (*Lateolabrax japonicus*) (Chen et al. 2007), Asian sea bass (*Lates calcarifer*) (Parameswaran et al. 2007), and Atlantic cod (*Gadus morhua*) (Holen et al. 2010). After cultured ESCs from medaka and gilthead sea bream were transplanted into recipient embryos, chimeras were formed in 90% and 44% of the surviving embryos, respectively.

PGCs, spermatogonia from testis and oogonia from ovary are germline stem cells (GSCs), which are capable of dividing and renewing themselves for long periods and can be used as initial material for germline stem cell culturing (Hou et al. 2008). GSCs provide a unique opportunity to study and control animal reproduction, and offer an excellent system for genetic manipulations of the germline cell in vertebrates. Long - term cultivation of GSCs into stable cell lines could be combined with the successful used of xenotransplantion of GSCs to generate fertile chimeras (Takeuchi et al. 2003; Yoshizaki et al. 2005; Okutsu et al. 2007). The genetically altered and selected GSCs in culture could be transplanted into a host of same or closely related species to generate transgenic offspring (Fletcher et al. 2012).

Fish GSCs culture research mainly focuses on spermatogomia stem cell (SSC) culture. The first established fish SSC line, SG3, retained stable proliferation, diploid karyotype, and phenotype and gene expression pattern characteristics of SSCs after 140 passages during two - year culture (Hong et al. 2004). The culture medium used for culturing SG3 was DMEM with fish serum, fish embryo extract and basic growth factor. Under appropriate culture conditions, SG3 undergoes meiosis and spermiogenesis to generate motile sperm *in vitro*. In zebrafish, male germ cells were cultured by co - culturing with a Sertoli cell feeder layer (Sakai. 2006).

Shikina and Yoshizaki (2010) enriched rainbow trout type A spermatogonia by using adherent difference sorting method. Bovine serum albumin, adenosine, and salmonid serum were added into the medium to enhance spermatogonia survival, mitotic

activity, and transplantability. Spermatogonia were successfully cultured in the modified medium for 42 days before transplantation. The cultured spermatogonia were able to colonize recipient gonads after the transplantation. Panda et al. (2011) isolated and enriched *Labeo rohita* SSCs by two - step Ficoll gradient centrifugation followed by magnetic activated cell sorting. The purified cells were cultured for more than two months.

For PGC culture studies, zebrafish PGCs from trans - RFP - gene embryos were sorted by flow cytometry, and grown in the optimized culture medium with Kit ligand a and stromal cell - derived factor 1b. PGCs doubled the number of colonies during the 4 - month of culture (Fan et al. 2004). Three days after the transplantation, the germ - line chimeras developed from the cultured PGCs were identified in the region of gonad.

In zebrafish, female germline stem cells from a transgenic line of fish that expressed Neo and DsRed under the control of the germ cell specific promoter - ziwi were isolated by using drug - G418 selection and cultured for more than 6 weeks (Wong et al. 2013). The cultured female germline stem cells were able to successfully colonize the gonad of sterile recipient fish and generate functional gametes. Up to 20% of surviving recipient fish were fertile and generated multiple batches of normal offspring for at least 6 months.

Fish Germline Stem Cell Cryopreservation

The process of cryopreservation is defined as the long - term storage of biological materials (cells, tissues and organs) at an ultra - low temperature (-196 ℃) to arrest the metabolism in a physical state (Routray et al. 2010). The cell type and size, growth phase and rate, pH, density at freezing, composition of the freezing medium, type and concentration of cryoprotectants, cooling rate, storage temperature and duration of storage, warming rate and recovery medium effects the cryopreservation efficiency (Garrisi et al. 1992; Frederick et al. 2004). Cryopreservation of the germ line could conserve genetic information of endangered fish species. The cryopreservation of sperm has been developed in many species (Chao et al. 2001). However, fish eggs cannot be cryopreserved because of their large size and high yolk content. Since GSCs can

differentiate into both egg and sperm, the cryopreserved GSCs can be interspecifically transplanted into the gonad of closely related species to regenerate offspring of the threatened species (Okutsu et al. 2006).

Cryopreservation has been used in fish PGCs conservation. The cryopreserved cultured PGC differentiated into either eggs or sperm after they were introduced into the gonads of recipient fish (Yoshizaki et al. 2005). Rainbow trout (*Oncorhynchus mykiss*) PGCs were successfully cryopreserved using a cryomedium containing ethylene glycol (Kobayashi et al. 2003). The thawed PGCs that were transplanted into the peritoneal cavities of allogenic trout hatchlings differentiated into mature spermatozoa and eggs in the recipient gonads. Furthermore, the fertilization of eggs derived from cryopreserved PGCs by cryopreserved spermatozoa resulted in the development of fertile F1 fish.

Cryopreservation technique was also used in fish SSCs conservation. Rainbow trout spermatogonia were cryopreserved by using a slow - freezing method (Yoshizaki et al. 2011). Functional eggs and sperm were derived from those spermatogonia after transplantation into the peritoneal cavity of triploid masu salmon (*Oncorhynchus masou*) hatchlings of both genders. Nile - tilapia SSCs tilapia were successfully cryopreserved and able to maintain their functionality after cryopreservation (Lacerda et al. 2010).

After the whole testes taken from rainbow trout were slowly frozen in a cryomedium, the viability of type A spermatogonia within testes did not decrease over a 728 - day cryopreservation. Thawed type A spermatogonia were intraperitoneally transplanted into sterile triploid hatchlings, and those type A spermatogonia migrated toward recipient genital ridges. Approximately, 50% of triploid recipients produced functional eggs or sperm derived from the type A spermatogonia isolated from the whole frozen testes (Lee et al. 2013).

The objective of the current research was to establish an *in vitro* blue catfish germ cell culture system and a germ cell cryopreservation methodology. A successful germ cell line can be used for cell transformation and transplantation studies.

Materials and Methods

Experimental Fish

Nine two - year - old male juvenile blue catfish and 3 two - year - old female juvenile blue catfish (TL: 25 - 30 cm) were collected from the Fish Genetics Research Unit, E. W. Shell Fisheries Center, Auburn University, AL. After anesthetized with tricaine methanesulfonate (MS - 222, Finquel), all catfish were incubated on ice and transferred to the lab. The surface of the fish was sterilized with 70% ethanol. The testes were carefully removed from the peritoneal cavity avoiding the connective tissue, including the peritoneum and blood vessels. Testes from one fish were placed in 5 mL anti - agent medium (Hanks' Balanced Salt solution, HBSS, with 1.0 μg/mL $NaHCO_3$, 100 unit/mL penicillin and 100 μg/mL streptomycin) in a one 15 mL centrifuge tube. Thereafter, the tubes were transferred into the biosafety cabinet (Labconco, Kansas City, MO). Every 3 fish from the same gender were considered as replications.

Culture Medium Buffering Ability

The basic medium L - 15 (Leibovitz's - 15) medium (Lonza) supplied with 25 mM HEPES, 100 unit/mL penicillin, 100 μg/mL streptomycin, 1.0 μg/mL $NaHCO_3$, 0.3 μg/mL L - glutamine, 20% FBS, 5% catfish serum and 1 ng/mL bFGF were used as culture medium. After all components were mixed together, the culture medium was filtered and sterilized with 0.22 μm filter (Millipore). Catfish serum was collected from blood of adult channel catfish. Channel catfish whole blood was collected and placed in a 50 mL centrifuge tube to allow the blood to clot by leaving the centrifuge tube undisturbed at 4 ℃ overnight, followed by centrifugation at 2,000g for 10 min at 4 ℃. The supernatant was serum.

L - 15 medium was compared to DMEM (Dulbecco's Modified Eagle Medium, Lonza). Instead of L - 15, DMEM was used as the basic medium and all the other components were same. The buffering ability of these two media was studied. Briefly,

two 6 - well plates (Falcon) with 2mL L - 15 based medium in each well were placed into two different incubators. One incubator was set at 27 ℃ with air; the other incubator was set at 37 ℃ with 5% CO_2. Media were taken out from one well of each incubator every day for six continuous days. The same procedure was performed for DMEM based medium.

SSC Culture

Testes from 3 two - year - old male juvenile blue catfish were used for SSC culture study. Within the biosafety cabinet, testes from each fish were transferred into a plastic petri dish and any connective tissue and blood was removed using a pair of blades. The testes were rinsed three times by 1 mL anti - agent medium, soaked in 5 mL of 0.5% bleach solution (prepared fresh at 0.5% in ddH_2O from a newly opened bottle) for 2 min, and rinsed three times by 1 mL HBSS. The testes were then minced into small pieces with a pair of sterilized blades. Minced testicular tissues of each replicate were transferred into one 50 mL autoclaved glass flask which contained a stir bar. Each sample were treated by 0.25% trypsin - EDTA (2.5 g/L trypsin and 0.38 g/L EDTA; Gibco), 50 times of the weight of the testicular tissue, followed by incubating on ice for 30 min and 1 hr at 22 ℃ with a magnetic stirrer to achieve higher digestion efficiency. The cell suspension from each replicate was then filtered using a 40 μm cell strainer (nylon mesh, Falcon) and centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet resuspended in 2 mL HBSS.

A discontinuous density gradient centrifugation made by using 70%, 45% and 35% of Percoll was used to enrich the percentage of spermatogonia. The top two cells bands which contained high concentrations of SSCs from an individual fish were pooled together and cultured in 6 - well plates. Plates were coated with attachment factor (0.1% gelatin, Gibco) at least 1 hr prior using. Each well contained 2 mL of culture medium and the initial cell culture concentration was $8 \sim 10 \times 10^6$ /mL. Cells were incubated in the incubator at 27 ℃ in air. After 2 - day (40 ~ 48 hr) culture, the cell suspension from each well was passed into a new gelatin coated well for another 2 - day (40 ~ 48 hr) culture. Then the cell suspension was passed again into a new gelatin coated well for long term

culture and partial media change was performed every four days. During culture, cells were observed using an inverted Nikon Eclipse TS100 microscope and imaged using a Nikon D40X DSLR camera.

Testicular Germ Cell Culture

Two different experiments were carried out by using different testicular tissue dissociation: enzymatic digestion and physical dispersion. Enzymatic digestion by 0.25% trypsin - EDTA was used in the first method, and dissociated cells isolated from testes of two - year - old male juvenile blue catfish were for initial cell culture. After filtration through a 40 μm cell strainer (nylon mesh, Falcon), the cell suspension from each fish was centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet was resuspended in culture medium. Cells were seeded in attachment factor coated 6 - well plates at the concentration of $8 \sim 10 \times 10^7$ /mL. Plates were incubated in the incubator at 27 ℃ in air.

For the physical dispersion method, after dissection and removal from the peritoneal cavity, testes were cleaned and sterilized in the biosafety cabinet. Testicular tissues were then minced into small pieces by a pair of sterilized blades, and the small pieces of tissue were directly placed in attachment factor coated 6 - well plates for culturing. Plates were also incubated in an incubator at 27 ℃ in air.

Cells and tissues were observed microscopically and imaged before changing medium. Media was partially changed every four days, and suspended cells or tissues were collected by centrifugation and returned to the same culture well. Cells were subcultured when 80 ~ 90% culture surface was covered by cells.

During subculture, the culture plates were first rinsed with warm PBS (Lonza) after culture medium was discarded. Cells were then incubated with 0.05% trypsin (Gibco) for 0.05 mL/cm^2 at 27 ℃ for 1 min. Cells were observed under the microscope, and when most cells became round in shape, the cells were loosened off the plate surface by gently tapping the side of the plates. Trypsin was neutralized by the addition of twice volume of culture media, followed by centrifugation (Eppendorf) at 500 g for 5 min.

Pelleted cells were resuspended in culture media and subcultured at a ratio of one well to one T - 25 flasks (Cellstar). Later, culture divisions of either 1 : 2 or 1 : 3 were employed.

Stable growing testicular germ cells at passage 6 were used in the growth curve study. Cells were seeded at 2×10^5 /mL in 12 - well culture plates (Falcon). After all plates were incubated at 27 ℃ for one day and all cells adhered to the plate surface, medium was changed for all wells every day for 7 days. Cells from three wells were removed everyday and cell number was measured with a hemocytometer for each well.

Ovarian Germ Cell Culture

Ovarian germ cells from three two - year - old female juvenile blue catfish were isolated separately by the 0.25% trypsin - EDTA digestion method used in testicular germ cell isolation. The cells and tissue suspension were filtered through a 40μm cell strainer (nylon mesh, Falcon) and centrifuged. The pellet was resuspended in culture medium. Cells were also seeded in the attachment factor coated 6 - well plates at a concentration of $1 \sim 2 \times 10^8$ /mL in culture medium. Plates were incubated at 27℃ in air. Cells were observed and imaged before changing medium. Partial media change was performed every four days and the suspended cells discarded. Subculture was needed when 80 ~ 90% of the culture surface was covered by cells.

Germ Cell Culture Identification

To confirm the germ cell characteristics of the testicular germ cell cultures and ovarian germ cell cultures, competitive PCR for the germ cell markers *nanos* and *vasa* (Xu et al. 2010) was evaluated. RNA from approximately 2×10^5 cultured testicular germ cells and cultured ovarian germ cells at 11th passage were extracted separately using TRIzol Reagent (Ambio) following the manufacturer's recommendation. The quality and concentration of all the samples were checked by gel electrophoresis and a ND - 1000 spectrophotometer (NanoDrop Technologies). All extracted samples should have an A260/280 ratio greater than 1.8. RNA was reverse transcribed into cDNAs by iScript Synthesis Kit (Bio - Rad). Each reaction consisted of a total volume of 10 μL containing 4.0 μL iScript reaction mix, 1.0 μL iScript reverse transcriptase, 500 ng RNA template,

and RNase / DNase - free water to reach the 10 μL volume. The reaction followed the protocol: 5 min at 25 ℃, 30 min at 42 ℃, 5 min at 85 ℃.

The *nanos* and *vasa* primers information is listed in Table 7. The PCR reactions were prepared in 10.0 μL volume containing 200ng cDNA, 1.0 μL of each primer (5 μM/l), 0.8 ul 2.5 mM of each dNTP, 1.0 μL 10 × PCR buffer, 0.4 μL 50 mM $MgCl_2$, RNase / DNase - free water. The PCR procedures were performed using the following thermo profile: after an initial denaturation at 95 ℃ for 5 min; PCR amplification was carried out at 95 ℃ for 30 s, 60℃ for 30 s, and 72 ℃ for 1 min for 35 cycles. A final extension at 72°C for 10 min was included. The seventeen amplification products were analyzed on an ethidium bromide stained 1.0 % agarose gel. Tracklt 100bp DNA Ladder (Invitrogen) was used to check the band size after electrophoresis.

Table 7 Primers used for PCR to confirm the germ cell characteristics of the 11th passage testicular germ cell cultures and ovarian germ cell cultures, which initially came from testes and ovaries of two - year - old juvenile blue catfish (*Ictalurus furcatus*). *Nanos*, *vasa* and 18S ribosome mRNA sequences were obtained with the NCBI accession number.

Gene	Accession No.	Forward Sequence	Reverse Sequence
Nanos	CK419278.1	TACGCCCGAGATTTCTCATC	ACCTTGAGGTGATGGAGGTG
Vasa	FD175080.1	CGTTATTGGTCGTGGAAAGG	GGCACTGAACATGAGGGTTT
18S	GQ465834.1	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG

Testicular Germ Cell Cryopreservation

Cultured testicular germ cells were at passage 6 when they were cryopreserved. Freezing medium was prepared by complete growth medium with 5% (v/v) DMSO. Briefly, after incubated in 0.05% trypsin, cells were collected by centrifugation (Eppendorf) at 500 g for 5 min. Instead of subculturing, cells were resuspended in freezing medium. All cells from one T - 25 flasks were resuspended in 1 mL freezing medium, and transferred into 1.5 mL freezing vials (CoolCell). Samples were placed in

Mr. Frosty containers (-1 ℃/minute, Nalgene) in a -80 ℃ ultrafreezer, and after 24 hr, were transferred to liquid nitrogen (-196 ℃).

Two weeks after cryopreservation, the cryotubes were taken out of liquid nitrogen and placed in a water bath for 1 to 2 min at 27 ℃ until the freezing medium was completely melted. The Trypan blue exclusion test was used to evaluate cell viability. After the cell suspension in freezing medium was diluted by culture medium, cells were seeded in T - 25 at 10×10^6 /mL and incubated at 27 ℃. Cell growth was observed with an inverted microscope.

Results

Culture Medium Buffering Ability

The pH of L - 15 and DMEM based culture media in both 27 ℃ with air and 37 ℃ with 5% CO_2 conditions during a six - day period are shown in Table 8. L - 15 based medium had better buffering ability than DMEM based medium in air. In 5% CO_2 condition, pH of both media remained approximately 7.23, which is permissive for cell growth.

Table 8 Buffering ability of L - 15 and DMEM based culture mediums in two different conditions.

		Culture medium pH						
Incubator	Medium	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
27℃ with air	L - 15 based medium	7.25	7.79	7.76	7.75	7.75	7.75	7.74
	DMEM based medium	7.19	8.45	8.55	8.56	8.56	8.56	8.56
37℃ with 5% CO_2	L - 15 based medium	7.25	7.18	7.19	7.23	7.23	7.23	7.23
	DMEM based medium	7.19	7.21	7.23	7.23	7.22	7.23	7.23

SSC Culture

During 16 - day culture, most of the spermatids in the cells from the top two bands after Percoll density gradient centrifugation were firstly adhering to the culture plate, and removed when cells were passed into a new gelatin coated well. Spermatogonia did not adhere to the plate and no cell proliferation was observed. Even though cells did not show proliferation during this short term culturing, cells were still alive when checked with Trypan blue exclusion test. Some of the spermatogonia adhered to each other (Fig. 12, arrow)

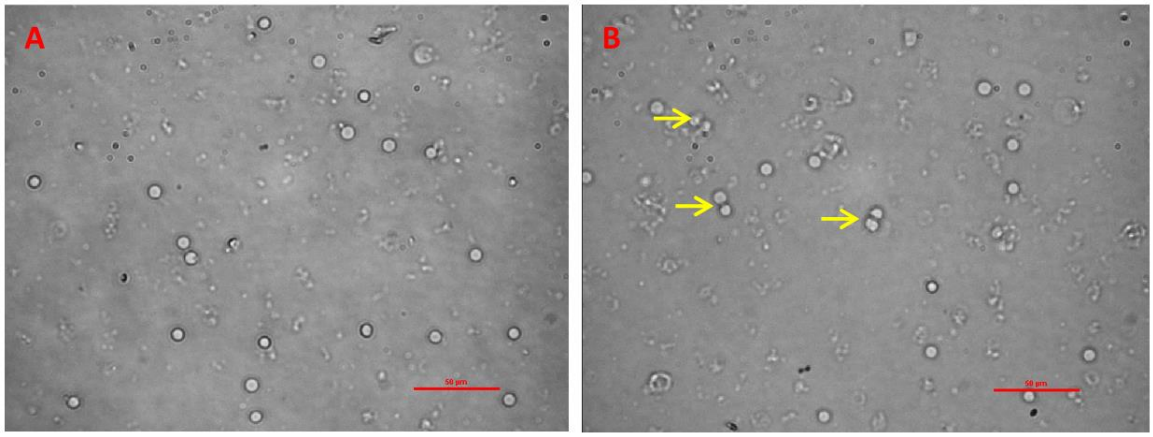

Fig. 12 Testes from two - year - old male juvenile blue catfish (*Ictalurus furcatus*) were digested by 0.25% trypsin - EDTA, spermatogonia were enriched by a discontinuous density gradient centrifugation made by using 70%, 45% and 35% of Percoll, followed by culturing in the basic medium L - 15 (Leibovitz's - 15) medium supplied with 25 mM HEPES, 100 unit/mL penicillin, 100 µg/mL streptomycin, 1.0 µg/mL $NaHCO_3$, 0.3 µg/mL L - glutamine, 20% FBS, 5% catfish serum and 1 ng/mL bFGF at 27 ℃ in air. A: spermatogonia and spermatocytes in the initial culture. B: spermatogonia adhered to each other (arrow, →) during culture. Bars A and B = 50 µm.

Testicular Germ Cell Culture

Dissociated Testicular Germ Cell Culture

After enzymatic digestion, isolated dissociated cells from testes of two - year - old male juvenile blue catfish were used for initial cell culture (Fig. 13, A). Testicular germ cells were cultured for one month. No spermatogonia attachment or proliferations were observed. Progressively, cell clusters formed during the culture (Fig. 13, B). When checked by Trypan blue exclusion test, > 70 % of the cells was still alive.

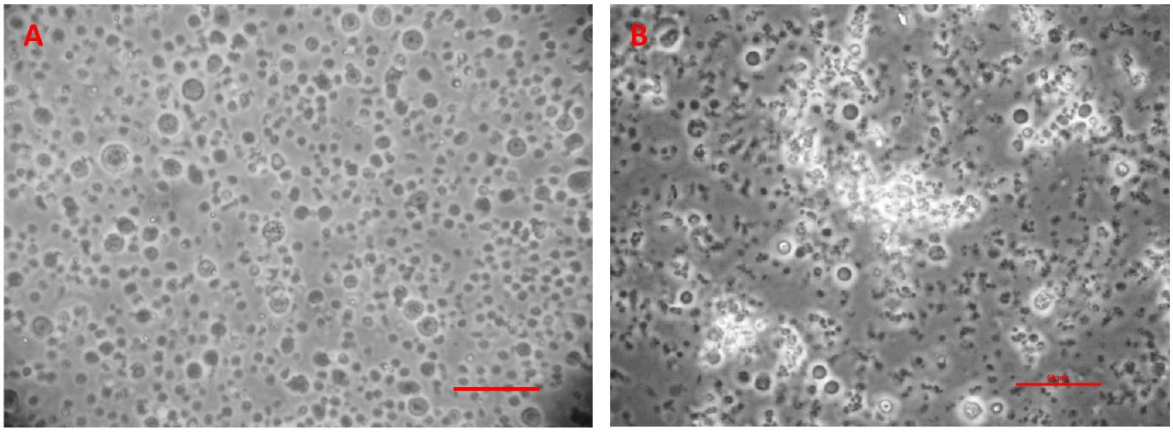

Fig. 13 Testes from two - year - old male juvenile blue catfish (*Ictalurus furcatus*) were digested by 0.25% trypsin - EDTA, dissociated cells were cultured in the basic medium L - 15 (Leibovitz's - 15) medium supplied with 25 mM HEPES, 100 unit/mL penicillin, 100 μg/mL streptomycin, 1.0 μg/mL $NaHCO_3$, 0.3 μg/mL L - glutamine, 20% FBS, 5% catfish serum and 1 ng/mL bFGF at 27 ℃ in air. A: testicular germ cell in the initial culture. B: cell clusters were formed during culture. Bars A and B = 50 μm.

Testicular Tissue Culture

During the initiation of the culture, testicular tissues were weakly attaching to the culture plate. Plates were handled very gently during the medium changing. The floating tissues were collected by centrifugation and returned to the same culture well. Tissues were completely settled after two weeks of culture. Cells began migrated outward from the tissue edges and formed monolayers (Fig. 14, A). Cells grew very slowly in the beginning, and when 80 - 90% of the culture surface was covered by cells, cells were subcultured to a new culture flask. Cells were fibroblast - like and grew fast. These testicular germ cell cultures might only come from one cell type, and might be the mix of somatic cell and spermatogonia. In the initial three passages, the cells were passaged once every 8 days with partial medium change every 4 days. After the third passage, cells grew very fast. The intervals of two passages were only 4 - 5 days with a 1 : 2 division ratio. Cells were at the 11th passage after 68 days of culture.

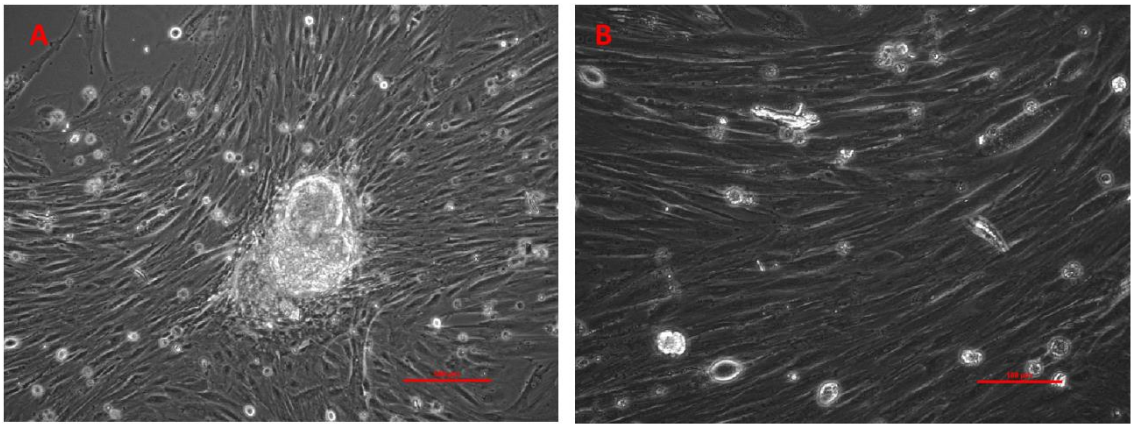

Fig. 14 Testicular tissues from two - year - old male juvenile blue catfish (*Ictalurus furcatus*) were minced into small pieces by a pair of sterilized blades, and the small pieces of tissue were directly placed in attachment factor coated 6 - well plates for culturing in the basic medium L - 15 (Leibovitz's - 15) medium supplied with 25 mM HEPES, 100 unit/mL penicillin, 100 μg/mL streptomycin, 1.0 μg/mL $NaHCO_3$, 0.3 μg/mL L - glutamine, 20% FBS, 5% catfish serum and 1 ng/mL bFGF at 27 ℃ in air. A: cells began migrating outward from the tissue edges and forming monolayers. B: cells of passage 6 formed monolayers during culture. Bars A and B = 100 μm.

Testicular Germ Cell Growth Curve

The cell growth curve is shown in Fig. 14. Cells adhered to the plate soon after the inoculation. At the fifth day, cell number reached 17.8×10^5 /mL. Cells number started dropping at day 6.

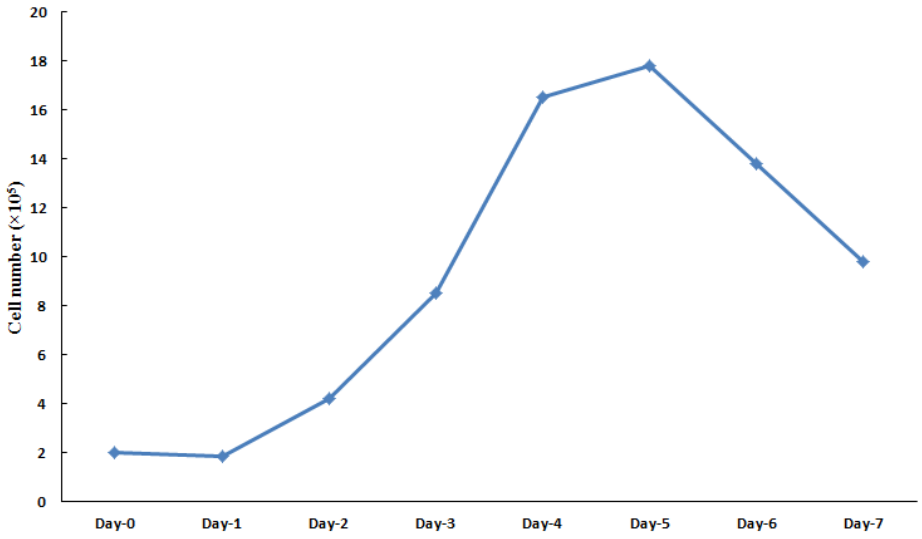

Fig. 15 Testicular germ cell growth curve of stable growing testicular germ cells from two - year - old male juvenile blue catfish (*Ictalurus furcatus*) at the 6th passage. Cells were cultured in the basic medium L - 15 (Leibovitz's - 15) medium supplied with 25 mM HEPES, 100 unit/mL penicillin, 100 µg/mL streptomycin, 1.0 µg/mL $NaHCO_3$, 0.3 µg/mL L - glutamine, 20% FBS, 5% catfish serum and 1 ng/mL bFGF at 27 ℃ in air.

Ovarian Germ Cell Culture

After enzymatic digestion, isolated dissociated cells from ovaries of two - year - old female juvenile blue catfish were used for initial cell culture, and cells started attaching to the culture plate in the first week of culture (Fig. 16, A). The unattached cells were discarded during medium changing. These ovarian germ cell cultures, might only come from one cell type, and also might be the mix of somatic cell and oogonia. When 80 - 90% of the culture surface was covered by cells, cells were subcultured to a new culture flask. Cells grew very fast after two passages. Cells were also fibroblast like. The intervals of two passages were 4 days with a 1 : 2 division ratio. Cells were at the 11th passage after 50 days of culture.

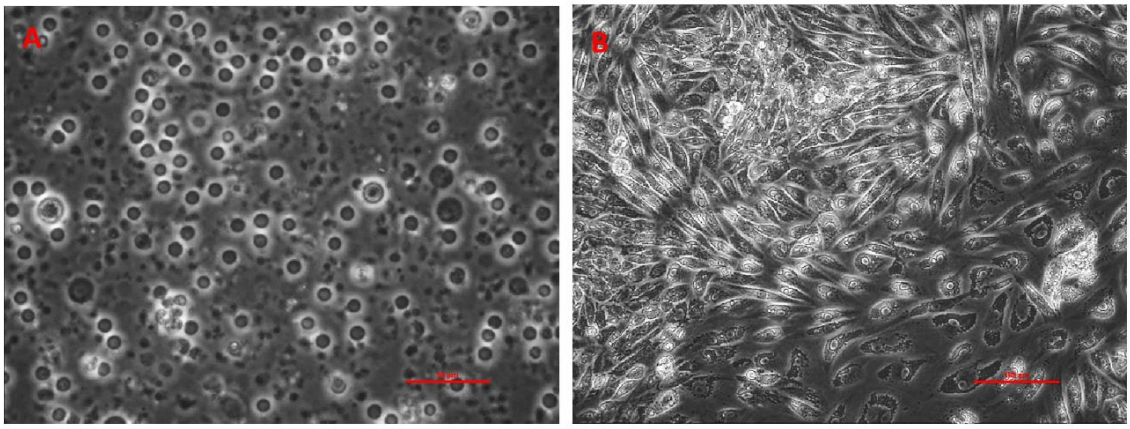

Fig. 16 Ovaries from two - year - old female juvenile blue catfish (*Ictalurus furcatus*) were digested by 0.25% trypsin - EDTA, dissociated cells were cultured in the basic medium L - 15 (Leibovitz's - 15) medium supplied with 25 mM HEPES, 100 unit/mL penicillin, 100 μg/mL streptomycin, 1.0 μg/mL $NaHCO_3$, 0.3 μg/mL L - glutamine, 20% FBS, 5% catfish serum and 1 ng/mL bFGF at 27 ℃ in air. A: dissociated ovarian germ cells in the initial culture. B: cells of passage 4 formed monolayers during culture. Bars A = 50 μm and B = 100 μm.

Identification of Germ Cell Cultures with Germ Cell Markers

The 11th passage testicular germ cell cultures and ovarian germ cell cultures, which came from testes and ovaries of two - year - old juvenile blue catfish, might only come from one cell type, and also might be the mix of somatic cell and GSC. *Nanos* and *vasa* genes expression were detected in both cultured testicular germ cells and cultured ovarian germ cells, which initially came from testes and ovaries of two - year - old juvenile blue catfish, at the 11th passage. The cultured testicular germ cells and cultured ovarian germ cells at 11th passage were germ cells.

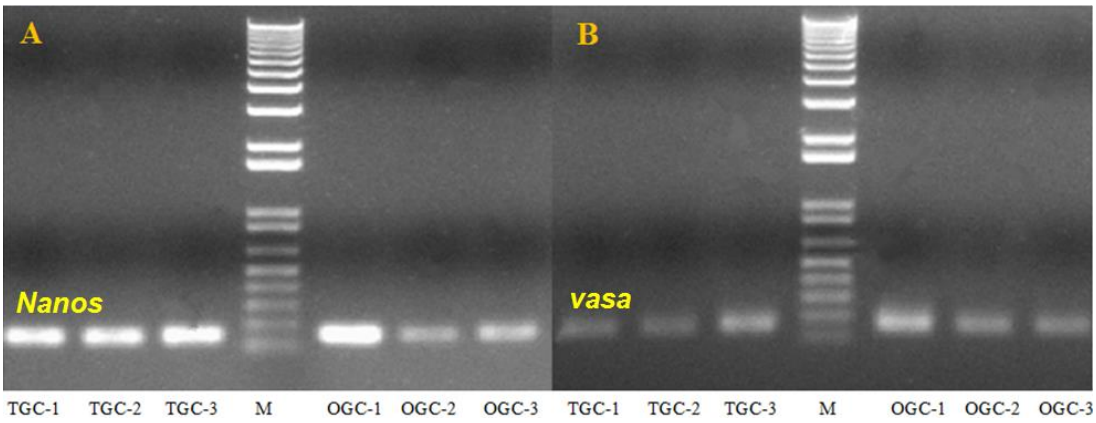

Fig. 17 *Nanos* and *vasa* amplification products for cultured testicular germ cells and cultured ovarian germ cells, which initially came from testes and ovaries of two - year - old juvenile blue catfish (*Ictalurus furcatus*), at 11th passage. A) *Nanos* gene expression was detected in cultured testicular germ cells and cultured ovarian germ cells. B) *Vasa* gene expression was detected in cultured testicular germ cells and cultured ovarian germ cells. TGC: short for testicular germ cells; OGC: short for ovarian germ cells.

Testicular Germ Cell Cryopreservation

After thawing, 77.4% of cryopreserved testicular germ cells cultures from two - year - old male juvenile blue catfish at passage of 6 were alive, which had been frozen for two weeks. Cell growth was stable during the recovery culture. After seven days of culture, the monolayer was formed.

Discussion

Testicular Germ Cell Culture

This is the first report of *in vitro* blue catfish testicular germ cell culture. To establish the cell culture, different types of cells and tissue were compared as initial culture materials. Small pieces of testicular tissues were the best material to initiate the cell culture. Cultured testicular germ cells, which initially came from testes of two - year - old juvenile blue catfish, at 11th passage were confirmed as germ cell by PCR for the competitive germ cell marker *nanos* and *vas* (Xu et al. 2010). The cultured testicular germ cells at 11th passage were germ cells. They might only come from one cell type, and also might be the mix of somatic cell and GSC. Based on the growth curve of the passage 6th testicular germ cells, cell number doubled during the first four days. At the fifth day, cell growth slowed down and cell number started dropping at day 6. Probably it was because of the nutrition limitation in the medium.

Testicular germ cell culture had been studied in zebrafish (*Danio rerio*) (Kurita et al. 2004 b) and rainbow trout (*Oncorhynchus mykiss*) (Shikina et al. 2008). Dissociated testicular cells containing spermatogonia, spermatocytes and spermatids, and testicular somatic cells, but few sperm from adult zebrafish, were dissociated with 500 U/mL of collagenase/dispase in L - 15 for 2 hr and followed by culturing on mitomycin C - treated feeder cells, in testicular cell culture medium supplemented with 3% FBS and incubated at 28 ℃ in air (Kurita et al. 2004 a, b). The *vasa* gene was still detectable after 12 - day culture. In rainbow trout testicular germ cell culture, spermatogonia were isolated form immature rainbow trout at age 8 ~ 10 month and cultured in Leibovitz's - 15 medium (pH 7.8) supplemented with 10% fetal bovine serum at 10℃ (Shikina et al. 2008). In addition, insulin, trout embryonic extract, and basic fibroblast growth factor were used to promote

the mitosis of spermatogonia. Testicular somatic cells were eliminated by adherent sorting. Spermatogonia were cultured for more than one month.

Even though we have confirmed that the cultured blue catfish testicular and ovarian germ cells cultures were germ cells, pluripotency of those testicular and ovarian germ cells cultures still needs to be proved by xenotransplantion and successful embryo production. For cells of testicular germ cells cultures, their pluripotency can be predicted if they have the specific gene expression as spermatogonia in Chapter 3. Long - term cultivation of SSCs into stable cell lines was combined with the successful xenotransplantion of SSCs to generate fertile chimeras in rainbow trout (Yoshizaki et al. 2008 Okutsu et al. 2007). Cultured spermatogonia could colonize and proliferate in recipient gonads following transplantation. Testicular germ cell culture also has great usefulness in creating sperm - based vectors. Transgenic zebrafish were successfully produced by cultured sperm which differentiated from spermatogonia *in vitro* (Kurita et al. 2004 b).

Enzymatic digestion is the most commonly used method for tissue dissociation. However, for some organs, embryos and larvae, enzymes are too strong, but gentle physical dispersion can be a better choice. Trypsin was too harsh for catfish testes digestion. After trypsin digestion, isolated testicular germ cell could not grow in culture medium. Weaker enzymes such as collagenase, dispase and actinase E also could be used in GSCs isolation (Yoshizaki et al. 2005).

Ovarian Germ Cell Culture

Blue catfish ovarian germ cells isolated from ovaries of two - year - old juvenile blue catfish were much easier to culture *in vitro* than testicular germ cells. Ovaries can be digested by trypsin, and the dissociated ovarian germ cells could be easily cultured in medium. Cultured cells have the same morphological characteristics as channel catfish ovary cell line (CCO). Cultured ovarian germ cells at 11th passage were confirmed as germ cells by PCR for the competitive germ cell markers *nanos* and *vasa*. The pluripotency of those ovarian germ cells also need to be proved by xenotransplantion.

Cultured female GSCs could be combined with the successful xenotransplantion in zebrafish. Female germline stem cells from a transgenic line of zebrafish were isolated and cultured for more than 6 weeks (Wong et al. 2013). The cultured female germline stem cells were able to successfully colonize the gonad of sterile recipient fish and generate functional gametes.

Testicular Germ Cell Cryopreservation

After two weeks cryopreservation, cultured testicular germ cells from two - year - old male juvenile blue catfish at passage 6 had high viability, 77.4%, and the growth of those cells was stable during the recovery culture. Cryopreservation of the testicular germ line could be effectively used to conserve different catfish species and strains. Cultured GSCs combined with successful cryopreservation and xenotransplantion forms a powerful tool in the catfish genetic enhancement and engineering which including hybridization and strain selection.

Cryopreservation has been used for fish PGC and SSC conservation. The cryopreserved cultured rainbow trout PGCs were differentiated into either eggs or sperm after they were introduced into the gonads of recipient fish (Yoshizaki et al. 2005). Rainbow trout, cryopreserved SSCs became functional eggs and sperm after transplantation into the peritoneal cavity of triploid masu salmon (*Oncorhynchus masou*) hatchlings of both genders (Yoshizaki et al. 2011).

In the current study, both testicular and ovarian germ cell culture was accomplished for more than two months. Optimization of Cell growth conditions and cell identification for long term culture need to be explored next.

Reference:

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell. 4th ed. New York: Garland Science. Isolating Cells and Growing Them in Culture. Available from: http://www.ncbi.nlm.nih.gov/books/NBK26851/

Anderson, D. P. 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Annual Review of Fish Diseases, 2: 281 - 307.

Bejar, J., Borrego, J. J., Alvarez, M. C. 1997. A continuous cell line from the cultured marine fish gilt - head sea bream (*Sparus aurata* L.). Aquaculture, 150: 143 - 153.

Bejar. J., Hong, Y., Alvarez, M.C. 2002. An ES - like cell line from the marine fish Sparus aurata: characterization and chimaera production. Transgenic Research, 11(3): 279 - 89.

Bradford, C. S., Sun, L., Collodi, P., Barnes, D. W. 1994. Cell cultures from zebrafish embryos and adult tissues. Journal of tissue culture methods, 16 (2): 99 - 107

Capecchi, M. R. 1989. Altering the genome by homologous recombination. Science, 244: 1288 -1292.

Chalmers, G. A. 2004. Aquaponics and Food Safety. Backyard Aquaponics. Online resource accessible at http://backyardaquaponics.com/Travis/Aquaponics-and-Food-Safety.pdf.

Chao, N. H., Liao, I. C. 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture, 197 (1 - 4): 161 - 189

Chaudry, A., 2004. Cell culture. The Science creative quarterly. Online resource accessible at http://www.scq.ubc.ca/cell-culture/

Chen, S. L., Sha, Z. X., Ye, H. Q. 2003a. Establishment of a pluripotent embryonic cell line from sea perch (*Lateolabrax japonicus*) embryos. Aquaculture, 218(1 - 4):141 - 151

Chen, S. L., Ye, H. Q., Sha, Z. X., Hong, Y. 2003b. Derivation of a pluripotent embryonic cell line from red sea bream blastulas. Journal of Fish Biology, 63(3): 795 - 805,

Chen, S. L., Sha, Z. X., Ye, H.Q., Liu, Y. et al. Pluripotency and chimera competence of an embryonic stem cell line from the sea perch (*Lateolabrax japonicus*). Marine Biotechnology, 9: 82 - 91.

Dayeh, V.R., Bols, N.C., Tanneberger, K., Schirmer, K., Lee, L.E. 2013.The use of fish - derived cell lines for investigation of environmental contaminants: an update following OECD's fish toxicity testing framework No. 171. Current Protocols in Toxicology, Chapter 1: Unit1.5.

Detrich, H. W., Weaterfield, M., Zon, L. I. 1999. The Zebrafish: Biology, Volume 1. Academic Press. p. 39 - 44.

Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S., Smithies, O. 1987. Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature, 330(6148): 576 - 8.

Evans, D. H., Claiborne, J. B. 2006. The Physiology of Fishes, Third Edition. CRC Press, Taylor & Francis Group, p. 551 - 556

Fan, M., Rhee, J., St - Pierre, J., Handschin, C., Puigserver, P., Lin, J., Jaeger, S., Erdjument - Bromage, H., Tempst, P., Spiegelman, B.M. 2004. Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC - 1α: modulation by p38 MAPK. Genes & Development, 18: 278 - 289.

Fan, L., Moon, J., Wong, T., Crodian, J., and Collodi, P. 2008. Zebrafish primordial germ cell cultures derived from *vasa*::RFP transgenic embryos. Stem Cells and Development, 17: 585 - 598

Fletcher, G. L., Rise, M. L. 2012. Aquaculture Biotechnology. Wiley - Blackwell, p.193 - 206.

Freshney, R. I., 2005. Chapter 12: Primary Culture of Animal cells: a munual of basic technique, 5th edn, John Wiley & Sons, Inc. pp175 - 197.

Frederick,V., Michiels, A., Goossens, E. 2004. Recovery, survival and functional evaluation by transplantation of frozen - thawed mouse germ cells. Human Reproduction, 19: 948 - 953.

Fryer, J., Lannan, C. 1994. Three decades of fish cell culture: a current listing of cell lines derived from fishes. Methods in Cell Science, 16: 87 - 94.

Garces, L. H., Larenas, J. J., Smith, P. A., Sandino, S., Lannan, C. N., Fryer, J. L. 1991. Infectivity of a rickettsia isolated from coho salmon *Oncorhynchus kisutch*. Disease of Aquatic Organisms, 11: 93 - 97

Garrisi, G.J., Navot, D. 1992. Cryopreservation of semen, oocytes and embryos. Current Opinion in Obstetrics and Gynecology, 4: 726 - 731.

Ghosh, C., Collodi, P. 1994. Culture of cells from zebrafish (*Brachydanio rerio*) blastula - stage embryos. Cytotechnology, 14(1): 21 - 6

Hasegawa, S., Nakayasu, C., Yoshitomi, T., Nakanishi, T., Okamoto, N. 1998. Specific cell - mediated cytotoxicity against an allogeneic target cell line in isogeneic ginbuna crucian carp. Fish and Shellfish Immunology, 8: 303 - 313.

Holen, E., Kausland, A., Skjaerven, K. 2010. Embryonic stem cells isolated from Atlantic cod (*Gadus morhua*) and the developmental expression of a stage - specific transcription factor ac - pou2. Fish Physiology and Biochemistry, 36(4): 1029 - 39.

Hong, Y., Winkler, C., Schartl, M. 1996. Pluripotency and differentiation of embryonic stem cell lines from the medakafish (*Oryzias latipes*). Mechanisms of Development, 60(1): 33 - 44.

Hong, Y., Winkler, C., Liu, T., Chai, G., Schartl, M. 2004. Activation of the mouse Oct4 promoter in medaka embryonic stem cells and its use for ablation of spontaneous differentiation. Mechanisms of Development, 121: 933 - 943

Hou, S. X., Singh, S. R. 2008. Germline Stem Cells. Methods in Molecular Biology, 450: v.

Kruse, P. F. Jr., Patterson, M. K. Jr. 1973. Tissue Culture: Methods and Applications. Academic Press, p. 12 - 14.

Kobayashi, T., Takeuchi, Y., Yoshizaki, G., and Takeuchi, T. 2003. Cryopreservation of trout primordial germ cells. Fish Physiology and Biochemistry, 28: 479 - 480.

Kumar, S. G. 2012. Development of Cell Culture Systems from Selected Species of Fish and Prawns. Doctoral theses. Cochin University of Science and Technology, India.

Kurita, K., Burgess, S. M., Sakai, N. 2004 a. Transgenic zebrafish produced by retroviral infection of in vitro - cultured sperm. Proceedings of the National Academy of Sciences, USA, 101(5): 1263 - 7.

Kurita, K., Sakai, N. 2004 b. Functionally distinctive testicular cell lines of zebrafish to support male germ cell development. Molecular Reproduction and Development, 67(4): 430 - 8.

Lacerda, S. M., Batlouni, S. R., Costa, G. M., Segatelli, T. M., Quirino, B. R., Queiroz, B. M., Kalapothakis, E., Franca, 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(5): e10740.

Lakra,W. S., Swaminathan, T. R., Joy, K. P. 2011. Development, characterization, conservation and storage of fish cell lines: a review. Fish Physiology and Biochemistry, 37: 1 - 20.

Lanza, R. P., Gearhart, J., Hogan, B., Melton, D., Pedersen, R., Thomson, J., West, M. 2004. Handbook of Stem Cells, Volume 1. Elsevier Academic Press, p. 493 - 495.

Lee, L. E. J., Dayeh, V. R., Schirmer, K., Bols, N. C. 2008. Applications and potential uses of fish gill cell lines: examples with RTGILL - W1. In Vitro Cellular & Developmental Biology - Animal, 45: 127 - 134.

Lee, S., Iwasaki, Y., Shikina, S., Yoshizaki, G. 2013. Generation of functional eggs and sperm from cryopreserved whole testes. Proceedings of the National Academy of Sciences, USA, 110(5):1640 - 5.

Ma, C., Fan, L., Ganassin, R., Bols, N., Collodi, P. 2001. Production of zebrafish germ - line chimeras from embryo cell cultures. Proceedings of the National Academy of Sciences, USA, 98(5): 2461 - 6.

Moon, T. W., Mommsen, T. P. 2005. Environmental Toxicology, Volume 6. Elsevier B.V., p. 43 - 84

Okutsu, T., Yano, A., Nagasawa, K., Shikina, S., Kobayashi, T., Takeuchi, Y., Yoshizaki, G. 2006. Manipulation of fish germ cell: visualization, cryopreservation and transplantation. Journal of Reproduction and Development, 52(6): 685 - 93.

Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y., Yoshizaki, G. 2007. Production of trout offspring from triploid salmon parents. Science, 317(5844): 1517.

Ostrander, G. K. 2000. The Laboratory Fish. Academic Press. p. 631 - 633.

Parameswaran, V., Shukla, R., Bhonde, R., Hameed, A. S. 2007. Development of a pluripotent ES - like cell line from Asian sea bass (*Lates calcarifer*) - an oviparous stem cell line mimicking viviparous es cells. Marine Biotechnology, 9: 766 - 775.

Peters, K., 2009. Chapter 11: Virology. In: Heil, N. (editor), National Wild Fish Health Survey - Laboratory procedures manual, 5th ed, U.S. Fish & Wildlife Service, Warm Springs, GA.

Piedrahita, J. A., Moore, K., Oetama, B., Lee, C. K., Scales, N., Ramsoondar, J., Bazer, F.W., Ott, T. 1998. Generation of transgenic porcine chimeras using primordial germ cell - derived colonies. Biology of Reproduction, 58(5): 1321 - 9.

Prater, M., Shehata, M., Watson, C. J., Stingl, J. 2013. Enzymatic dissociation, flow cytometric analysis, and culture of normal mouse mammary tissue. Methods in Molecular Biology, 946: 395 - 409.

Routray, P., Dash, C., Dash, S. N., Tripathy, S., Verma, D. K., Swain, S. K., Swain, P., Guru, B.C. 2010. Cryopreservation of isolated blastomeres and embryonic stem - like cells of Leopard danio, *Brachydanio frankei*. Aquaculture Research 41(4): 579 - 589

Sakai, N.2006. In vitro male germ cell cultures of zebrafish. Methods, 39: 239 - 45.

Sansom, B., 2010. Toxicity assessment of oil sands process - affected water using fish cell lines. MS thesis, Waterloo, Ontario, Canada.

Sashikumar, A., Desai, P.V. 2008. Primary cell cultures from *Scylla serrate*. Cytotechnology. 56: 161 - 169.

Segner, H.1998. Fish cell lines as a tool in aquatic toxicology. EXS. 86: 1 - 38.

Sen, A., Kallos, M. S., Behie, L. A. 2004. New tissue dissociation protocol for scaled - up production of neural stem cells in suspension bioreactors. Tissue Engineering. 10: 904 - 913.

Shikina, S., Ihara, S., Yoshizaki, G. 2008. Culture conditions for maintaining the survival and mitotic activity of rainbow trout transplantable type A spermatogonia. Molecular Reproduction and Development, 75(3): 529 - 37.

Shikina, S., Yoshizaki, G. 2010. Improved in - vitro culture conditions to enhance the survival, mitotic activity and transplatability of rainbow trout type A spermatogonia. Biology of Reproduction, 83: 268 - 276

Sommerset, I., Krossøy, B., Biering, E., Frost, P. 2005. Vaccines for fish in aquaculture. Expert Review of Vaccines, 4: 89 - 101.

Takeuchi, Y., Yoshizaki, G., Takeuchi, T. 2003. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biology of Reproduction, 69(4): 1142 - 1149.

Thomson, J. A., Itskovitz - Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., Jones, J. M. 1998. Embryonic Stem Cell Lines Derived from Human Blastocysts. Science, 282: 1145 - 1147.

Uribe, C., Folch, H., Enriquez, R., Moran, G. 2011. Innate and adaptive immunity in teleost fish: a review. Veterinarni Medicina, 56: 486 - 503

van de Lavoir, M. C., Diamond, J. H., Leighton, P. A, Mather - Love, C., Heyer, B. S., Bradshaw, R., Kerchner, A., Hooi, L. T., Gessaro, T. M., Swanberg, S. E., Delany, M. E., Etches, R. J. 2006. Germline transmission of genetically modified primordial germ cells. Nature, 441(7094): 766 - 9.

Wakamatsu, Y., Ozato, K., Sasado, T. 1994. Establishment of a pluripotent cell line derived from a medaka (*Oryzias latipes*) blastula embryo. Molecular Marine Biology and 3(4): 185 - 91.

Wolf, K., Ahne, W.1982. Fish cell culture. In: Advances in Cell Culture: Volume 2, Maramorosch, K (editor). Academic Press, p. 305 - 328.

Wolf, K., Mann, J. A. 1980. Poikilotherm vertebrate cell - lines and viruses a current listing for fishes. In Vitro, 16: 168 - 179.

Wolf, K., Quimby, M. C. 1962. Established Eurythermic Line of Fish Cells in vitro. Science 135: 1065 - 1066

Wongtavatchai, J., Conrad, P.A., Hedrick, R.P. 1994. In vitro cultivation of the microsporidian: Enterocytozoon salmonis using a newly developed medium for salmonid lymphocytes. Journal of Tissue Culture Methods, 16:125 - 131

Wong, T. T., Tesfamichael, A., Collodi, P. 2013. Production of zebrafish offspring from cultured female germline stem cells. PLoS One, 8(5): e62660.

Xu, H.Y., Li, M.Y., Gui, J.F., Hong, Y.H., 2010. Fish germ cells. Science China, Life Sciences, 53: 435 - 446.

Yoshizaki, G., Tago, Y., Takeuchi, Y., Sawatari, E., Kobayashi, T., Takeuchi, T. 2005. Green fluorescent protein labeling of primordial germ cells using a nontransgenic method and its application for germ cell transplantation in salmonidae. Biology of Reproduction, 73: 88 - 93.

Yoshizaki, G., Fujinuma, K., Iwasaki, Y., Okutsu, T., Shikina, S., Yazawa, R., Takeuchi, Y. 2011. Spermatogonial transplantation in fish: A novel method for the preservation of genetic resources. Comp Biochem Physiol Part D Genomics Proteomics - Part D Genomics Proteomics, 6(1): 55 - 61.

Zhu, L.Y., Nie, L., Zhu, G., Xiang, L.X., Shao, J.Z. 2013. Advances in research of fish immune - relevant genes: a comparative overview of innate and adaptive immunity in teleosts. Developmental & Comparative Immunology, 39: 39 - 62.

CHAPTER FIVE

Blue Catfish (*Ictalurus furcatus*) and Channel Catfish (*Ictalurus punctatus*) Germ Cell Genetic Transformation and Transplantation

Abstract

This is the first report of blue catfish (*Ictalurus furcatus*) and channel catfish (*Ictalurus punctatus*) germ cell genetic transformation. Cells were transformed with construct FRMwg, which was constructed by fusing the carp β - actin promoter, wg - GFP coding sequence and first intron to the ocean pout antifreeze protein terminator and putative boundary element, using electroporation. Both blue catfish ovarian germ cells and channel catfish testicular germ cells expressed green florescence protein (GFP) for a 26 days Blue florescence protein (BFP) expression construct - FRM2bl was also used to transform catfish germ cells, but no cells exhibited BFP expression. Two transplantation techniques, surgery and catheterization, were studied. Both blue catfish SSCs enriched by Percoll density gradient centrifugation and dissociated testicular germ cells were used as donor cells to transplant into recipient fish, triploid channel catfish. Ten months after the transplantation, eight male triploid channel catfish were evaluated. Three of the recipient fish were transplanted by surgery and blue catfish cells were detected in the testes of all three fish. Five of the recipient fish were transplanted by catheterization and blue catfish cell were detected in the testes of four fish. Both surgery and catheterization were efficient transplantation methods.

Keywords: transformation, transplantation, testicular germ cell culture, ovarian germ cell culture

Introduction

Transgenic Fish

Transgenesis has been utilized as a tool in genetic and genome research, and has been successfully carried out in animals, plants, fungi and microbes. In most cases the aim of gene transfer to aquatic species is to introduce a new trait to the organism to increase economic benefits in aquaculture food production, which includes improvement of growth rate, feed conversion, disease resistance, enhanced nutritional and processing qualities, coloration, recreational traits and other desirable traits (Dunham, 2003; Van Eenennaam, 2006).

Producing transgenic fish is relatively easy compared with mammals. Most fish species can produce a large number of embryos each ovulation, have a lower possibility of carrying human pathogens and have large eggs that are readily pierced by a suitable glass needle during the microinjection (Liu, et al. 1990; Houdebine, et al. 1991; Maclean, 1998; Van Eenennaam, 2006). Transgenic fish have been produced in at least 14 species of fish, including zebrafish, medaka, salmon, tilapia, trout, catfish, carp, goldfish and loach (Dunham, 2003; Devlin et al. 2006). Growth (growth hormone) (Du et al. 1992; Rahman et al. 2001), freeze tolerance (antifreeze protein) (Shears et al. 1991), disease resistance (cecropin and lactorferrin) (Dunham et al. 2002; Zhong et al. 2002; Sarmasik et al. 2002), carbohydrate metabolism (glucose transporter and hexokinase) (Pitkanen et al. 1999), lipid metabolism (D6 - desaturase) (Alimuddin et al. 2005), phosphorus metabolism (phytase) (Hostetler et al. 2005), and vitamin C metabolism (L - gulono - gamma - lactone oxidase) (Toyohara et al. 1996; Krasnov, et al. 1998) are among the target traits that have been modified in fish.

Microinjection of the germinal vesicle or one - cell embryo, electroporation of early embryos or sperm, retroviral infection and particle gun bombardment are some of the genetic engineering techniques used in fish transgenics (Gibbs et al. 2000). Microinjection is the traditional method of transgenic fish production, but it is a complicated operation, requires high skill, and is tedious and slow. Electroporation is

more efficient as a large number of cells can receive the DNA construct instantly (Miller and Nickoloff, 1995). However, transport of material into and out of the cell during electropermeability is relatively nonspecific, which can cause ion imbalance and even cell death (Weaver, 1995). Transposon - based transgenesis has been widely used in fish cell transformation (Suster et al. 2011). Theoretically, homologous recombination is the key to successful gene transfer allowing insertion of foreign DNA.

Transgene Expression

Sometimes, random integration can cause unpredictable transgene expression pattern, and obviously choice of promoters can strongly influence the expression of transgenes. In some cases, heterologous transcription units derived from non - piscine species may not be optimally recognized by fish transcriptional regulatory factors, and expression level would be reduced (Gibbs et al. 2000). Highly methylated CG dinucleotide content can cause transcription repression and even the silencing of transgenic expression (Boyes et al. 1991). Components without introns show a higher expression level in fish cell culture (Liu et al. 1990; Betancourt et al. 1993). Boundary elements may enable position - independent expression (Eissenberg et al. 1991).

Fluorescent Marker Genes

Fish modified with fluorescent protein marker genes, which can make certain living organs or cells visible by fluorescence, have been widely used to study gene expression, gene interaction, protein function, and embryonic development. Green fluorescent protein (GFP) which was originally derived from the jellyfish (*Aquorea Victoria*, Shimomura et al. 1962) does not require any chemical substrate for visualization (Okabe et al. 1997). The GFP gene is often used in fish germ cell transformation studies (Xu et al. 2010). Red fluorescent proteins derived from a protein isolated from *Discosoma* sp. is also commonly used in transgenic fish studies (Xu et al. 2010).

By using transgene constructs using the germline - specific promoters, *vasa* and *nanos*: *vas - gfp, vas - rfp* and *nos - gfp,* which combined with GFP or RFP gene, florescent transgenic PGCs were raised in embryos of medaka (*Oryzias latipes*), zebrafish

(*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) (Xu et al. 2010). In these transgenic embryos, PGCs were visible and easy to detect. This allows for the easy isolation and manipulation of these cells (Kobayashi et al. 2004). Reporter mRNA, which is a fusion of GFP or RFP coding region and the 3' - untranslated regions (UTR) of the *vasa* or *nanos* gene, was injected into early developing embryos (Koprunner et al. 2001; Ciruna et al. 2004) and PGCs also can express florescence proteins. In this case, cis - acting elements within the 3' - UTR mediated interaction between trans - acting protein factors and RNAs determined the localization of reporter mRNA. Synthetic RNAs have been microinjected into early embryos. Reporter RNAs allowed PGCs labeling to examine PGC migration, proliferation and survival studies during fish embryonic development (Hashimoto et al. 2004; Herpin et al. 2007).

Fish Germ Cell Transplantation

Germ cell transplantation is a basic germline engineering technique and also an important tool in understanding germ cell development. It has been successfully applied in a number of animal species including mammals, birds and fish Honaramooz et al. 2002; Kang et al. 2008; Xu et al. 2010). Germline stem cells have several important functional aspects, which include capacity of giving rise to donor gametes in the gonad of recipient species (syngeneic and xenogeneic transplantation), sex reversal plasticity of the spermatogonia and oogonia, and the possibility of genetically manipulating GSCs before transplantation to produce transgenic fish (Lacerda et al. 2012).

PGCs have been successfully transplanted into blastula - stage and newly hatched fish embryos. The lack of immune system function in recipient embryos, allows donor PGCs to completely replace the host germ line. PGC transplantation was reported first in the rainbow trout (*Oncorhynchus mykiss*) by transplanting GFP - labeled PGCs into newly hatched fish embryos of the wild type of rainbow trout (Takeuchi, 2003). GFP - labeled PGCs were sorted by flow cytometry from transgenic rainbow trout donor fish, which had green fluorescent protein (GFP) expression in their PGCs. After transplantation, donor PGCs were able to migrate to the gonadal ridge in the recipient, proliferate and develop into gametes which could be used to produce offspring. Intraspecific and interspecific transplantation of single PGCs from pearl danio (*Danio*

albolineatus), goldfish (*Carassius auratus*) and loach (*Misgurnus anguillicaudatus*) into sterilized blastula - stage zebrafish (*Danio rerio*) embryos was studied by Saito et al. (2008). These PGCs formed donor gonads in the recipient fish.

Testicular germ cells containing SSCs from transgenic rainbow trout have been successfully transplanted into newly hatched embryos (Okutsu et al. 2006; Okutsu et al. 2007). The testicular germ cells could differentiate into gametes in the gonads of recipients. Furthermore, the donor - derived spermatozoa and eggs obtained from the recipient fish were able to produce normal offspring. SSCs have also been transplanted into adult fish gonads. After suppression with busulfan (1, 4 - butanediol dimethanesulfonate) and high temperature (35 ℃), type A spermatogonial cells from the Nile tilapia (*Oreochromis niloticus*) testes enriched by Percoll density gradient centrifuge and labeled with the fluorescent lipophilic dye PKH26 - GL were injected into the adult recipient's testes through the common spermatic duct (Lacerda et al. 2006). Further study demonstrated that donor type A spermatogonia completed spermatogenesis in a tilapia recipient after 8 - 9 weeks post - transplantation and donor spermatozoa were produced in recipient testes (Lacerda et al. 2010).

The plasticity of oogonia was also studied by transplanting donor female germ cells into the newly hatched embryos. Oogonia differentiated into functional eggs in the female recipient and sperm in male recipients, which means oogonia also have germline stem cell function (Yoshizaki et al. 2010).

The hybrid resulting from the mating of channel catfish female × blue catfish male (C × B hybrid) is the best catfish for pond culture (Dunham, 2011). Channel catfish female and blue catfish male cannot mate naturally due to interspecific reproductive barriers. The current technology, artificial fertilization and handstripping is economical and commercially feasible; but is labor intensive, requires 3 - 4 year - old, sexually mature channel catfish females and sacrifice of the blue catfish males, which do not reach full maturity and usefulness until 5 - 6 years of age. If we can artificially produce xenogenic channel catfish male which can produce blue catfish sperm, those male can mate with a normal channel catfish female naturally and the progeny would be all C × B hybrid catfish. The efficiency of hybrid catfish producing can be improved tremendously.

Furthermore, combining the germ stem cell xenotransplantion and germ stem cells gene modification can increase the efficiency of produce transgenic catfish gametes which can be used in catfish genetic enhancement programs. Most of the transgenic fish were started with microinjection or electroporation genes into fish embryo, but this first transgenic generation is mosaic, and not every cell contains the foreign gene (Gibbs et al. 2000). The 100% transgenic fish only can be achieved in F1 generation after fertilizing the transgenic gametes. But if we start with transgenic stem cells gene and use them to produce xenogenic fish. Those xenogenic fish will produce transgenic gametes; upon mating the transgenic fish or by combining the gametes in vitro, we can obtain 100% transgenic fish.

The objective of this research is to carry out interspecific transplantation of blue catfish germ stem cells into sterilized triploid channel catfish to produce xenogenic channel catfish males which can produce blue catfish sperm. The resultant males will be able to naturally mate with a normal channel catfish females. The fish produced by this process could lead to increased efficiency of reproduction for catfish hybridization. Another objective is to produce genetically transformed catfish germ cells by electroporation to accomplish interspecific catfish germ stem cell transplantation. Combining these two techniques could result in xenogenic channel catfish which can produce transgenic catfish gametes. One hundred percent transgenic catfish can be achieved by combining the transgenic gametes.

.

Materials and Methods

Construct Preparation, Extraction and Linearization

The GFP expression plasmid FRMwg (GenBank: AF170915.1) and the blue florescence protein (BFP) expression plasmid FRM2bl (Gibbs and Schmale, 2000) were used in this experiment. FRMwg was constructed by fusing the carp β - actin promoter, the wg - GFP coding sequence and first intron to the ocean pout antifreeze protein terminator and putative boundary element.

Both FRMwg and FRM2bl plasmids were amplified by One Shot® Top10 chemically competent *Escherichia coli* cells. After transformation with the plasmids following the procedures recommended by the protocol, *E.coli* cells were cultured on the solid Lysogeny Broth (LB) selection medium (10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, 100 μg ampicillin, 15.0 g agar in 1.0 L water). A single colony was picked up from the plate and plasmid DNAs were extracted by use of a Qiagen mini - prep kit. Plasmids were linearized with Sfi I (20,000 unit/mL; BioLabs). For each plasmid digestion, a 50 μL reaction system, which contains1 μg of plasmid DNA, 8 units of Sfi I, NEBuffer 4 (10 × stocks), BSA (100 × stocks) and water was placed in 50 ℃ water bath for 16 hr. Both unlinearized and linearized plasmid DNAs' size were confirmed by DNA agarose gel electrophoresis.

Once the size of plasmid was confirmed, further amplification was accomplished by culturing the *E.coli* colony in liquid LB medium at 37 ℃ overnight (10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl and 100 μg Ampicillin. in 1.0 L water). Plasmid DNA extraction was extracted with a Qiagen maxi - prep kit, stored in -80℃ ultrafreezer and linearized before electroporation. Plasmid DNA was linearized following the same digestion protocol, but the volume of the reaction system was expanded to 500 μL per reaction. After the linearization, phenol - chloroform - ethanol method was used to inactivate Sfi I enzyme and purify the DNA. The DNA quantity was measured using a ND - 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware).

Both FRMwg and FRM2bl plasmids were prepared using the same procedure. The pure linearized plasmids were diluted in TE buffer (5 mM Tris - HCl, 0.5 M EDTA, pH = 8.0) at the concentration of 10 μg/μL and filter sterilized with 0.22 μm filter (Millipore) before use.

Germ Cell Transformation and Electroporation Procedures

Both testicular germ cells and ovarian germ cells used in the electroporation study were isolated from two - year - old juvenile blue catfish and channel catfish by trypsin digestion. Every 3 fish from the same gender and species were considered as three replications. For each electroporation procedure, 0.75 mL of 6×10^6 /mL germ cell in PBS and 50 μL 10 μg/μL construct in TE buffer were used. Before electroporation, cell suspension and construct solution were first added to the electroporation 60 ×15mm Petri dish (Falcon), and mixed well. Electroporation was performed with the Baekon 2000 macromolecule transfer system with the parameters: 300 V, 2^7 pulses, 0.05 sec burst, 4 cycles, and 160 μsec. 1 min after the electroporation, cells were transferred into 6 - well plates with culture medium and cultured at 27 ℃ in air. Partial media change was performed every two days. Cells were observed everyday using an inverted Nikon Eclipse TS100 microscope and imaged using a Nikon D40X DSLR camera. GFP expression was detected by using a FITC fluorescent filter and BFP expression was detected by using a DAPI fluorescent filter.

Triploid Induction

Channel catfish females were induced to ovulate using the general procedures of et al. (1999), Dunham et al. (2000), Hutson (2006), and Kristanto et al. (2009). Luteinizing hormone releasing hormone analog, LHRHa (85% active ingredient) was administered intramuscularly as a single 90μg/kg body weight dose with EVAC implants (Center of Marine Biotechnology, University of Maryland, Baltimore, MD).

When females began to ovulate, thirty - gram aliquots of eggs were hand - stripped, placed in small pie pans and dry fertilized. Water was added to the pie pan to activate sperm and eggs for fertilization. At three minutes post fertilization, the eggs were placed in an egg basket, and the basket placed in a hydrostatic pressure chamber

(Lilyestrom et al. 1999). At five minutes post fertilization, the fertilized eggs were pressure shocked using a hydrostatic pressure of 6000psi (408.27 atm) for 5 minutes. After pressure shocking, the eggs were removed from the chamber and placed in a hatching trough fitted with a paddle wheel and flow through.

Hatched fry were grown to 8 ~ 10cm fingerlings within indoor tanks, then were stocked in a 0.04ha pond and allowed to grow for three years. In April 2012, the fish were harvested, and transported to American Sport Fish, Montgomery, Alabama for ploidy analysis. Each fish was analyzed for ploidy individually using blood from a tail prick. The blood was placed in a cuvette containing 5mL of Isoton diluent and 2 drops of Zap - Oglobin II (a digestive enzyme) (Beckman Coulter). Each sample was analyzed for erythrocyte nuclei volume with a Coulter - counter (Beckman Coulter), as described by Beck and Biggers (1983). Triploid and diploid individuals were separated, and transported to Auburn University. The triploid individuals were given unique individualized brands and placed in an earthen pond.

Testicular Germ Cell Transplantation

Two types of testicular germ cells from two - year - old male juvenile blue catfish were used as donor cells in transplantation study. They were SSCs enriched by Percoll density gradient centrifuge and dissociated testicular germ cells isolated after trypsin digestion.

Recipient fish were adult male triploid channel catfish. For surgical transplantation, two male recipient fish were transplanted with dissociated testicular germ cells and one male recipient fish was transplanted with Percoll density gradient centrifugation enriched SSCs. For transplantation by catheterization, all recipient fish were transplanted with SSCs only.

For the surgical procedure, recipient fish were anesthetized with tricaine methanesulfonate (MS - 222, Finquel). A 5 ~ 7cm incision was made from the center of the stomach to the pelvic bone. The Gastrointestinal tract was carefully moved outside, and the deformed gonads were revealed at the top of the truck kidney and swim bladder and attached to the mesorchia. One mL of testicular germ cells (approximate 1.2×10^6

cells) or SSCs solution (approximate 9×10^4 cells) was delivered with a 1mL syringe fitted with a 30 gauge needle. For a pair of gonads, each side was injected with 0.5mL cell solution. The Gastrointestinal tract was returned to its original position in the body cavity and the incision closed with biodegradable sutures. Fish were branded and placed in a tank to recover for 10 days, and then stocked into an earthen pond.

For the catheterization procedure, an 8 cm polyethylene tube (0.8 mm interior diameter, 1.2 mm outside diameter) was carefully inserted into genital tract though the genital opening in females and the urogenital opening in males until at least half of the tube was inserted into the fish. The uninserted portion was shortened to 1 cm. One mL of donor cell isolate was injected into the gonads of the fish through the polyethylene tube (Fig. 18). After the injection, the tubing was removed directly. Fish were also branded and placed in the tank for 10 days, and then moved into an earthen pond.

Ten months after the transplantation, eight male recipient fish were recaptured from the pond and transferred to indoor tanks. The brands had faded, however, the surgical scars indicated that three of them were transplanted by surgery and five were transplanted by catheterization. One fourth of the testes tissues were biopsied from all recaptured recipient fish. Testes samples were placed into a 1.5 mL microfuge tube on ice during sampling, and then stored in a -80 ℃ ultrafreezer before DNA extraction.

DNA Extraction from Gonadal Tissue

DNA was extracted using the protocol of Waldbieser et al. (2008). Briefly, cell lysis solution which contained 600 µL of DNA extraction cocktail buffer (100 mM NaCl, 10 mM Tris - HCl, pH 8, 25 mM/L EDTA, 0.5% /L sodium dodecyl sulfate) and 3 µL 20 mg/mL freshly made proteinase K (Sigma) were used for gonad sample digestion. The gonad samples were incubated and digested in the cell lysis solution overnight at 55 ℃. Extracted proteins were precipitated with a protein precipitation solution (Qiagen) and DNA was precipitated by isopropanol. DNA was washed twice with cold 70% ethanol, air - dried, resuspended in RNase / DNase free water, and quantified with an ND - 1000 spectrophotometer (NanoDrop Technologies). DNA from control samples of diploid

channel catfish, blue catfish and C × B hybrid catfish were extracted by the same procedure.

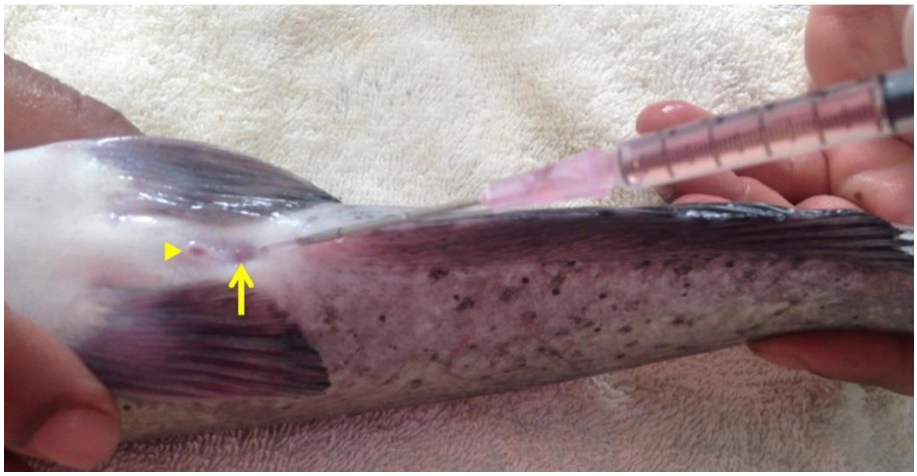

Fig. 18 Transplantation by catheterization. Polyethylene tube (0.8 mm i.d., 1.2 mm o.d.) was inserted into genital tract though the genital opening in females of triploid channel catfish (*Ictalurus punctatus*) and the urogenital opening in males of triploid channel catfish until at least half of the tube was inserted into the fish. One mL testicular germ cells (approximate 1.2×10^6 cells) or SSCs solution (approximate 9×10^4 cells) isolated from blue catfish (*I. furcatus*) testes were injected into the gonads of the recipient triploid channel catfish through the polyethylene tube. Arrow is pointed toward the urogenital opening, and arrowhead is pointed toward the anal opening.

PCR Detection

PCR reaction was used to differentiate channel catfish, blue catfish, hybrid catfish and xenogenic catfish following the protocol from Waldbieser and Bosworth (2008). Marker genes were follistatin, *Fst* and hepcidin, *Hamp* antimicrobial protein of channel catfish and blue catfish. Primers are listed in Table 9. PCR was carried out in a 10.0 μL reaction containing 20 - 250 ng genomic DNA in TE buffer (1.0 μL 10 mM Tris - HCl, pH 9.0), 0.4 μL 50 mM $MgCl_2$, 0.8 μL 2.5 mM of each dNTP, 0.6 μL 10 pmM each *Fst* primer, 0.3 μL 10 pmM each *Hamp* primer, 0.1 μL 5 U/μL platinum Taq polymerase and

3.9 μL water. The PCR procedures were performed using the following thermo profile: after an initial denaturation at 95 ℃ for 3 min; the first PCR amplification were carried out at 95 ℃ for 1min, 65 ℃ for 1 min, and 70 ℃ for 1 min for 35 cycles; followed by a second PCR amplification at 95 ℃ for 30 s, 63 ℃ for 30 s, and 72 ℃ for 1 min for 35 cycles; and the final extension at 72 °C for 10 min. The *Fst* and *Hamp* amplification products were analyzed on an ethidium - bromide - stained 2.5% agarose gel. Tracklt 100 bp DNA Ladder (Invitrogen) was used to check the band size. All the reactions for each sample were repeated three times.

Table 9 Primers for channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*) and hybrid catfish differentiation using follistatin, *Fst* and hepcidin antimicrobial protein, *Hamp* genes.

Gene	Forward Primer	Reverse Primer	Size of Amplication Product (bp) Channel Catfish	Blue Catfish
Fst	ATAGATGTAGAGGAGCATTTGAG	GTAACACTGCTGTACGGTTGAG	348	399
Hamp	ATACACCGAGGTGGAAAAGG	AAACAGAAATGGAGGCTGGAC	222	262

Results

Blue Catfish Germ Cell Transformation

Seventy - two hours after the electroporation, culture medium was totally changed very gently, and cells were observed under a fluorescent microscope. A few ovarian germ cells were weakly attaching to the culture plate (Fig. 19 A). Green fluorescence was observed in ovarian germ cells (Fig. 19 B). No testicular germ cells attached to the plate during the culture, and no green fluorescence was observed. Neither testicular germ cells nor ovarian germ cells had blue fluorescent protein expression.

After 26 days, some cultured ovarian germ cells still showed strong green fluorescent protein expression (Fig. 19 C and D).

Channel Catfish Germ Cell Transformation

Seventy - two hours after the electroporation, a few ovarian germ cells were weakly attaching to the culture plate, but no fluorescent protein expression was observed in testicular germ cells or ovarian germ cells. A few testicular germ cells attached to the plate at day 8, and some showed green fluorescent protein expression. During culture, no cell division was observed, and all attached cells contained two nuclei in one cell (Fig. 20).

Green fluorescent protein expression still could be observed in some testicular germ cells at day 26 (Fig. 21).

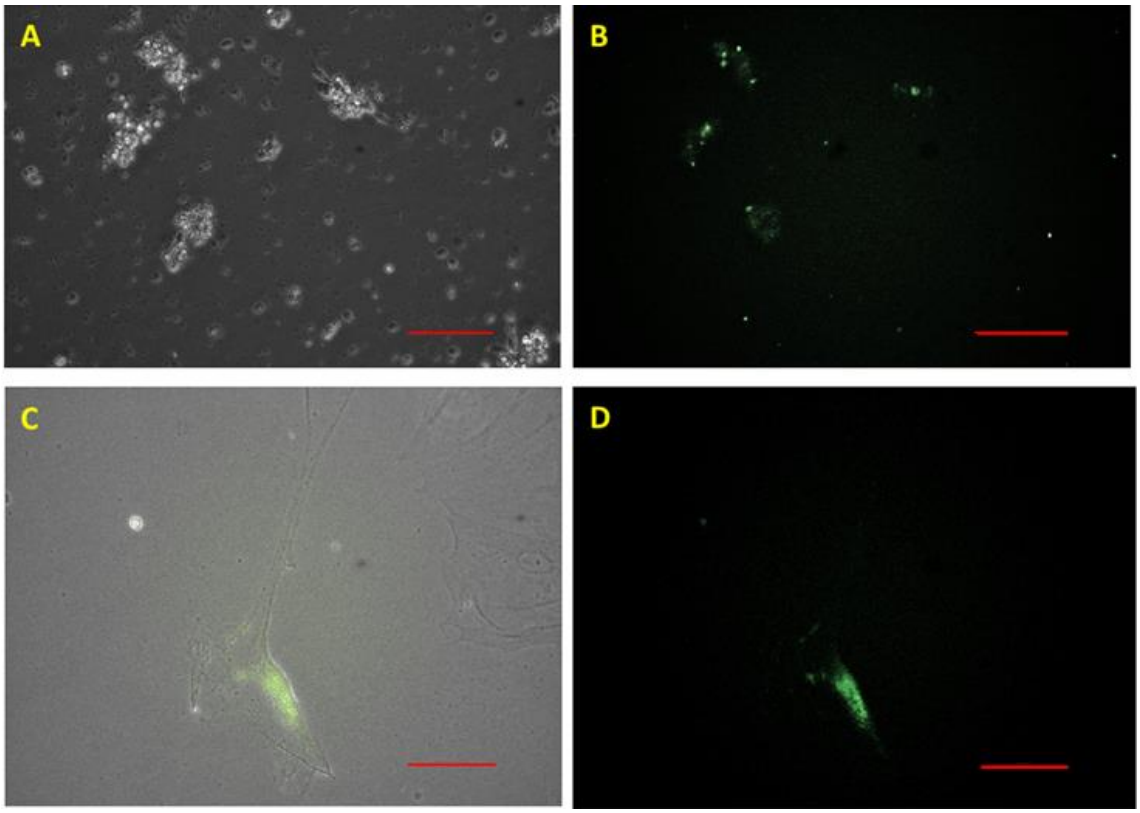

Fig. 19 Green fluorescent protein expression in blue catfish (*Ictalurus furcatus*) ovarian germ cells which were transformed with GFP expression construct FRMwg (GenBank: AF170915.1) by electroporation. A) Three - day (72 hr) after the electroporation, few ovarian germ cells were weakly attaching to the culture plate. B) When observed by using a FITC fluorescent filter, cells showed green fluorescence. C) The fluorescent cell could be recognized by overlapping cultured ovarian germ cells picture with green fluorescence picture. After 26 - day culture, some ovarian germ cells still show strong green fluorescent protein expression. D) The green fluorescent protein expression was observed in the ovarian germ cell by using a FITC fluorescent filter, 26 days after the electroporation. Bars A, B, C and D = 100 μm.

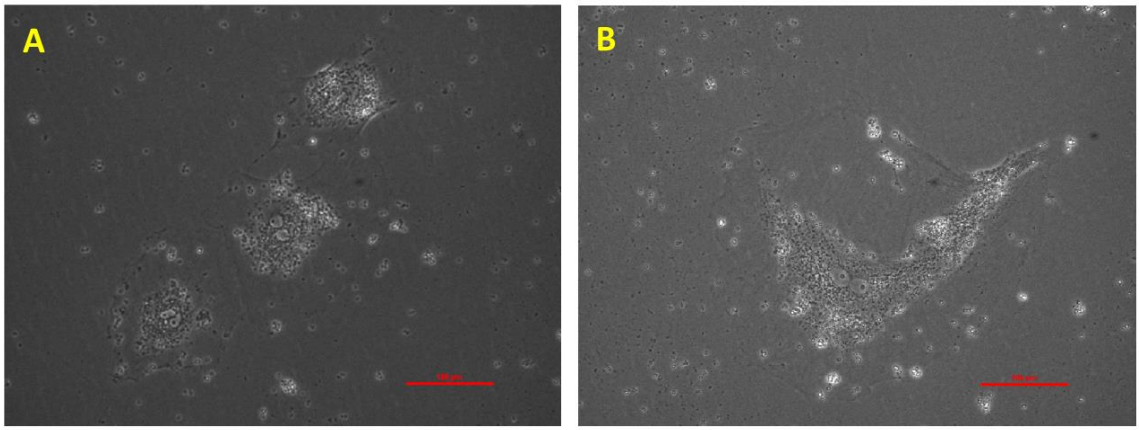

Fig. 20 Channel catfish (*Ictalurus punctatus*) testicular germ cells were transformed with GFP expression construct FRMwg (GenBank: AF170915.1) by electroporation. During culture, cells attached to the plate. However, each cell contained two nuclei. Bars A and B = 100 μm.

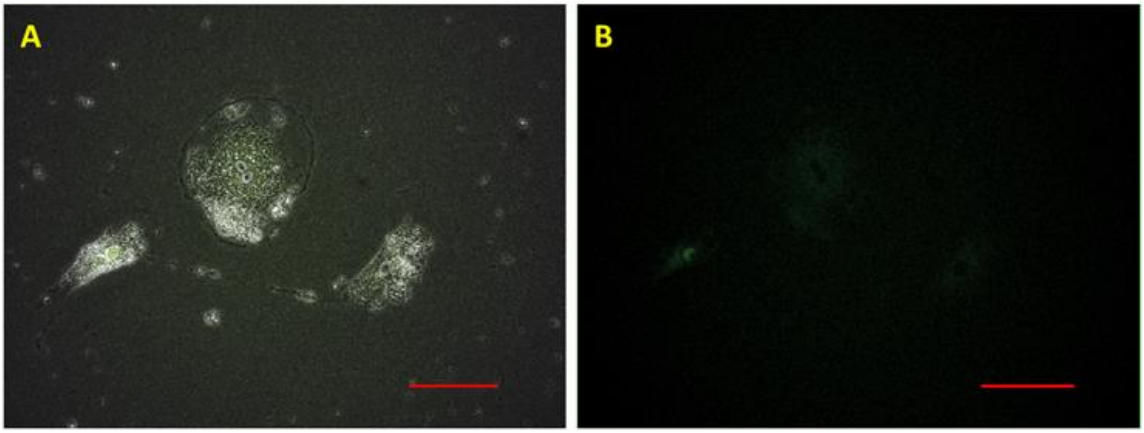

Fig. 21 After 26 - day culture, green fluorescent protein expression still could be observed in channel catfish (*Ictalurus punctatus*) testicular germ cells which were transformed with GFP expression construct FRMwg (GenBank: AF170915.1) by electroporation. A) The fluorescent cell could be recognized by overlapping cultured testicular germ cells picture with green fluorescence picture. B) The green fluorescent protein expression was observed in the testicular germ cell by using a FITC fluorescent filter, 26 days after the electroporation. Bars A and B = 100 μm.

Testicular Germ Cell Transplantation

Ten months after the transplantation, eight male triploid channel catfish were recaptured. The brand was unrecognizable on those fish, but the surgery transplanted fish could be separated from the catheterization transplanted fish because of the surgical scar. Three had been transplanted by surgery and five transplanted by catheterization. Testicular traits characters of all the eight recipient fish were record and showed in Table 10.

PCR results for detection of blue catfish cells in the testes of triploid channel catfish are shown in Fig. 22. For surgical transplantation, three recipient fish, A3, A26 and A29, all possessed blue catfish cells in the testes. For transplantation by catheterization, four recipient fish - A1, A2, A5 and A25 possessed blue catfish cells in the testes, but A30 did not.

Table 10 Testicular traits of the eight recipient triploid channel catfish (*Ictalurus punctatus*) 10 months after they were transplanted with blue catfish (*I. furcatus*) SSCs enriched by Percoll density gradient centrifugation or dissociated testicular germ cells isolated after trypsin digestion. Presence of blue catfish cells was determined with PCR, and primers were designed based on follistatin, *Fst* and hepcidin antimicrobial protein, *Hamp* genes.

Transplantation Method	Recipient fish	Testes characteristics	Blue catfish (*I. furcatus*) cells present
Surgery	A3	testis only existed on the left side and was very small	Yes
	A26	paired testes, right side testis was larger than the left side	Yes
	A29	paired testes were well developed and milky color	Yes
Catheterization	A1	paired testes were very small	Yes
	A2	paired testes were very small	Yes
	A5	paired testes, right side testis was larger than the left side	Yes
	A25	paired testes were very small	Yes
	A30	paired testes were well developed and milky color	No

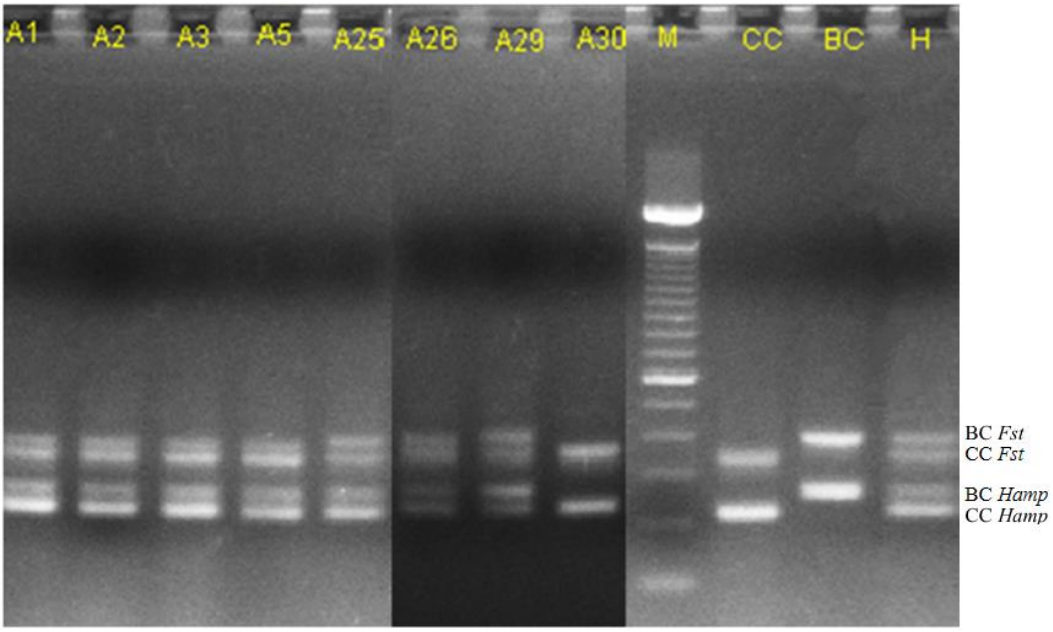

Fig. 22 PCR results for detecting blue catfish (*Ictalurus furcatus*) cells in the testes of triploid channel catfish (*Ictalurus punctatus*). Blue catfish and channel catfish cells were differentiated with PCR using follistatin, *Fst* and hepcidin antimicrobial protein, *Hamp* genes as markers. CC was channel catfish control; BC was blue catfish control; H was channel catfish female × blue catfish male hybrid control. Three recipient fish - A3, A26 and A29 were transplanted by the surgical method and blue catfish cells were detected in the testes of all three fish. Five recipient fish - A1, A2, A5, A25 and A30 were transplanted by the catheterization method and blue catfish cells were detected in the testes of four fish, A1, A2, A5 and A25; blue catfish cells were not detected in fish A30.

Discussion

Catfish Germ Cells Transformation

The FRMwg construct has been previously used in zebrafish transgenic research (Gibbs et al. 2000). Approximately 90% of zebrafish embryos microinjected with FRMwg construct, had one or more fluorescent cells screenable by epifluorescence microscopy at day 5 (120 hours) of development. From 24 to 120 hours of development the intensity of fluorescent marking appeared stable, with a general increase in the number of screenable cells and cell types. In our case, the GFP expression from construct FRMwg was obtained in blue catfish ovarian germ cells and channel catfish testicular germ cells. The construct FRMwg can be used in catfish cell catfish germ cell labeling. The labeled germ cell could be used for further transplantation studies. The migration, proliferation and survival of the donor germ cell could be easily identified due to the visible labeling. Since cultured transgenic cells were produced, the possibility exists to produce transgenic catfish by transplanting transgenic germ cells.

The germ cells used in this experiment were freshly isolated germ cells. Compared to cultured cell lines, the freshly isolated germ cells *in vitro* growth was unpredictable. Culture may have been made even more difficult because of potential damage during isolation and electroporation.

FRM2bl construct encoding blue florescence protein (BFP) did not express in germ cells of channel catfish nor blue catfish. When both FRMwg and FRM2bl constructs were compared in zebrafish, the null class of non - expressing embryos was 26.3% higher for FRM2bl compared to FRMwg (Gibbs et al. 2000). The FRM2bl construct did not express as strongly as FRMwg. In the current study, transformation did not occur for FRM2bl or it did not express. The results cannot be fully interpreted without verifying that the cells integrated these constructs.

Some testicular germ cells from channel catfish attached to the culture plate, but no cell division was observed, and all attached cells contained two nuclei. Even though,

these cells could not proliferate normally, it was a step forward for culturing transgenic catfish cells. Different culture media and growth factors should be evaluated to improve normal proliferation of these cultured cells.

Furthermore, since interspecific transplantation of germ stem cells between different species of catfish is a potentially ground breaking tool that can artificially reduce the generation interval of the catfish, combining germ stem cells transformation with the transplantation can increase the efficiency of produce transgenic fish by one generation, which in catfish is about 3 years. With normal embryo transformation the first transgenic generation is mosaic. The transgene is in every cell once the F1 generation is reached. By transferring transgenic germ stem cells into the host, the xenogenic host will produce transgenic gametes, and subsequently 100% transgenic fish can be directly produced by mixing the transgenic gametes.

Testicular Germ Cell Transplantation

Two transplantation techniques, surgery and catheterization were compared. Both surgery and catheterization were efficient and had a very high transformation rates, 80 ~ 100%. A 30 ~ 70% success rate was achieved when spermatogonia of rainbow trout (*Oncorhynchus mykiss*) were intraperitoneally microinjected into newly hatched embryos of triploid sterile masu salmon (*O.masou*) (Okutsu et al. 2007). However catheterization is much easier than surgery and causes less damage to the recipient fish. Both putative SSCs from density gradient centrifugation and dissociated testicular germ cells can be used in transplantation.

Blue catfish DNA could be detected in the gonads of triploid channel catfish for at least 10 months. This indicated that the SSCs and testicular cells were able to colonize and survive in the interspecific gonads for an extended period of time.

In the case of rainbow trout, 2 years after the transplantation, sterile triploid salmon recipients only produced rainbow trout sperm and eggs (Okutsu et al. 2007). Fertilization with the sperm and eggs was able to successfully produce rainbow trout offspring.

If a xenogenic triploid channel catfish male can produce blue catfish sperm, those male can mate with a normal channel catfish female to produce progeny that would all be C × B hybrid catfish. The interspecific reproductive barriers between channel catfish and blue catfish would be broken. The efficiency of production of hybrid catfish, which are optimal for fish farming, should be able to be greatly improved.

.

Reference

Alimuddin, Yoshizaki, G., Kiron, V., Satoh, S., Takeuchi, T. 2005. Enhancement of EPA and DHA biosynthesis by over - expression of masu salmon D6 - desaturase - like gene in zebrafish. Transgenic Research, 14(2): 159 - 65.

Betancourt, O. H., Attal, J., Theron, M. C., Puissant, C., Houdebine, L. M., Bearzotti, M. 1993. Efficiency of introns from various origins in fish cells. Molecular Marine Biology and Biotechnology, 2: 181 - 188.

Boyes, J., Bird, A. 1991. DNA methylation inhibits transcription indirectly via a methyl - CpG binding protein. Cell, 64: 1123 - 1134.

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., Schier, A.F. 2002. Production of maternal - zygotic mutant zebrafish by germ - line replacement. Proceedings of the National Academy of Sciences, USA, 99: 14919 - 14924.

Devlin, R. H., Sundstrom, L. F., Muir, W. M. 2006. Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends in Biotechnology. 24: 89 - 97.

Du, S. J., Gong, Z. Y., Fletcher, G. L., Shears, M. A., King, M. J., Idler, D. R., Hew, C. L. 1992 Growth enhancement in transgenic Atlantic salmon by use of an “all fish” chimeric growth hormone gene construct. Biotechnology (N. Y.), 10(2): 176 - 181.

Dunham, R. A., Warr, G. W., Nichols, A., Duncan, P. L., Argue, B., Middleton, D., Kucuktas, H. 2002. Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. Marine Biotechnology, 4(3): 338 - 44.

Dunham, R. A. 2003. Status of genetically modified (transgenic fish): research and application. http:// www.fao.org/es/ESN/food/risk_biotech_animal_en.stm

Dunham R. A. 2011. Aquaculture and Fisheries Biotechnology: Genetic Approaches, 2nd Edition 93.

Eissenberg, J. C., Elgin, S. C. R. 1991. Boundary functions in the control of gene expression. Trends in Genetics, 7: 335 - 340.

Gibbs, P. D., Schmale, M. C. 2000. GFP as a genetic marker scorable throughout the life cycle of transgenic zebra fish. Marine Biotechnology, 2: 107 - 125.

Hashimoto, Y., Maegawa, S., Nagai, T., Yamaha, E., Suzuki, H., Yasuda, K., and Inoue, K. 2004. Localized maternal factors are required for zebrafish germ cell formation. Developmental Biology, 268(1): 152 - 161.

Herpin, A., Schindler, D., Kraiss, A., Hornung, U., Winkler, C., Schart, M., 2007. Inhibition of primordial germ cell proliferation by the medaka male determining gene Dmrt1by. BMC Developmental Biology. 7: 99.

Honaramooz, A., Megee, S. O., Dobrinski, I. 2002. Germ cell transplantation in pigs. Biology of Reproduction, 66(1): 21 - 28.

Hostetler, H. A., Collodi, P., Devlin, R. H., Muir W. M. 2005. Improved phytate phosphorus utilization by Japanese medaka transgenic for the Aspergillus niger phytase gene. Zebrafish, 2(1): 19 - 31.

Houdebine, L. M., Chourrout, D. 1991. Transgenesis in fish. Experientia, 47: 891 - 897.

Hutson, A. M. 2006. Evaluation of LHRHa implants and injections on the production of channel catfish (Ictalurus punctatus) female x blue catfish (Ictalurus furcatus) male fry. M.S. Thesis, Auburn University, Auburn, AL.

Kang, S. J., Choi, J. W., Kim, S. Y., Park, K. J., Kim, T. M., Lee, Y. M., Kim, H., Lim, J. M., Han, J. Y. 2008. Reproduction of wild birds via interspecies germ cell transplantation. Biology of Reproduction, 79(5): 931 - 937.

Kobayashi, M., Kato, Y., Yoshiura, Y., Aida, K. 1997. Molecular cloning of the cDNA encoding two types of pituitary gonadotropin a subunit from the goldfish, *Carassius auratus*. General and Comparative Endocrinology, 105: 372 - 378.

Koprunner, M., Thisse, C., Thisse, B., Raz, E., 2001. A zebrafish *nanos* - related gene is essential for the development of primordial germ cells. Genes & Development. 15: 2877 - 2885.

Krasnov, A., Reinisalo, M., Pitkanen, T. I., Nishikimi, M., Molsa, H. 1998. Expression of rat gene for L - gulono - gammalactone oxidase, the key enzyme of L - ascorbic acid biosynthesis, in guinea pig cells and in teleost fish rainbow trout (*Oncorhynchus mykiss*). Biochimica et Biophysica Acta, 1381(2): 241 - 8.

Kristanto, A. H., Umali, G., Beam, D. R., and Dunham, R. A. 2009. Effects of post - anifacturing processing and shipping of luteinizing hormone releasing hormone agonist on induced ovulation for producing of channel catfish female x blue catfish male hybrid fry. North American Journal of Aquaculture, 71: 307 - 311.

Lacerda, S. M., Batlouni, S. R., Silva, S. B. G., Homem, C. S. P., França, L. R. 2006. Germ cells transplantation in fish: the Nile - tilapia model. Animal Reproduction Science, 3: 146 - 159.

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.

Lacerda, S. M., Costa, G. M., Campos - Junior, P. H., Segatelli, T. M., Yazawa, R., Takeuchi, Y., Morita, T., Yoshizaki, G., França, L. R. 2013. Germ cell transplantation as a potential biotechnological approach to fish reproduction. Fish Physiology and Biochemistry, 39: 3 - 11.

Lambert, D. M., Argue, B. J., Liu, Z., and Dunham, R. A. 1999. Effects of seasonal variations, thyroid and steroid hormones and carp pituitary extract on the artificial production of channel catfish (Ictalurus punctatus) x blue catfish (I. furcatus) hybrid embryos. Journal of the World Aquaculture Society, 30: 80 - 89.

Liu, Z. J., Moav, B., Faras, A. J., Guise, K. S., Kapuscinski, A. R., Hackett, P. B., 1990. Functional analysis of elements affecting expression of the beta - actin gene of carp. Molecular and Cellular Biology, 10: 3432 - 3440.

Maclean, N. 1998. Regulation and exploitation of transgenes in fish. Mutation Research, 399: 255 - 266.

Miller, E. M., Nickoloff, J. A. 1995. Escherichia coli electrotransformation. In: Nickoloff J.A., editor. Electroporation protocols for microorganisms. Totowa, New Jersey: Humana Press. p: 105 - 114.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., Nishimune, Y. 1997. 'Green mice' as a source of ubiquitous green cells. FEBS Letters, 407: 313 - 319.

Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., Yoshizaki, G. 2006a. Testicular germ cell can colonize sexually undifferentiated embryonic gonad and produce functional egg in fish. Proceedings of the National Academy of Sciences, USA, 103: 2725 - 2729.

Okutsu, T., Yano, A., Nagasawa, K., Shikina, S., Kobayashi, T., Takeuchi, T., Yoshizaki, G. 2006b. Manipulation of fish germ - cell: visualization, cryopreservation and transplantation. Journal of Reproduction and Development, 52: 685 - 693.

Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y., Yoshizaki, G. 2007. Production of trout offspring from triploid salmon parents. Science, 317: 1517.

Pitkanen, T. I., Krasnov, A., Reinisalo, M., Molsa, H. 1999. Transfer and expression of glucose transporter and hexokinase genes in salmonid fish. Aquaculture, 173 (1 - 4): 319 - 332.

Rahman, M. A., Ronyai, A., Engidaw, B. Z., Jauncey, K., Hwang, G - L., Smith, A., Roderick, E., Penman, D., Varadi, L., Maclean, N. 2001. Growth and nutritional trials on transgenic Nile tilapia containing an exogenous fish growth hormone gene. Journal of Fish Biology, 59(1): 62 - 78.

Saito, T., Goto - Kazeto, R., Arai, K., Yamaha, E., 2008. Xenogenesis in teleost fish through generation of germ - line chimeras by single primordial germ cell transplantation. Biology of Reproduction, 78: 159 - 166.

Sarmasik, A., Warr, G., Chen, T.T. 2002. Production of transgenic medaka with increased resistance to bacterial pathogens. Marine Biotechnology, 4(3): 310 - 322.

Shears, M. A., Fletcher, G. L., Hew, C. L., Gauthier, S., Davies, P. L. 1991. Transfer, expression, and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). Moecular Marine Biology and Biotechnology, 1: 58 - 63.

Shimomura, O., Johnson, F., Saiga, Y. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. Journal of Comparative Physiology, 59 (3): 223 - 39.

Suster, M. L., Abe, G., Schouw, A., Kawakami, K. 2011. Transposon - mediated BAC transgenesis in zebrafish. Nature Protocols, 6(12): 1998 - 2021.

Takeuchi, K., Sereemaspun, A., Inagaki, T., Hakamata, Y., Kaneko, T., Murakami, T., Takahashi, M., Kobayashi, E., Ookawara, S. 2003. Morphologic characterization of green fluorescent protein in embryonic, neonatal, and adult transgenic rats. The Anatomical Record. Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology, 274: 883 - 886.

Toyohara, H., Nakata, T., Touhata, K., Hashimoto, H., Kinoshita, M., Sakaguchi, M., Nishikimi, M., Yagi, K., Wakamatsu, Y., Ozato, K. 1996. Transgenic expression of L - gulono - gammalactone oxidase in medaka (*Oryzias latipes*), a teleost fish that lacks this enzyme necessary for L - ascorbic acid biosynthesis. Biochemical and Biophysical Research Communications, 223(3): 650 - 3.

Uzbekova, S., Chyb, J., Ferriere, F., Bailhache, T., Prunet, P., Alestrom, P., Breton, B. 2000. Transgenic rainbow trout expressed sGnRH - antisense RNA under the control of sGnRH promoter of Atlantic salmon. Journal of Molecular Endocrinology, 25(3): 337 - 50.

Van Eenennaam, A. L., Olin, P. G. 2006. Careful risk assessment needed to evaluate transgenic fish. California Agriculture, 60: 126 - 131.

Waldbieser, G. C., B. G. Bosworth. 2008. Utilization of a rapid DNA - based assay for molecular verification of channel catfish, blue catfish, F1 hybrids, and backcross offspring at several life stages. North American Journal of Aquaculture, 70: 4, 388 - 395.

Weaver, V. M., Howlett, A. R., Langton - Webster, B., Petersen, O. W., Bissell, M. J. 1995. The development of a functionally relevant cell culture model of progressive human breast cancer. Seminars in Cancer Biology, 6: 175 - 184.

Xu, H. Y., Li, M. Y., Gui, J. F., Hong, Y. H., 2010. Fish germ cells. Science China, Life Sciences. 53: 435 - 446.

Yoshizaki, G., Ichikawa, M., Hayashi, M., Iwasaki, Y., Miwa, M., Shikina, S., Okutsu, T. 2010a. Sexual plasticity of ovarian germ cells in rainbow trout. Development, 137: 1584.

Yoshizaki, G., Okutsu, T., Ichikawa, M., Hayashi, M., Takeuchi, Y. 2010b. Sexual plasticity of rainbow trout germ cells. Animal Reproduction Science, 7: 187 - 196.

Zhong, J. Y., Wang, Y. P., Zhu, Z. Y. 2002. Introduction of the human lactoferrin gene into grass carp (*Ctenopharyngodon idellus*) to increase resistance against GCH virus. Aquaculture 214(1 - 4): 93 - 101.

Conclusion

The hybrid channel catfish female × blue catfish male, which has faster growth, better feed conversion, higher tolerance to low oxygen, increased resistance to many common diseases, higher tolerance to crowded growth conditions in ponds, more uniformity in size and shape and higher dress out percentage, is the ideal catfish for pond culture. Our long - term goal is to develop a protocol for producing xenogenic catfish by interspecifically transplant the blue catfish germ stem cells into sterilized triploid channel catfish. If xenogenic channel catfish males carrying blue catfish sperm can mate with normal channel catfish females, a greatly simplified system would be available to commercially produce hybrid catfish. Additionally, combining xenotransplantion with germ stem cell gene modification could speed transgenic catfish research and increase the efficiency of catfish genetic enhancement programs. Several studies were conducted to progress towards, these long - term goals.

To achieve our goal, gonadal characteristics of different ages of blue catfish and channel catfish were studied histologically, and germline stem cells (GSCs) were identified in the gonads. Both oogonia (12 ~ 15 μm diameter, distinct nucleus 7 ~ 8 μm) and spermatogonia (12 ~ 15 μm, distinct nucleus 6 ~ 7.5 μm) were found in all ages of fish. The percentage of germline stem cells was higher in younger blue catfish of both sexes. Testes of two - year - old male juvenile blue catfish contained a high percentage of spermatogonia, and were used for spermatogonial stem cells (SSCs) isolation in the following study.

A discontinuous density gradient centrifugation made by using 70%, 45% and 35% of Percoll was used to enrich the percentage of spermatogonia. Four distinct cell bands were generated after the centrifugation. It was estimated that 55% of the total

cells in top band - cell band I were type A spermatogonia (diameter 12 ~ 15 μm) and type B spermatogonia (diameter 10 ~ 11 μm), 35% were spermatocytes (diameter 5 ~ 9 μm), 10% were spermatids (diameter < 1 μm). In the second band - cell band II, most of the cells were spermatocytes (60%, diameter 5 ~ 9 μm), some were type B spermatogonia (30%), a few were spermatids (10%) and cell clusters could also be observed. The third band - cell band III contained most of the spermatids (95%) and very few secondary spermatocytes (5%, diameter 5 ~ 8 μm). Red blood cells (95%) and a few spermatids (5%) were the predominant cells in the fourth cell band - cell band IV.

SSCs specific makers were identified by determining gene expression in different types of testicular germ cells of both channel catfish and blue catfish. The same expression change pattern were found for gene pfkfb4, urod, Plzf, Integrin6, ID - 4, integrinV, Thy1 and CDH1, in purified SSCs of both channel and blue catfish, and they were identified as spermatogonia markers. The SOX2 gene was indicated as the spermatid marker.

To establish cell culture, SSCs enriched form Percoll density gradient centrifugation, dissociated testicular germ cell and minced testicular tissue were compared as initial culture materials. During the culture of enriched SSCs, most of the spermatids were firstly adhering to the culture plate, and removed when cells were passed into a new gelatin coated well. Spermatogonia did not adhere to the plate and no cell proliferation was observed. Enzymatically dissociated testicular germ cells did not attach or proliferate during one month culture. Small pieces of testicular tissues from testes of two - year - old juvenile blue catfish were the best material to initiate the cell culture. The ovarian germ cells isolated from ovaries of two - year - old juvenile blue catfish were very easy to culture *in vitro*. Both cultured testicular and ovarian germ cells at 11th passage were confirmed as germ cells by PCR for the competitive germ cell markers *nanos* and *vasa*. Cultured testicular germ cells at passage of 6 were used in a cryopreservation study. Approximately, 77.4% of the cells were alive after two weeks of cryopreservation, and the growth of those cells was stable during the recovery culture.

The GFP expression construct FRMwg was successfully transferred into blue catfish ovarian germ cells and channel catfish testicular germ cells by electroporation. The success of the electroporated GFP construct implies that this method can be used in catfish germ cell labeling. The labeled germ cell could be used for further transplantation study for determining the donor GSCs location and proliferation in recipient. It also proved the possibility to produce transgenic catfish by using the FRMwg construct to target gene elements, such as growth or disease resistance.

Surgery and catheterization transplantation techniques were studied for transforming putative SSCs from density gradient centrifugation or dissociated testicular germ cells into the gonads of triploid channel catfish. Both surgery and catheterization were efficient and had very high transformation rates, 80 ~ 100%. The blue catfish DNA was detected in the gonads of triploid channel catfish 10 months after the transplantation indicating that the SSCs and testicular cells were able to colonize and survive in the interspecific triploid gonads. Cultured GSCs combined with successful cryopreservation, transformation and xeno - transplantation forms a powerful tool kit for controlled catfish genetic enhancement and engineering. These methods open the door to improved hybridization, strain selection and gene modification.

Future work will focus on: 1) identification of a suitable SSC surface marker that can be used for SSC enrichment and labeling; 2) optimization of the SSC *in vitro* culture system. This will include improvements in the culture medium and growth factor selection. In addition, I will seek to 3) optimize the SSC electroporation transformation system to achieve a higher percentage of healthy transgenic cells.