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Production of fertile sperm from *in vitro* propagating enriched spermatogonial stem cells of farmed catfish, *Clarias batrachus*

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Summary

Spermatogenesis is a highly co-ordinated and complex process. *In vitro* propagation of spermatogonial stem cells (SSCs) could provide an avenue in which to undertake *in vivo* studies of spermatogenesis. Very little information is known about the SSC biology of teleosts. In this study, collagenase-treated testicular cells of farmed catfish (*Clarias batrachus*, popularly known as magur) were purified by Ficoll gradient centrifugation followed by magnetic activated cell sorting using Thy1.2 (CD90.2) antibody to enrich for the spermatogonial cell population. The sorted spermatogonial cells were counted and gave ~3 × 10^6 cells from 6 × 10^6 pre-sorted cells. The purified cells were cultured *in vitro* for >2 months in L-15 medium containing fetal bovine serum (10%), carp serum (1%) and other supplements. Microscopic observations depicted typical morphological SSC features, bearing a larger nuclear compartment (with visible perinuclear bodies) within a thin rim of cytoplasm. Cells proliferated *in vitro* forming clumps/colonies. mRNA expression profiling by qPCR documented that proliferating cells were Plzf $^+$ and Pou2$^+$, indicative of stem cells. From 60 days onwards of cultivation, the self-renewing population differentiated to produce spermatids (~6 × 10^7 on day 75). *In vitro*-produced sperm (2260 sperm/SSC) were free swimming in medium and hence motile (non-progressive) in nature. Of those, 2% were capable of fertilizing and generated healthy diploid fingerlings. Our documented evidence provides the basis for producing fertile magur sperm *in vitro* from cultured magur SSCs. Our established techniques of SSC propagation and *in vitro* sperm production together should trigger future *in vivo* experiments towards basic and applied biology research.

Keywords: *Clarias batrachus*, Fertile sperm, *In vitro*, Magnetic activated cell sorting, SSC

Introduction

Spermatogonial stem cells (SSCs) originate from primordial germ cells (PGCs) in mammals (before birth) and teleostean fish (generally after hatching). SSCs reside within the seminiferous tubules of testis. SSCs undergo the tightly regulated and complex processes of spermatogenesis, involving their self-renewal to maintain their own pool and differentiation to form mature spermatids. Unlike in mammals, spermatogenesis in fish takes place in a cyst within seminiferous tubules (Schulz *et al.*, 2005; Panda *et al.*, 2011). The cysts are formed when a Sertoli cell(s) encapsulates a single primary spermatogonium that

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subsequently divides synchronously to constitute an isogenic germ cell clone. The numbers of differentiated spermatogonia are also much higher in teleosts as compared with their mammalian counterpart (Vilela *et al.*, 2003; Schulz *et al.*, 2005).

SSCs are indeed unique adult stem cells – capable of transmitting genetic information in a Mendelian fashion. Hence, *in vitro* propagation could provide the opportunistic platform for targeted genetic modifications, having implications in basic as well as applied biology research. The establishment of pure or significantly enriched populations of male germ line stem cells is an important aspect for *in vitro* propagation to undertake biological experimentation and male germ-cell manipulations. A major problem associated with *in vitro* propagation has been the lack of sufficient knowledge regarding their candidate phenotypic and genotypic features. A developmental stage-specific understanding of those signatures, linked with either self-renewal or differentiation, is essential for efficient purification and accurate characterization during *in vitro* propagation. Ample evidence on isolation, culture and maintenance of rat (*Rattus norvegicus*), sheep (*Ovis aries*), mouse (*Mus musculus*), human (*Homo sapiens*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*) and chicken (variety: Hy Line-36) (*Gallus gallus*) SSCs *in vitro* is available (Buomyong *et al.*, 2004; Rodriguez-Sosa *et al.*, 2006; Dym *et al.*, 2009; He *et al.*, 2010; Hua *et al.*, 2011; Liu *et al.*, 2011; Kala *et al.*, 2012; Vlajkovi'c *et al.*, 2012; Baazm *et al.*, 2013; Wu *et al.*, 2013; Momeni-Moghaddam *et al.*, 2014).

There is a very little information about spermatogonial self-renewal and differentiation in fish testis. Male germ cell markers such as Pou2 (Pit-Oct-Unc-2, an orthologue of mammalian Pou5f1, expressed in proliferating SSCs), Plzf (promyelocytic leukemia zinc finger as an undifferentiated marker) and c-Kit (the transmembrane tyrosine kinase receptor for stem cell factor, a candidate differentiation gene) have been documented in rohu (*Labeo rohita*), medaka fish (*Oryzias latipes*) and dogfish (*Scyliorhinus canicula*) (Hong *et al.*, 2004; Loppion *et al.*, 2008; Mohapatra *et al.*, 2010; Sanchez-Sanchez *et al.*, 2010; Panda *et al.*, 2011; Mohapatra & Barman, 2014; Mohapatra *et al.*, 2014; Zhao *et al.*, 2015).

Unlike in laboratory animals, evidence regarding a well defined culture system for highly purified SSCs is not available for any fish species. The enrichment of SSCs from total testicular cells by magnetic activated cell sorting (MACS) using Gfrα1 antibody has been demonstrated in mammalian species including human and rodents (Meng *et al.*, 2000; Buageaw *et al.*, 2005; Ebata *et al.*, 2005; Hofmann *et al.*, 2005; Gassei *et al.*, 2009; Kossack *et al.*, 2009; Yuan *et al.*, 2009; He *et al.*, 2010). It has also been possible to sort spermatogonial cells of rodents, non-human primates and bull by fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) using Thy1 (CD90) antibody (Kubota *et al.*, 2003; Ryu *et al.*, 2004; Hermann *et al.*, 2009; Reding *et al.*, 2010). Other markers such as CD9 and a6-integrin have been identified as phenotypic surface markers in the case of rodents (Shinohara *et al.*, 1999; Kanatsu-Shinohara *et al.*, 2004). We have previously devised a modified two-step protocol (Ficoll gradient centrifugation followed by Thy1 antibody-labelled MACS sorting) for enriching SSCs of a commercially important farmed rohu carp, *Labeo rohita* (Panda *et al.*, 2011). We demonstrated that Thy1-mediated purification is better than Gfrα1-mediated purification to obtain highly pure rohu SSCs, these could be cultured for longer periods in an undifferentiated state. Subsequently, Thy1-mediated purification of spermatogonial cells has been adopted successfully for other species such as dairy goat (*Capra hircus*), mammals and mouse (Niu *et al.*, 2011; Kaucher *et al.*, 2012; Wu *et al.*, 2013). Recently, isolation and transplantation of spermatogonia of Siberian sturgeon (*Acipenser baerii*) in newly hatched sterlet *Acipenser ruthenus* larvae was used successfully to produce germline chimera in sturgeon (Pšenička *et al.*, 2015).

Induced breeding technology has been successfully demonstrated for both sexes of commercially important freshwater fishes, except that of male catfish (*Clarias batrachus*), commonly known as magur. While stripping-based milt collection is possible in the majority of commercially important freshwater fishes, male magur is mandatorily required to be sacrificed to collect milt for external fertilization. The sacrifice of highly valued male magur for breeding purposes has been a concern. *In vitro* production of sperm from the cultured spermatogonial cells under culture condition could be an alternative strategy for producing male gametes to be used in magur breeding programmes. *In vitro* production of spermatids from cultured spermatogonial in the presence of feeder cells has been documented in bovine, Japanese eel and mouse (Miura *et al.*, 1991; Izadyar *et al.*, 2003; Kanatsu-Shinohara *et al.*, 2003; Marh *et al.*, 2003). Immortalized mouse germ cells were also capable of generating haploid sperm (Hofmann *et al.*, 1994; Feng *et al.*, 2002). Medakafish (*O. latipes*) spermatogonial cell line co-cultured with stromal cells of the embryonic rainbow trout gonad cell line were capable of differentiating into haploid sperm-like cells in culture (Hong *et al.*, 2004). Recently, SSCs generated from the hyperplastic testis of zebrafish and sperms were produced *in vitro* in the presence of Sertoli feeder cells (Kawasaki *et al.*, 2016). However, it would be of interest to produce fertile sperm *in vitro* from the enriched spermatogonial cells in the absence of any feeder cells.

In our previous study, a population of enriched and proliferating SSCs of rohu carp (*L. rohita*)

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generated/produced fertile sperm (Panda *et al.*, 2011). This technique provided an unique opportunity to generate fertile male gamete throughout the year, instead of being normally dependent on the breeding (monsoon) season. Keeping these aspects in mind, we intended to explore SSC-mediated sperm production *in vitro* from the highly valued magur, *C. batrachus*. We demonstrated efficient enrichment, cultivation and characterization of magur spermatogonial cells, and the successful production of fertile sperm from cultured magur SSCs in the absence of any feeder cells.

Materials and methods

Fish and sample collection

Live adult male and female magur fish (*Clarias batrachus*) of ~100 g body weight (and about 1 year old) were collected during the month of June from the hatcheries of ICAR – Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, Odisha, India. Different tissue samples such as muscle, kidney, heart, spleen, liver, gill, brain, intestine, testis and ovary were collected aseptically and stored at −80°C until further use.

Gradient separation of magur spermatogonial cells from testicular cells

Primary culture of magur testicular cells and Ficoll gradient separation of spermatogonial cells were performed following an established protocol as described (Panda *et al.*, 2011). Briefly, two-thirds of aseptically dissected testis [~70 mg; Gonadosomatic Index (GSI) is 0.07] were treated with 0.5% of sodium hypochlorite, chopped and digested with collagenase type II (500 units/ml in 20 mM HEPES, 0.5% BSA, 5% FBS and penicillin/streptomycin antibiotics) for 2 h at 28°C. Dissociated cells were pelleted and the pellet was resuspended in 4 ml of 1× PBS containing 2 mM EDTA, and 0.5% BSA and antibiotics; and layered onto 3 ml of Ficoll-Paque PLUS (GE Healthcare). Gradient separation was performed by centrifugation at 800 *g* for 30 min at room temperature. Another part of the testis was frozen to extract total RNA as described below.

Purification and culture of pure spermatogonial stem cells by magnetic activated cell sorting

MACS was carried out as described (Panda *et al.*, 2011). Briefly, the gradient-separated cells (total ~6 × 10^6 cells/ml, counted under a microscope using a haemocytometer), were resuspended in MACS buffer (1× PBS, 0.5% BSA, 2 mM EDTA and 1× penicillin/streptomycin antibiotics), and were then incubated with mouse anti-Thy1.2 (also known as CD90.2)-conjugated microbeads (Miltenyi Biotech, Germany, order no.: 130–049–101). Thy1 is a glycosyl phosphatidylinositol (GPI)-anchored membrane glycoprotein of the Ig superfamily, involved in adhesion mechanisms and signal transduction and was originally discovered as a thymocyte antigen. Microbeads containing the cell suspension were passed through a MACS column placed in a strong magnetic field separator and the flow through was collected as the depleted fraction. After repeated washing, the Thy1.2-labelled cells (sorted fraction) were collected and maintained in L-15 complete medium containing 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× minimum essential medium (MEM) non-essential amino acid solution, 100 µM 2-mercaptoethanol, 2 nM sodium selenite, 6 mg/mL D(+)- glucose, 0.5% BSA (fraction V), 1% carp serum, 10% FBS, 1× N-2 supplement (Invitrogen, USA) at 28°C in a humidified chamber containing 5% CO_2 in air for *in vitro* propagation.

Sperm motility test

The sperm motility test was performed manually as described in the World Health Organization (WHO) manual (WHO, 2010). Briefly, sperm-containing medium was further diluted with L-15 complete medium. About 10 µl diluted sperm sample was overlaid onto a haemocytometer containing a coverslip and examined under a bright-field microscope.

Total RNA isolation and cDNA synthesis

DNase I-treated total RNA was extracted from magur muscle, kidney, heart, spleen, liver, gill, brain, intestine, testis and ovary by the TRIzol (Invitrogen, USA) method. Quality checks and quantification were performed following established methods of gel electrophoresis and NanoDrop spectrophotometer readings. RNA was reverse-transcribed to cDNA using the SMART Scribe Reverse Transcriptase cDNA Synthesis Kit (Clontech, USA) as per the manufacturer's guidelines (Mohanta *et al.*, 2014; Panda *et al.*, 2014; Patra *et al.*, 2015). RNA was also extracted from the purified SSCs (up to 3 × 10^6 cells) by the TRIzol method as mentioned above and cDNA synthesis was achieved using the Smarter Pico cDNA Synthesis Kit (Clontech, USA) according to the manufacturer's instructions.

Quantitative real-time PCR (polymerase chain reaction) (qPCR) and statistical analysis

mRNA expression profiling for Plzf (Promyelocytic Leukemia Zinc Finger), Pou2 (Pit-Oct-Unc-2, an ortholog of mammalian Pou5f1) and Gfrα1 (GDNF

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family receptor alpha 1) in different organs including proliferating SSCs was evaluated by qPCR. Sequence information for Plzf, Gfrα1 and Pou2 of *C. batrachus* was lacking. Therefore, for the purpose of accuracy, we first cloned and characterized full-length cDNA sequences for the *Plzf* and *Gfrα1* genes (unpublished data, submitted GenBank accession numbers: KU215904 and KU215905 respectively) including the partial cDNA sequence of *Pou2* (GenBank accession number: KU215906) following which, primer sets of these three genes for qPCR analysis were designed as shown in Table S1. PCR amplification was performed using various primer combinations to verify single band amplification; these were confirmed by bi-directional sequencing. qPCR and data analysis of different marker genes and the β-*actin* house-keeping gene were performed in triplicate for each cDNA sample using the SYBR Green 1 Kit (Roche Diagnostics, Germany) in a Light Cycler 480 Real-Time PCR system (Roche Diagnostics, Germany) as per the manufacturer's instructions (Panda *et al.*, 2011; Mohapatra *et al.*, 2013; Mohanta *et al.*, 2014; Panda *et al.*, 2014; Patra *et al.*, 2015). Negative control reactions with respective RNA templates were performed to rule out the possibility of genomic DNA contamination.

All data of triplicate experiments were normalized with respect to β-*actin*, which was used as the house-keeping gene. The threshold cycle (C_t) value (the PCR cycle number at which fluorescence was detected above threshold and decreased linearly with increasing input target quantity) was obtained using the qPCR system software (Roche Diagnostics, Germany) and used to calculate fold change for relative gene expression using the standardized $\log_2 \Delta\Delta C_t$ method. The significance of expression of target genes was analyzed using the Kruskal–Wallis test using SAS (v 6.12) without checking the normal distribution of expression level. A *P*-value of 0.05 was used to reject the null hypothesis.

Karyotypic analysis

Chromosome preparation from the kidney tissue of magur hatchlings (about 20 g) was performed following a standard protocol as described previously (Ida *et al.*, 1978; Pradeep *et al.*, 2011). Briefly, 200 µl of 0.5% colchicine was injected intramuscularly per fish for a period of 2 h. Subsequently, fish were anaesthetized with tricaine methanesulfonate MS222 (Sigma Aldrich) (Panda *et al.*, 2014) and kidney tissues were collected. Tissues were homogenized in hypotonic solution (0.56% KCl) and allowed to swell for 20–25 min at room temperature (25°C). Swollen cells were fixed with chilled Carnoy's solution (methanol:glacial acetic acid, at a ratio of 3:1). Fixed cells were dropped onto glass slides from a height, air-dried, stained (5% Giemsa stain in phosphate-buffered saline) and observed under a microscope.

Results

Spermatogonial cell enrichment from testicular cells

The percentage of SSCs in a testis is very low in mammalian and non-mammalian species including fish species (Mardanpour *et al.*, 2008; Panda *et al.*, 2011; Shang *et al.*, 2015). Hence, we tried to purify spermatogonial cells from the primary culture of total testicular cells. Total testicular cells prepared from magur (*C. batrachus*) by enzymatic digestion were cultured in L-15 complete medium. Microscopic observations of primary testicular culture contained a mixed population of somatic cells and spermatogonial cells (various stages of spermatogenesis) including motile sperm.

Based on an established enrichment protocol (Panda *et al.*, 2011), attempts were made to purify magur spermatogonial cells from the total testicular cells. First, a primary culture of enzymatically digested testicular cells was separated by gradient (over 3 ml of Ficoll-Paque PLUS; GE Healthcare) that resulted in partly removing unwanted somatic cells and spermatids (data not shown). Ficoll gradient centrifugation-separated spermatogonia remained at the top as well as in the middle layers, whereas spermatids, spermatozoa, cell debris, etc. were pelleted down at the bottom of the tube. Using this procedure, we could not achieve a highly purified sample of spermatogonia as per our requirement. Second, these gradient-separated cells (top and middle layers) were subsequently subjected to MACS using Thy1.2 (CD90.2) antibody. MACS generated about 3×10^6 spermatogonial cells from the gradient-separated 6×10^6 cells (Fig. 1*A*, *B*). Microscopic observation confirmed that sorted cells were free from (decontaminated) spermatids, etc. (Fig. 1*A*). The morphology of sorted cells was like that of spermatogonial cells, bearing the typical characteristics of a larger nucleus within a thin cytoplasmic rim. The nucleus also contained dark perinuclear bodies, the typical features of spermatogonial cells.

Such morphological features of purified cells provided the clue that Thy1 (CD90) is likely to be present on the plasma membrane of magur SSCs. This result is in line with rat, non-human primates, human, bull, mouse, rohu, mammals and goat (Kubota *et al.*, 2003; Buom-yong *et al.*, 2004; Ryu *et al.*, 2004; Hermann *et al.*, 2009; He *et al.*, 2010; Reding *et al.*, 2010; Niu *et al.*, 2011; Panda *et al.*, 2011; Kaucher *et al.*, 2012; Wu *et al.*, 2013).

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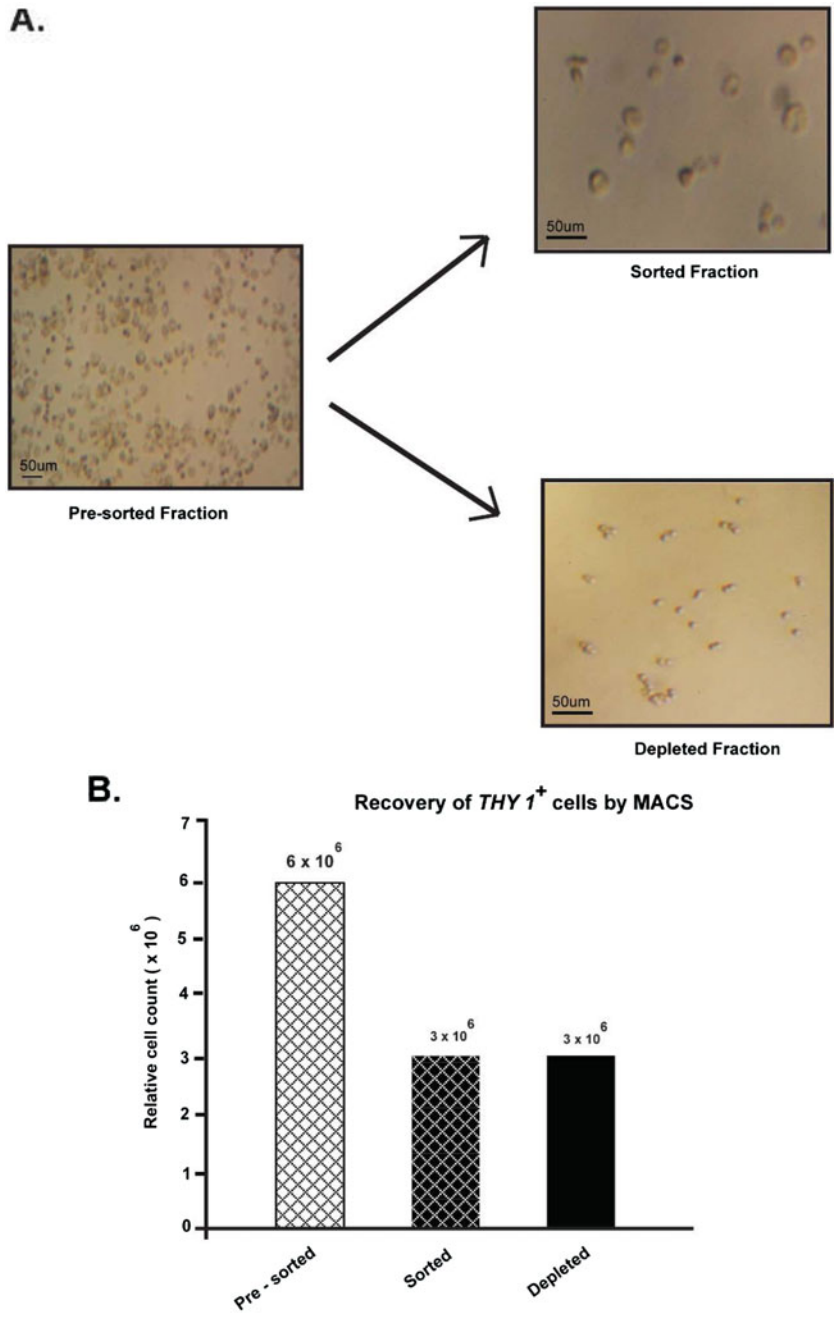

Figure 1 Enrichment of spermatogonial cells by magnetic activated cell sorting (MACS). (*A*) A representative figure showing the Ficoll gradient separated cell suspension known as pre-sorted fraction (left side). Gradient-separated cells were further subjected to MACS using Thy1.2 (CD90.2) labelled microbeads. Magnetically sorted (top right side) and depleted cell fractions (bottom right side) are shown. (*B*) On an average 6×10^6 pre-sorted cells were loaded onto a MAC separator column of which 50% cells were recovered in the sorted fraction using the Thy1.2 (CD90.2) antibody.

Enriched spermatogonial cells were proliferative and undifferentiated in nature

The purified cells were cultured *in vitro* for 2 months in L-15 complete medium under the conditions listed in Materials and methods. The sorted cells proliferated, forming characteristic clumps/colonies and remained loosely attached to the culture dish. The morphology of these cells looked like SSCs, with the feature of a larger nuclear compartment as compared with the cytosolic compartment. The darker perinuclear bodies, the typical characteristics of SSC, were also observed under a microscope. Even though the cells were not tightly attached, they seemed to have affinity for each other; and thereby proliferating cells formed clumps (Fig. 2). The initial 3×10^6 cells resulted in

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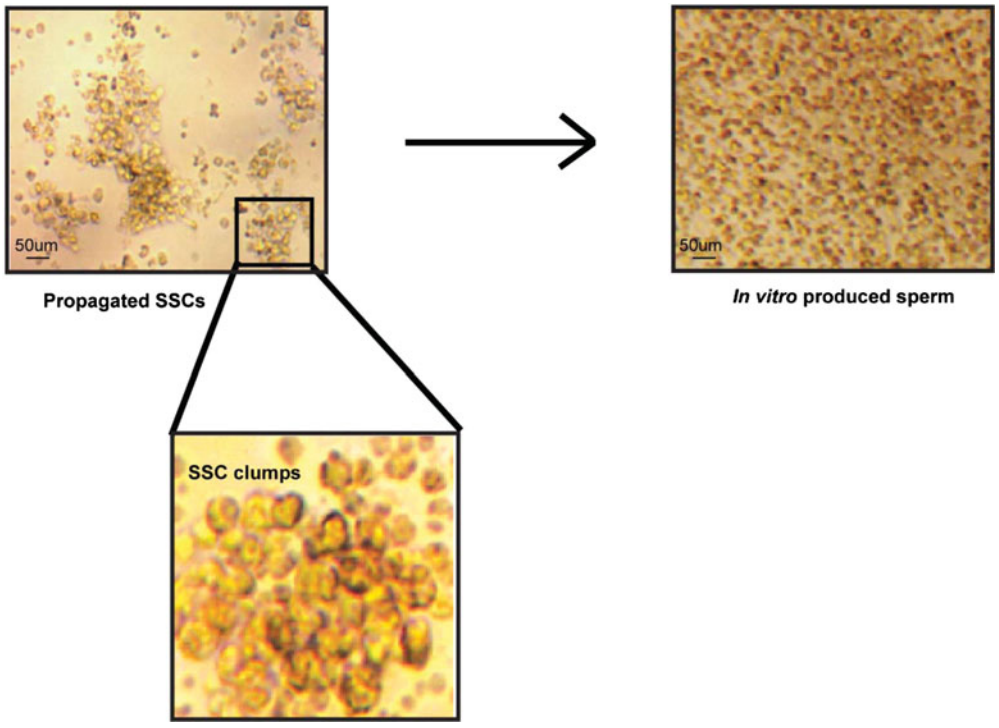

Figure 2 *In vitro* culture of magur enriched spermatogonial stem cells (SSCs) forming clumps that are loosely attached to the surface and *in vitro* production of motile/fertile sperm.

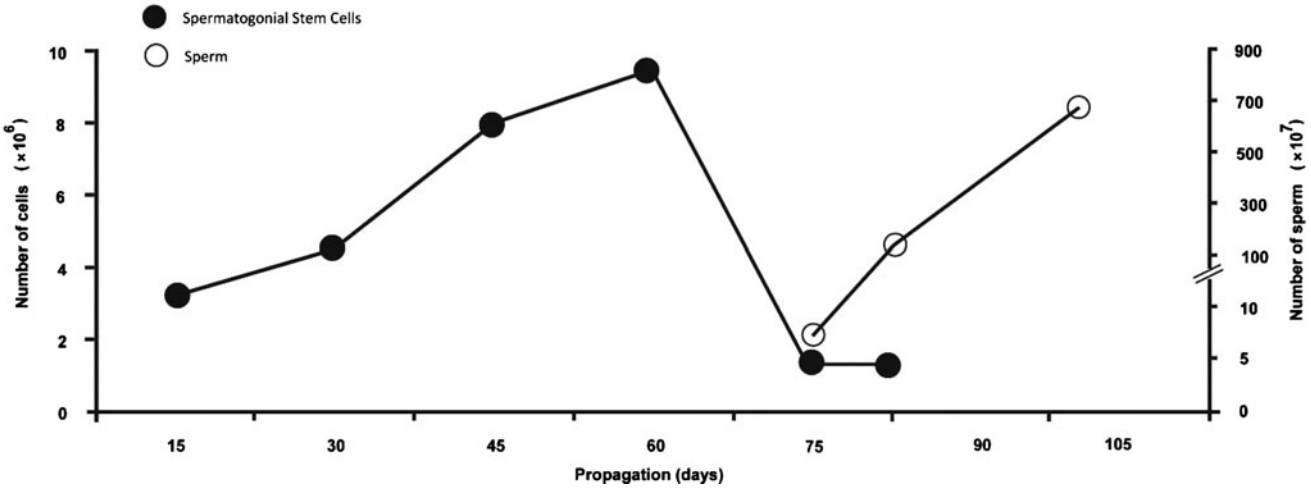

Figure 3 Graphical representation of spermatogonial stem cell proliferation (approximately 1×10^6) with respect to sperm production (approximately 1×10^7). The sorted spermatogonial stem cells (3×10^6) resulted in about (9×10^6) cells on 60 day of culture; the self-renewing population produced motile sperm, approximately $\sim 6 \times 10^7$ on day 75. The SSC population was reduced from day 60 onwards. Sperm were produced, approximately $\sim 102 \times 10^7$ and $\sim 678 \times 10^7$ on days 80 and 100, respectively. Approximately, 678×10^7 sperm were produced from 3×10^6 enriched SSCs within a span of 3½ months.

about 9×10^6 cells after 60 days of culture (Fig. 3). The counted dead cells were negligible as estimated by trypan-blue dye exclusion examination (data not presented).

In order to verify the stemness properties of the proliferating purified cells, the mRNA expression levels for *Plzf* and *Pou2* were quantified at 55 days of culture using qPCR. The heightened mRNA expression levels of *Pou2* and *Plzf* were noticed in cultured SSCs as compared with muscle, heart, brain, kidney, spleen, gill, liver and intestine (Fig. 4). The heightened expression of *Pou2* and *Plzf* on the proliferating spermatogonial cells revealed that the SSCs were undifferentiated in nature.

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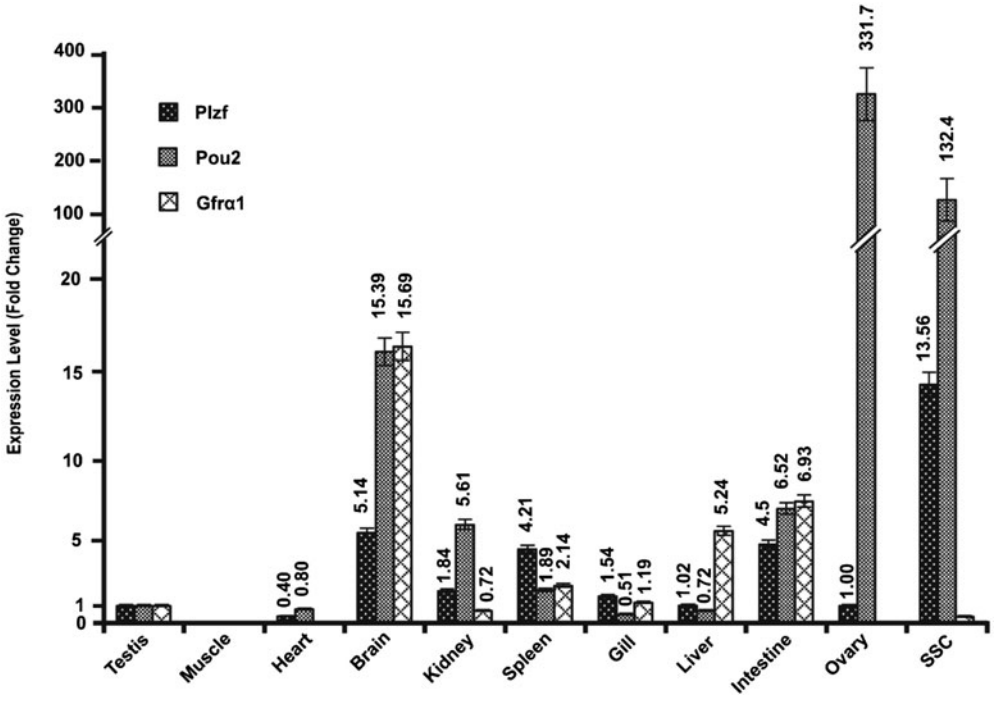

Figure 4 Differential expression profiles of stem cell markers in different tissues including enriched spermatogonial cells of *C. batrachus* detected by quantitative real-time PCR (qPCR).

Production of motile/fertile sperm *in vitro* from proliferating magur spermatogonial stem cells

The major population of SSCs proliferated for a period of 2 months with negligible sperm counts. To our surprise, after 60 days of culture, the SSC population started depleting dramatically together with a change in morphological characteristics as observed under the microscope. The clumped cells started dissociating from each other and were relatively larger in shape and size. It appeared that cells started to differentiate. We also observed spermatids, including free-swimming (with non-progressive motility) sperm in the culture dish. About 70% sperm showed a swimming pattern confined to a tight circle, but not in a forward straight line as observed in progressive motility, and hence were classified as the non-progressive motile category. The remaining 30% of *in vitro*-produced sperm were non-motile, having no movement. As shown in Fig. 3, the self-renewing population of magur spermatogonial cells produced motile sperm of approximately ~6 $\times$ 10^7 on day 75. The SSC population depleted dramatically (1 $\times$ 10^6) from day 60 onwards. At the same time, the *in vitro*-produced sperm increased by about 20-fold on day 80. Enormous numbers of sperm were produced in the region of ~102 $\times$ 10^7 and ~678 $\times$ 10^7 on days 80 and 100, respectively. On average, 678 $\times$ 10^7 sperm were produced from 3 $\times$ 10^6 enriched spermatogonial cells (2260 sperm/SSC) within a span of 3½ months.

These results indicated that proliferating SSCs differentiated in the undefined media formulation. The major population of the *in vitro*-produced sperm showed non-progressive movement in the culture medium, demonstrating that they were motile in nature. To examine the fertile nature of these motile sperm, the pelleted sperm were fertilized with magur eggs stripped from a matured female following a standard induced breeding technique. The success rate of fertilization was about 2%. The newly hatched off-springs were quite active and their morphology seemed to be similar to that of the control hatchlings (Fig. 5). The test hatchlings grew well in our hatchery with the artificial feed, up to fingerling stage.

The metaphase chromosomes were prepared from kidney cells of the magur hatchlings. As shown in Fig 5(*C*), the cytogenetic profile of magur hatchlings (*C. batrachus*) was found to be diploid (2n = 52 to 54) (Siraj *et al.*, 2009) in number, revealing possible contributions from sperm produced *in vitro.*

Discussion

Because of seasonal breeding and other difficulties in breeding male magur (*C. batrachus*) by hormonal manipulation, we undertook studies linked to culture of SSCs, aiming to produce sperms *in vitro.* Our established protocol of purifying spermatogonial cells

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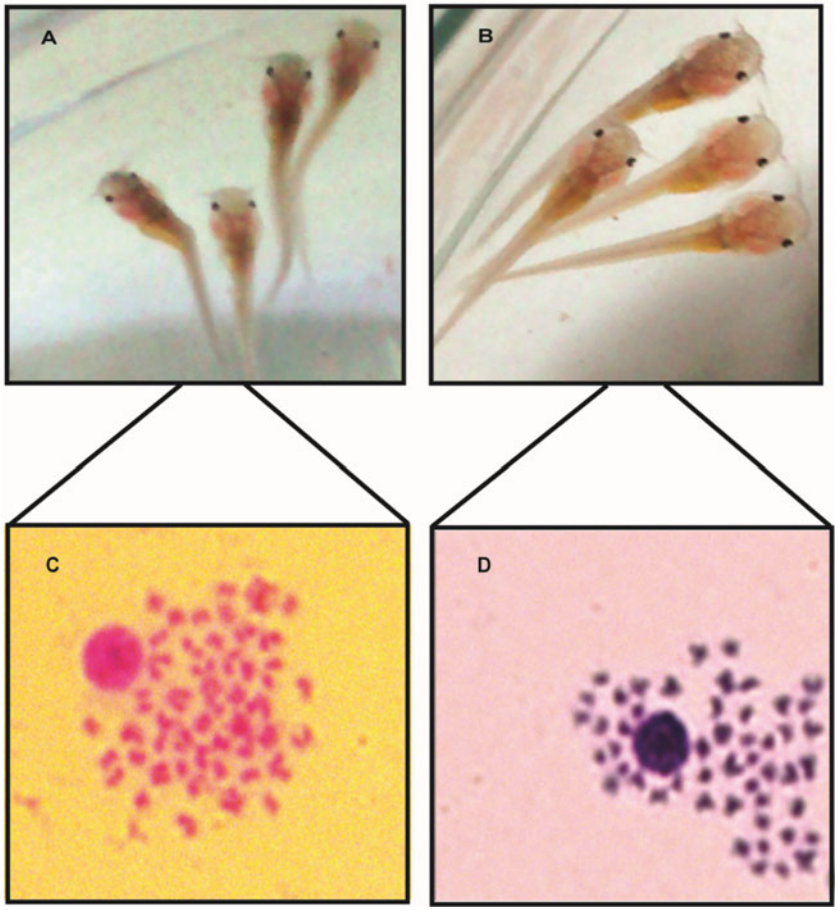

Figure 5 Fertilized magur (*C. batrachus*) off-spring (diploid) derived from *in vitro*-produced sperm. (*A*) Experimental hatchlings and (*B*) control hatchlings. (*C*) Representative figures showing diploid metaphase chromosomes of experimental hatchlings ($n = 4$) and (*D*) control hatchlings ($n = 2$).

(Panda *et al.*, 2011) was implemented to enrich magur spermatogonial cells. The dramatically heightened recovery rate, in the region of 50% sorted magur spermatogonial cells using Thy1 immuno-magnetic beads, was comparable with that of rohu (*L. rohita*) spermatogonial cells. This finding that Thy1 is expressed on the plasma membrane of spermatogonial cells was consistent with earlier findings (Kubota *et al.*, 2003; Buom-yong *et al.*, 2004; Ryu *et al.*, 2004; Hermann *et al.*, 2009; He *et al.*, 2010; Reding *et al.*, 2010; Niu *et al.*, 2011; Panda *et al.*, 2011; Kaucher *et al.*, 2012; Wu *et al.*, 2013). This result also implied that Thy1 (CD90) is likely to be conserved across eukaryotes and could be considered as a potential candidate marker for spermatogonial cells.

Purified cells could be cultured for a period of 2 months in an undifferentiated state, as documented by typical morphological features and heightened mRNA expression profiles for Plzf and Pou2. The *Pou2* gene expression level was high in the case of ovary, compared with other genes, which is in the line with the rohu *Pou2* expression pattern (Mohapatra *et al.*, 2014). These results also implied that Plzf and Pou2 play major roles in SSC proliferation and maintenance in line with earlier findings (Hong *et al.*, 2004; Loppion *et al.*, 2008; Mohapatra *et al.*, 2010; Sanchez-Sanchez *et al.*, 2010; Panda *et al.*, 2011; Kala *et al.*, 2012; Mohapatra & Barman, 2014; Shang *et al.*, 2015; Zhao *et al.*, 2015). Gfrα1 could not be detected in the proliferating cells, which is in line with previous reports of rohu SSCs (Panda *et al.*, 2011). Our study reconfirmed that Pou2 and Plzf could be used as spermatogonial stem cell markers in wide range of fish species. Consensus is also being developed that Thy1 could effectively be used to enrich for proliferative spermatogonial cells that are undifferentiated in nature.

The major population of proliferating SSCs differentiated to produce free-swimming sperm with non-progressive motility from 60 days onwards. This result is in line with earlier findings of *in vitro* sperm production from male germ cells in culture (Hofmann *et al.*, 1994; Izadyar *et al.*, 2003; Hong *et al.*, 2004). The exact reason for the dramatic shift from

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the undifferentiated to the differentiated state is not known. One reason could be that the medium formulation for mitotic activity was not properly defined, especially in the presence of FBS and carp serum. A population of proliferating rohu spermatogonial cells also produced fertile sperm *in vitro* (Panda *et al.*, 2011). Interestingly, it was possible to produce about 2260 live sperm per spermatogonia (sorted) over the period of 3½ months. Out of those, 2% were capable of fertilizing eggs to generate healthy fingerlings. Even though the rate of fertile sperm production is very low, our documented evidence provided a way of *in vitro* fertile sperm production from magur SSCs in culture. Future studies could be undertaken to fine tune improved fertile and quality sperm production using defined media formulations supplemented with specific growth factor(s). Interestingly, these hatchlings contained a 2n chromosomal complement providing a pointer of both parental contributions. However, the possibility of diploid gynogens (maternal) cannot be ruled out at this stage. Both parental contributions could be clarified by genotyping (depending upon the availability of DNA markers) in future. This study also provided an avenue for gene manipulation. Recently, we established a highly efficient nucleofection protocol for rohu SSCs (Barman *et al.*, 2015). Enriched and proliferating magur SSCs (2n complement) could be transfected with donor DNA construct within 60 days of cultivation to produce genetically modified fertile sperm (n) from 60 days onwards. These manipulated sperm could be fertilized to generate transgenic magur. Due to the only small success rate in producing transgenic farm fishes using conventional methods, mainly due to mosaic expression (Stuart *et al.*, 1990; Rahman *et al.*, 2000), spermatogonial stem cell-mediated transgenesis could be an effective alternative transgenesis. Established techniques of SSC propagation and transfection protocol together should trigger experiments towards the basic understanding of complex spermatogenesis processes.

In conclusion, this is the first evidence of successful enrichment, characterization and *in vitro* propagation of magur (*C. batrachus*) spermatogonial cells in the undifferentiated state. Furthermore, evidence was provided with regard to *in vitro* production of fertile sperm from proliferating SSCs. The medium formulation used in this study was not clearly defined either linked to self-renewal or differentiation. Future studies could be undertaken to uncover unique essential growth factors required for self-renewal versus differentiation to facilitate the developmental stages of spermatogenesis *in vitro*. *In vitro* sperm production could mitigate the problem of sacrificing the male magur during milt collection. Sperm could also be produced all year round, and therefore not restricted to the breeding season. Although the success rate of fertile sperm production was only 2%, this study has provided the platform for future basic and applied biology research in this area.

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Supporting information

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0967199416000149

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