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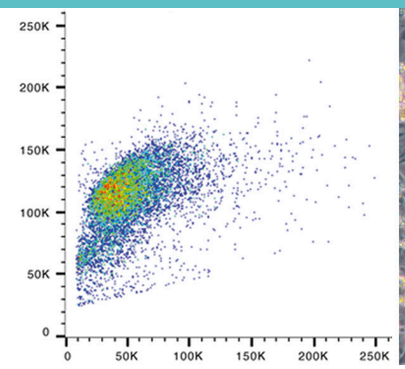
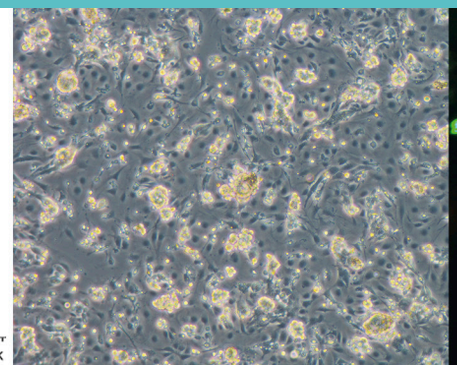
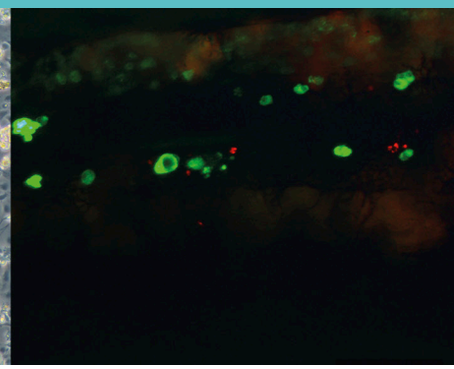
# *In vitro* culture of sturgeon germ stem cells

## *In vitro* kultivace zárodečných kmenových buněk jesetera





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## CONTENT

# CHAPTER 1

## GENERAL INTRODUCTION

## General introduction

With the development of various applications of sturgeon germ cells, such as surrogate reproduction and gene editing, some strategies are needed to be developed to obtain a pure germ cell population. In the present thesis, the main idea is to establish efficient methods to identify, isolate, and culture spermatogonia in sturgeon. We reviewed fish spermatogonia characterization and the regulation of spermatogenesis, also summarized the available protocols and advances in enriching and *in vitro* culture of germ cells (Chapter 2). Moreover, based on current techniques, we developed some methods in sturgeon germ cells, such as germ cell *in vitro* culture (Chapter 3), enrichment of type A spermatogonia (Chapter 4), and identification of type A spermatogonia (Chapter 5). Chapters presented in this thesis could provide an available platform to further characterization and wide manipulations of sturgeon germ cells.

### Germ stem cells

#### Introduction

Germ stem cells (GSC) is the only stem cell population among tissue stem cells that possess the ability of self-renewal to maintain undifferentiated state and differentiation that finally generate gametes to transmit genetic information to the next generation. The balance of self-renewal and differentiation can be synergistically regulated by GSC themselves and somatic cells which formed a microenvironmental structure named "niche." In fish, germline stem cells are also known as spermatogonial stem cells (SSCs) and oogonial stem cells (OSC) or female germline stem cells (FGSC). Progression in aspects of GSC morphology, identification, and gametogenesis were reported in past decades. Moreover, GSCs present very high biotechnological potential in aquaculture. In the subsequent sections, we will review the current knowledge of GSCs morphology and gametogenesis in the fish, the limitation and future trend of GSC study, as well as the relevant developments regarding sturgeon germ cells.

#### Origination and development of fish germ cells

In fishes, GSCs are formed as primordial germ cells (PGCs) early in embryonic development and then migrate toward the genital ridge. The migration pattern of PGCs toward the gonadal ridge depends on species. In a primitive fish species such as sturgeon, PGCs are originated from vegetal pole in the embryo and migrate dorsally when the tail rudiment bulged out. Finally, PGCs localize at developing gonadal ridges (Saito et al. 2014).

Germ cells begin to proliferate when the single gonadal primordium splits into two bilateral primordia (Nishimura and Tanaka 2014). In teleost testes, undifferentiated type A spermatogonia ($A_{und}$, Figure 1), which remains "stemness," are believed to self-renew and arise to differentiated type A spermatogonia ($A_{diff}$), which share the similar morphology with $A_{und}$ (Schulz et al. 2010). $A_{diff}$ can be observed in cysts with two to eight germ cells in Nile-tilapia (*Oreochromis niloticus*) and zebrafish (*Danio rerio*). After several generations, type B spermatogonia are present in cysts with 16 or more cells, depending on the species-specific cell cycle generations. Then the last type B spermatogonia differentiate into spermatocytes to enter meiosis.

The development of germ cells in testes is regulated by endocrine and autocrine. Chapter 2 reviewed the impact of estrogens and androgens on spermatogenesis. Spermatogenesis in

different fish requires different time (Amer et al. 2001; Vázquez et al. 2012; Billard 1983). It also presumed that it can be influenced by the somatic environment. Growth factors, such as GDNF (Meng et al. 2000), Wnt5a (Safian et al. 2018) and insulin-like peptide 3 (Insl3) (Assis et al. 2016) secreted by Sertoli cells, Leydig cells, and peritubular myoid cells can modulate SSC self-renewal or differentiation (Oatley et al. 2009). More molecular and functional studies were reported to reveal the specific morpho-function or molecular characteristics of the SSC niche in the teleost testis (de Rooij 2006; Nóbrega et al. 2010; Savvulidi et al. 2019; de Siqueira-Silva et al. 2019).

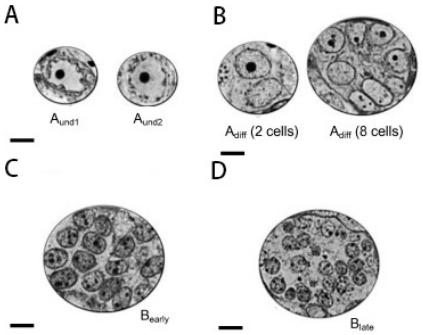


***Figure 1.*** ***Main morphological characteristics presented by fish spermatogonia, evaluated by high-resolution light microscopy.*** *A. Type A undifferentiated spermatogonia ($A_{und}$); B. Type A differentiated spermatogonia ($A_{diff}$); C. type B spermatogonia early ($B_{early}$); D. type B spermatogonia late ($B_{late}$). (Lacerda et al. 2014).*

In ovary, OSCs also derive from PGCs. With the migration of PGCs to the gonadal ridge, the development of female reproductive system starts (Lubzens et al. 2010). Generally, OSCs were mostly similar to SSCs. Structure changes during the transformation of PGC into oogonia. The isolated OSCs have similar morphology to freshly isolated SSCs, with large cell bodies with little cytoplasm, helical nuclei with slight staining, a large ratio of nuclear plasma, and their nuclear diameter of 12–20 mm (Wu et al. 2015). Nuage has also been found in oogonia. In teleost, as (Saito et al. 2007) described, oogonia could be characterized as type I and type II. Type I are the single isolated germ cells (Gs), responsible for self-renew, surrounded by supporting cells after division. Type II oogonia are the cyst-forming germ cell (Gcys) that is surrounded by supporting cells to form a cyst. The Gcys include both mitotic and meiotic germ cells. Subsequently, Gcys enter meiosis and differentiate into diplotene oocytes, which are surrounded individually by supporting cells and exit from cradles to form follicles.

## Identification of germ stem cells

Identifying germ cell populations by morphology is not an accurate and effective way. In this regard, specific molecular markers can give great help to identify and isolate germ cell populations, especially germ stem cells. In past decades, the expression of *SGSA-1* was observed in type A spermatogonia ($A_{und}$ and $A_{diff}$) in Japanese eel (*Anguilla japonica*) (Kobayashi et al. 1998); medaka (*Oryzias luzonensis*) (Sánchez-Sánchez et al. 2010). *Dead end* and *nanos2* were also identified as a specific marker for type A spermatogonia in medaka (Liu et al. 2009), rainbow trout (*Oncorhynchus mykiss*) (Bellaiche et al. 2014) and Pacific blue-fin tuna (*Thunnus orientalis*) (Yazawa et al. 2013). However, so far, there are not many studies about oogonia stem cell marker. The expression of *nanos2* was found in oogonia in medaka and zebrafish (Beer and Draper 2013; Nakamura et al. 2010). Besides, it also

investigated the effect of some hormones on fish testicular gene expression patterns, and on the molecular mechanisms to regulate specific candidate genes during early stages of testis maturation (Schulz et al. 2010). Moreover, the detection of markers, which are expressed by sturgeon germ stem cells, is essential to develop new methods for purification and culture of germ stem cells *in vitro*. In addition to the importance of germ stem cells, they are also valuable cells with different applications in transgenesis, transplantation, and biotechnology.

During the past decade, gene expression studies using PCR- and immunochemistry-based methods and high-throughput gene expression profiling have provided valuable information about the key factors which are crucial for the biology and function of germ cells. Nevertheless, these studies have technical limitations. These techniques require a considerable amount of material from hundreds of thousands to millions of cells which may be limited, especially at early developmental stages or with small populations of a given cell type. Moreover, investigation of whole tissue results in the measurement of gene expression levels that are averaged over a cell population. Recent developments in low-input RNA extraction protocols have significantly reduced the minimum necessary material. High-throughput RNA sequencing technology is possible to investigate and measure a large number of genes in a low amount of cells at same time.

## Fish germ cell manipulations

As various fish germ cell markers were identified, a new perspective of biotechnologies has been processing connected with germline cells. Surrogate reproduction is one of the most useful technologies of GSC that produce donor-derived gametes in a recipient by transplanting germ cells of a donor into a recipient of a different strain or species. This technique is a complex strategy, involving amplification and cryopreservation of germ cells, preparation of sterile recipients, transplantation, and production of donor-derived offspring in recipients. Surrogate reproduction provides a rapid and low-cost alternative to enhance the propagation of high value and endangered fish species, such rainbow trout (Okutsu et al. 2007; Yoshizaki et al. 2010), carp (*Cyprinus carpio*, Franěk et al. 2019) and sturgeon (Pšenička et al. 2015; Ye et al. 2017; Octavera and Yoshizaki 2018).

To accomplish surrogate reproduction strategy, a continuous supply of large amounts of GSCs is required for the transplantation system. Instead of using fresh GSCs from individual donor fish, GSCs expanding by *in vitro* culture condition can overcome the limitation of GSCs in individual fish. Studies regarding sturgeon germ cell *in vitro* culture, isolation, and identification were performed in the present thesis (Chapter 3–5).

Isolation of fish germ cells by enzymatic digestion has been widely applied in past decades for both mammalian and teleost species (Bellve et al. 1977; Sakai 2002; Kossack et al. 2009; Kaul et al. 2012). This method is achieved by decapsulating and mechanically mincing the gonads followed with dissociation with enzymatic incubation. Several germ cell purification methods have been established to isolate homogenous populations such as centrifugal elutriation (Bellaïche et al. 2014), density gradient centrifugation (Lacerda et al. 2010; Yoshikawa et al. 2009; Wong et al. 2013), differential plating (Shikina et al. 2013), fluorescence-activated cell sorting (FACS) (Kise et al. 2012; Ichida et al. 2017), magnetic-activated cell sorting (MACS) (Ichida et al. 2019) and a combination of these methods (Panda et al. 2011; Lacerda et al. 2013). In chapter 2, available protocols and advances in enriching undifferentiated spermatogonia were introduced. *In vitro* culture of germ cells can be used as an approach to study cell proliferation, metabolism, and *ex vivo* interaction with hormones or growth factors. It would also expand the limited population of spermatogonia for genetic manipulation and expanding the potential use of the male germ cell lineage (Loir and Sourdaine 1994; Sakai 2002). Developments of fish germ cell culture in past decades have been summarized in Chapter 2.

## Sturgeon

### Introduction

Sturgeons belong to Osteichthyes, Actinopterygii, Chondrostei, which is the representation of an ancient Actinopterygian lineage, regarding as "living fossils" (Gardiner 1984). It is generally believed that there are 27 species of sturgeons and paddlefishes in Order Acipenseriformes. Compared with other fish, sturgeon are known as their large size and long lifecycle. Most species of sturgeon perform high similarity in morphological structure. The body is elongated with flat abdomen. Sturgeon also have flattened rostra, distinctive scutes and barbels, and elongated upper tail lobes. They are unique among bony fishes because their skeletons are almost entirely cartilaginous. These relatively primitive characteristics are generally considered to be the features of early fish evolution (Grande and Bemis 1996; Bemis and Kynard 1997; Billard and Lecointre 2000).

Sturgeon live in the rivers, estuaries, near-shore oceanic environments, and inland seas of the northern hemisphere (Birstein 1993; Bemis and Kynard 1997; Birstein et al. 2006). Most of sturgeon species migrate for reproduction and feeding. Most species of Acipenseriforms grow at sea and return to fresh water for breeding (Myers 1949; Bemis and Kynard 1997), such as European sturgeon (*Acipenser sturio*), white sturgeon (*A. transmontanus*) and Chinese sturgeon (*A. sinesis*). Some species live in freshwater in their whole life, such as sterlet (*A. ruthenus*) and Siberian sturgeon (*A. baerii*). Other species, such as Russian sturgeon (*A. gueldenstaedtii*) and Beluga (*Huso huso*), migrate between freshwater and brackish water (Bemis and Kynard et al. 1997).

To date, most species in Acipenseriformes are considered to be threatened or endangered (Billard and Lecointre 2000; Pikitch et al. 2005; Pourkazemi 2006; Carmona et al. 2009) according to IUCN 2020 (https://www.iucnredlist.org). Chinese Paddlefish (*Psephurus gladius*), one of the world's largest freshwater fishes, was declared extinct in 2020 (Zhang et al. 2020). Estimation of sturgeon resources is difficult that usually evaluate through their catch. Since the end of the 1970s, the sturgeon catch has decreased dramatically. By the mid-1990s, they collapsed to their lowest level (Bronzi et al. 2011). Overfishing, increasing global demand of caviar, water pollution, damming of rivers in Acipenseriformes habitat, deterioration of spawning habitat are believed to be the main factors leading to the rapid decline of the natural population of sturgeon (Billard and Lecointre 2000; Secor 2002; Parsley et al. 2002; Zhang et al. 2017; 2011).

### Reproduction biology of sturgeon

The reproduction of sturgeon possesses special characteristics. The life cycle of Acipenseriformes is generally quite long with late sexual maturity. The age of the sexual maturation of the natural population of most species needs to be more than eight years old. The individual of sexual maturity is different. Most females cannot lay eggs every year, which even ranges from 1 to 8 years. Sturgeons can release a large number of eggs, approximately 100,000 to 3 million. Besides, the maturing period and breeding interval in female sturgeon are longer than males (Grande and Bemis 1996). For example, Beluga can live for 100 years and exceed 1,000 kg in mass, and males need 12–16 years to attain sexual maturity of age, whereas females need 16–22 years (Billard and Lecointre 2000). Notably, compared with other fishes, sperm and egg of sturgeons have capability to survive long time *in vitro*, even for several hours. Under aquaculture conditions, cultured sturgeon reach sexual maturity earlier than natural populations due to the suitable aquaculture environment and adequate supply of

nutrients. For example, under natural conditions, sterlet, as the earliest maturity and smallest species of Acipenseridae family, need 3–5 years in male and 4–7 years of female to maturation. Cultured sterlet can be sexually mature in 3 years (Billard and Lecointre 2000). Due to the characteristics of late sexual maturity and long lifespan, sturgeon resources recover relatively slow. It is indispensable to explore the mechanism of gonad maturation. Large-scale artificial breeding and reproduction are essential ways to increase sturgeon resources. However, rearing sturgeon broodstock is costly and time-consuming. Therefore, it is indispensable to seek a new way to protect sturgeon, such as the isolation, freezing, preservation, and transplantation of germ cells. Surrogate reproduction shortens sexual maturity time, and speeding up its reproduction cycle also brings new ideas and ways to preserve sturgeon resources (Yoshizaki and Yazawa, 2019).

***Table 1.*** *Age, weight and total length at puberty, average time (years) between two spawnings and longevity in the wild sturgeon species.*

| Species | River | Age and size at puberty | | | | | | Years between two spawnings | | Longevity (years) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Years | | Total length (cm) | | kg | | | | | |
| | | Male | Female | Male | Female | Male | Female | Male | Female | Historic | Present |
| *A. sturio* | Gironde | 7–15 | 16–20 | 145 | 165 | 20 | 30 | | | 48 | |
| *H. dauricus* | Amur | 14–21 | 17–23 | | 230 | | | 3–4 | 4–5 | | 55 |
| *H. huso* | Volga | 14–16 | 19–22 | 160 | 200 | 30 | 60 | 3–4 | 5–6 | 107–118 | 60 |
| *P. kaufmanni* | Amu-Darya | 5–7 | 6–8 | | | | | | | | |
| *A. nudiventris* | Kura | 6–9 | 12–14 | 100–130 | 140–150 | 10 | 30 | | 2–3 | | |
| *A. sinesis* | Yangtze | 9–17 | 14–26 | | | 40 | 120 | | | | |
| *A. brevirostrum* | Georgia | 2–3 | 4–6 | 46–50 | | | | | 2–4 | | 67 |
| *A. fulvescens* | St. Lawrence | 15–17 | 20–24 | 85–95 | 90–120 | 4–5 | 4–9 | 2–3 | 4–6 | 152 | 40 |
| *A. gueldenstaedtii* | Volga/ Danube | 11–13 | 12–16 | 100 | 120 | 3 | 9 | 2–3 | 5 | ≥50 | 38 |
| *A. stellatus* | Volga | 7 | 9 | 105 | 120 | 3–4 | 9–10 | | 3–4 | 41 | 30 |
| *A. ruthenus* | Danube | 3–5 | 4–7 | 35 | 40–45 | | | 1 | 1–2 | 26 | 22 |
| *A. persicus* | Volga, Ural | 15 | 18 | 122 | 162 | 12 | 19 | 2–4 | 2–4 | 38 | |
| *A. baerii* | Ob, Yenissei | 11–13 | 17–18 | 75–80 | 85–90 | | | 1–4 | 3–6 | 60 | |
| | Farms | 3–4 | 7–8 | | | | | 1–3 | 2–4 | | |
| *A. oxyrinchus* | St. Lawrence | 22–24 | 27–28 | 165 | 190 | | | 1–5 | 3–5 | 60 | |
| *A. transmontanus* | Colombia | 12 | 16–35 | 120 | 150 | | | 3 | 2–11 | | 104 |

Data mostly from (Holcik 1986; Rochard et al. 1991, Billard and Lecointre 2000).

To date, Studies on sturgeon focus on gonadal transcriptome profiling between sexes or different gonadal development stages (Yue et al. 2015; Wang et al. 2017; Berbejillo et al. 2012; Hagihara et al. 2014). However, the characterization of germ cell subpopulations still lacks knowledge, especially germ stem cells. Thus, an efficient isolation method and useful tool to identify germ stem cells are still required.

The expression characteristics of fertility genes, such as *nanos1*, *dazl*, *dead end*, *piwi*, *vasa,* and *boule*, were observed in germ cells and gonads of different stages. The localization of Nanos1, Vasa and Dead end protein in gonad cells were detected, which is useful for identifying germ cell marker genes, screening and isolating germ cells (Ye et al. 2016; 2015; Yang et al. 2015; Ye et al. 2012; Linhartová et al. 2015). Nevertheless, it is unclear that there is a marker gene only express in sturgeon GSCs. What's more, the morphology and cellular and molecular mechanisms of sturgeon germ cell development have been unclear and have remained to be a question of broad interest.

On the other hand, visualizing and tracing PGCs were demonstrated by Saito et al. It confirmed that the PGCs of sturgeon (the hybrid offspring of Beluga and Sterlet) originated from the vegetal pole of fertilized eggs. Studies revealed that the mode of PGCs specification

in sturgeon is similar to that of anurans, but the migration pattern resembles that of teleosts (Saito et al. 2014). Later on, Saito and Psenicka. (2015) established a method to visualize sterlet PGCs *in vivo*, which is feasible by simply labeling the vegetal hemisphere with fluorescein isothiocyanate (FITC)-dextran. Pšenička (Pšenička et al. 2015) firstly established a germ cell transplantation technique for Siberian sturgeon (*A. baerii*) using sterlet as recipients and showed colonization of donor cells in the gonads of recipients. Ye et al. demonstrated that isolated germ cells from Chinese sturgeon could be colonized in Dabry's sturgeon (*A. dabryanus*) larvae (Ye et al. 2017). What's more, more manipulation of germ cell has also been established, such as cryopreservation (Pšenička et al. 2016) and gene editing techniques CRISPR/Cas (Baloch et al. 2019a,b). Surrogate reproduction technology is expected to be a powerful method to preserve and recover endangered sturgeon species.

Therefore, a sturgeon germ cell culture condition is essential to overcome the limitations of low germ cell numbers for cryopreservation and transplantation as well as to provide a platform investigating germ cell regulation. In Chapter 3, we tried to establish a sturgeon germ cell culture condition to support germ cell proliferation *in vitro*. Moreover, as mention in "Fish germ cell manipulations", a large amount of pure germ stem cells are expected to improve the success rate of surrogate reproduction. The traditional techniques, such as Ficoll and Percoll, offer lower resolution capacity since it could not avoid contamination by other germ cell types, Sertoli cells, peritubular myoid cells, and Leydig cells. This contamination with other cell types have been reported in rainbow trout (Sato et al. 2014), sturgeon (Pšenička et al. 2015) and catfish (Shang et al. 2015). To the best of our knowledge, FACS has not been widely utilized to isolate subpopulations of germ cells in fishes, especially commercial and endangered species which do not carry transgenes or lack of specific antibodies to recognize germ stem cells. Thus, in chapter 4, we applied FACS on sturgeon testicular cells expected to purify spermatogonia stem cells properly. What's more, as mentioned above, genes expressed in germ stem cells specifically has not been found yet. In chapter 5, we found sturgeon spermatogonial stem cells were able to identify in proteomic level, that some epitopes could be recognized by monoclonal antibodies.

**The aims of the thesis**

The overall aim of this thesis was to establish efficient methods with respect to identification, enrichment, and long-term culture condition of germ cells in sturgeon, in order to provide pure germ cells from donors for long term to complete a complex surrogate reproduction of sturgeon.

The specific objectives were to:

1) Optimize *in vitro* culture conditions of sturgeon germ cells for long term supply of surrogate production.
2) Isolate undifferentiated type A spermatogonia from different developmental stages of sterlet testes using fluorescence-activated cell sorting (FACS) based on light scatter properties.
3) Detect monoclonal antibodies and find suitable antibodies that can specifically recognize sterlet ASGs.

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## CHAPTER 2

# SPERMATOGONIAL STEM CELLS IN FISH: CHARACTERIZATION, ISOLATION, ENRICHMENT AND RECENT ADVANCES OF *IN VITRO* CULTURE SYSTEMS

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My share on this work was about 70%.

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*Review*

# Spermatogonial Stem Cells in Fish: Characterization, Isolation, Enrichment, and Recent Advances of In Vitro Culture Systems

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**Abstract:** Spermatogenesis is a continuous and dynamic developmental process, in which a single diploid spermatogonial stem cell (SSC) proliferates and differentiates to form a mature spermatozoon. Herein, we summarize the accumulated knowledge of SSCs and their distribution in the testes of teleosts. We also reviewed the primary endocrine and paracrine influence on spermatogonium self-renewal vs. differentiation in fish. To provide insight into techniques and research related to SSCs, we review available protocols and advances in enriching undifferentiated spermatogonia based on their unique physiochemical and biochemical properties, such as size, density, and differential expression of specific surface markers. We summarize in vitro germ cell culture conditions developed to maintain proliferation and survival of spermatogonia in selected fish species. In traditional culture systems, sera and feeder cells were considered to be essential for SSC self-renewal, in contrast to recently developed systems with well-defined media and growth factors to induce either SSC self-renewal or differentiation in long-term cultures. The establishment of a germ cell culture contributes to efficient SSC propagation in rare, endangered, or commercially cultured fish species for use in biotechnological manipulation, such as cryopreservation and transplantation. Finally, we discuss organ culture and three-dimensional models for in vitro investigation of fish spermatogenesis.

**Keywords:** spermatogonial stem cell (SSC); fish; spermatogenesis; florescence-activated cell sorting (FACS); magnetic-activated cell sorting (MACS); germ cell culture

## 1. Overview of Fish Germ Cell Biology

Spermatogenesis is a complex and orderly developmental process in which a single diploid spermatogonial stem cell (SSC) proliferates and differentiates to form mature spermatozoa. Spermatogenesis depends on the activity of the SSC, which can both self-renew to produce more stem cells or differentiate into daughter cells committed to spermatogenesis [1–5]. The proper balance between SSC self-renewal and differentiation is essential to assure the continuous homeostasis of spermatogenesis. The decision as to a SSC's self-renewal or differentiation is mediated by cell–cell communication, and in vitro germ cell culture provides a novel platform with which to investigate the regulatory network that determines cell fate. Furthermore, germ cell culture can be combined with gene editing techniques such as clustered regularly interspaced shortpalindromic repeats (CRISPR)/CRISPR-associated(Cas) for germ-line transmission, cell transplantation, nuclear transfer, and in vitro spermatogenesis [6–9]. In the following section, we will review fish spermatogonial cell morphology, distribution, identification, and niche, and the endocrine and paracrine regulation of

spermatogenesis. In the subsequent section, we will summarize the available protocols and advances in enriching undifferentiated spermatogonia according to their unique physiochemical and biochemical properties. Finally, we will review developments of traditional in vitro germ cell culture conditions to maintain proliferation and survival of spermatogonia in selected fish species, as well as organ culture and three-dimensional models for in vitro investigation of fish spermatogenesis.

### *1.1. Spermatogenesis—an Overview*

Spermatogenesis is a continuous and dynamic developmental process which can be divided into three phases: the mitotic, or spermatogonial, phase with the generation of spermatogonia; the meiotic phase with primary and secondary spermatocytes; and the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating into motile, flagellated, haploid spermatozoa.

In the spermatogonial phase, the primary increase in germ cell numbers occurs during successive rounds of mitotic duplication of the spermatogonia. The number of spermatogonial generations, and hence the number of mitotic divisions before differentiation into spermatocytes, varies among, but not within, species. There can be as few as two (humans) and as many as 14 (guppy) generations, but, most commonly, five to eight generations are reported [10]. In this phase, in all invertebrates and vertebrates, at the end of mitosis, incomplete cytokinesis occurs, and the two newly-generated spermatogonia remain connected by a cytoplasmic bridge, instead of forming individual cells (Figure 1A,B). However, the cytoplasmic bridge is not present in the descendants of an SSC that enters a self-renewal pathway in which two single, isolated daughter cells are generated. Therefore, cytoplasmic bridges are considered a sign of SSC differentiation and are present during all subsequent germ cell divisions (Figure 1B). All differentiated descendants of an SSC form clones connected by cytoplasmic bridges through which their developmental steps are synchronized (Figure 1A). These bridges are cleaved when spermatogenesis is complete, and germ cells leave the germinal epithelium as spermatozoa [11].

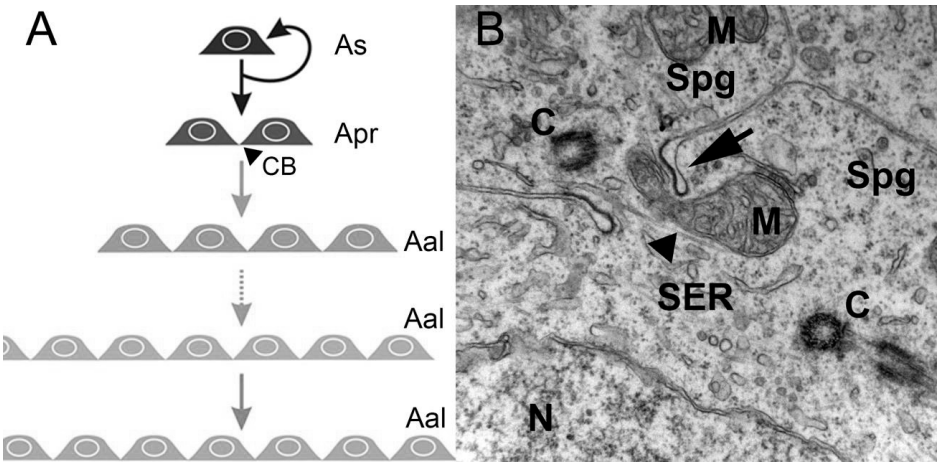


**Figure 1.** Mitotic/spermatogonial phase. (**A**) Mitosis produces spermatogonium clones. In rodents, type A single spermatogonia (As) harbor the spermatogonial stem cell population, which can either self-renew or generate two interconnected cells named type A-paired spermatogonia (Apr). The A-paired spermatogonia are interconnected by cytoplasmic bridge (CB) as a consequence of incomplete cytokinesis during cell division. Amplifying divisions beyond the A-paired also do not complete cytokinesis and continue to generate longer syncytial chains, termed A-aligned spermatogonia (Aal). (**B**). An electron micrograph showing a cytoplasmic bridge (arrow) connecting daughter cells resulting from spermatogonium division. The mitochondria (M), nucleus (N), smooth endoplasmic reticulum (SER),

centrioles (C), and microtubules (arrowhead) are depicted in the cytoplasms of two interconnected spermatogonia (Spg) (illustration and data: Nóbrega—unpublished observations).

During the meiotic phase, the spermatogonia differentiate into spermatocytes that go through two meiotic divisions characterized by reshuffling of the parental genetic material during the first division and the reduction to a haploid genome at the second division [10].

In the spermiogenic phase, the haploid spermatids emerging from meiosis differentiate into flagellated spermatozoa without further proliferation. The morphological changes in germ cells occurring during spermiogenesis involve reduction in cytoplasmic volume and organelles, maximum DNA condensation, and differentiation of the flagellum, and are similar among species. However, the final spermatozoon morphology can differ and sometimes provides taxonomic discrimination [10].

Following the general vertebrate scheme, the testes of fish are composed of the interstitial or intertubular compartment, and the germinal or tubular compartment, separated by a basement membrane [12]. The interstitial compartment contains the steroid-producing Leydig cells, blood/lymph vessels, macrophages, granulocytes, and connective tissue elements [13]. The peritubular myoid cells form a single layer of flattened cells surrounding the seminiferous tubules [14]. These cells are contractile and involved in the transport of spermatozoa and testicular fluid in the tubule [14]. The germinal compartment is composed of germ cells at various stages of development and their associated somatic Sertoli cells, which together form the germinal epithelium [12]. In the epithelium, germ cell survival and development depend on constant close contact with Sertoli cells [15]. Although many features are conserved in vertebrate spermatogenesis, the Sertoli/germ cell association differs among vertebrates. While anamniotes (fishes and amphibians) exhibit so-called cystic spermatogenesis, the amniotes (reptiles, birds, and mammals) present non-cystic spermatogenesis [12], in which the seminiferous epithelium can be divided into separate stages according to the cell associations observed in each tubular cross-section [16].

The germinal epithelium of amniote adult testes is composed of a fixed number of "immortal" Sertoli cells that support successive waves of spermatogenesis [15,16]. During these waves, a given Sertoli cell simultaneously supports several germ cell developmental stages (i.e., cells belonging to different germ cell clones). The Sertoli cell base may contact spermatogonia, with lateral segments contacting spermatocytes and early spermatids, and adluminal segments contacting late spermatids. In this type of spermatogenesis, Sertoli cells have been shown to be terminally differentiated in the testes, ceasing division during the early pre-pubertal phase, from approximately 10 and 20 days after birth in mice and rats, respectively [15,17]. However, recent studies have demonstrated that Sertoli cells from the transition region between the seminiferous tubules and the rete testis of adult testes remain undifferentiated for a longer period and are able to proliferate, although at a lower rate [17].

In anamniote vertebrates, the germinal epithelium is composed of spermatogenic cysts. The cyst as a morpho-functional unit is formed when a group of Sertoli cells envelope a single SSC [18]. As spermatogonia divide, the derived cells remain interconnected by cytoplasmic bridges [10,12,15]. Thus, the anaminiote Sertoli cell supports a single germ cell clone, while in amniote testes, depending on species, at least five germ cell clones at different stages of development are supported by a single Sertoli cell [10,12,15]. Sertoli cells from anaminiote testes are able to continuously proliferate, even after the onset of puberty [19].

The structural differences in the Sertoli/germ cell relationship of anamniotes and amniotes result in a less complex situation in fish than exists in mammals. Cystic spermatogenesis proceeds in a sequential manner, while, in non-cystic spermatogenesis, several processes occur simultaneously [15]. In vertebrates, the endocrine system has evolved as the master control system of spermatogenesis, and the somatic Sertoli, Leydig, and peritubular myoid cells became the primary targets for the major reproductive hormones, acting as paracrine relay stations for these signals in the testes [20,21].

### *1.2. Spermatogonial Stem Cell Niche*

Spermatogenesis relies on the activity of SSCs, which are capable of self-renewal to produce more stem cells or differentiation into daughter cells dedicated to spermatogenesis [1–5]. The balance between SSC self-renewal and differentiation is the basis of maintaining the homeostasis of spermatogenesis. If one process takes precedence over the other, testicular cancer (in case of self-renewal) or a depletion of spermatogenesis (in differentiation) is the outcome (Figure 2).

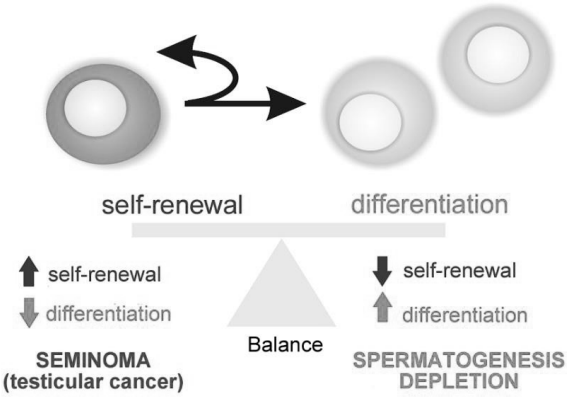


**Figure 2.** Balance between spermatogonial stem cell (SSC) self-renewal and differentiation. An imbalance results in testicular cancer or depletion of spermatogenesis.

The simultaneous process occurs in vertebrates showing continuous spermatogenesis, while, in seasonal breeding species, a switch from self-renewal to differentiation is observed as gonads begin to mature [10].

Spermatogonial stem cells are maintained in a specialized microenvironment in the testes known as the testicular niche (Figure 3) [2–4,22–25]. The niche provides growth factors and cell-to-cell interactions that regulate SSC activity in the testes: cell-cycle quiescence, maintenance of the undifferentiated state, proliferation via self-renewal, and apoptosis (Figure 3) [2–4].

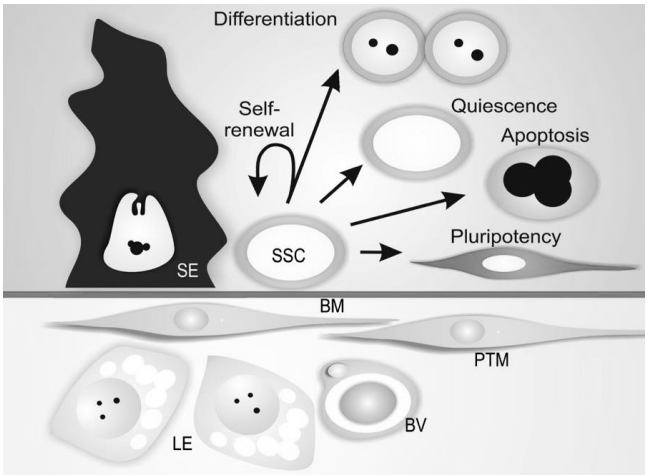


**Figure 3.** Mammalian SSC niche. Spermatogonial stem cells (SSC) reside along the basement membrane (BM) in proximity to interstitial Leydig cells (LE) and blood vessels (BV), and are in contact with Sertoli

cells (SE). In this microenvironment, physical and paracrine interactions regulate SSC self-renewal, differentiation, quiescence, apoptosis, and the ability to transform into different cell types (pluripotency). Peritubular myoid cells (PTM) are also depicted in the figure.

The niche can be defined as a microenvironment that maintains the undifferentiated state of a stem cell by preventing its differentiation, and is usually composed of (1) supporting cells; (2) stem cells; and (3) the surrounding extracellular matrix (Figure 3) [26–28]. In mammals, SSCs lie on the basement membrane of the seminiferous epithelium and are in contact with Sertoli cells, which control the fate of the SSCs via physical and paracrine interactions [4,29–31]. In addition to Sertoli cells, peritubular myoid cells and Leydig cells may contribute soluble growth factors to the niche environment [32–34]. Mammalian SSCs are preferentially located in those areas of the seminiferous tubules near the interstitial tissue where Leydig cells and blood vessels reside (Figure 3) [33,35–38]. Recent studies have shown that type A undifferentiated spermatogonia are uniformly distributed on the basement membrane of seminiferous mouse epithelium [39]. It has been demonstrated that SSC self-renewal and proliferation are intensified in areas of high fibroblast growth factor (FGF), which corresponds to vasculature-proximal and interstitium-proximal regions, and SSCs must be exposed to a sufficiently high level of FGF in order to maintain the self-renewal state [40].

In anamniote species, SSCs and their niche remain poorly investigated. In fish and other anamniotes, SSCs are single cells, not lying directly on the basement membrane, but completely enclosed by Sertoli cell cytoplasmic extensions [10]. Similarly to mammals, fish have SSCs that are considered type A undifferentiated spermatogonia ($A_{und}$) [1,10]. There is evidence for two sub-types of single, undifferentiated spermatogonia, type $A_{und*}$ and type $A_{und}$, in several fish species (Figure 4A–C), including ancient species, such as sterlet *Acipenser ruthenus* (Figure 4D–G) [10,22,41]. The $A_{und*}$ spermatogonia exhibit a large nucleus with little heterochromatin; a high volume of the cytoplasm; a convoluted nuclear envelope; evident nucleoli; and particularly relevantly, darkly staining material near invaginations of the convoluted nuclear envelope [10,41]. These patches are known as "nuage," material composed of ribonucleic acid (RNA) and RNA-processing proteins [10]. The $A_{und}$ spermatogonia show a smooth nuclear envelope, darker chromatin with abundant patches of heterochromatin distributed in the nucleus, and less nuage [10,41].

These cells also exhibit differences in the cell cycle, as shown by bromodeoxyuridine 5-bromo-2′-deoxyuridine (BrdU), a marker of S-phase in pulse-chase experiments [22]. Nóbrega and collaborators [22], using BrdU pulse-chase, reported that BrdU was rapidly diluted in type $A_{und}$ spermatogonia, while the percentage of BrdU-positive $A_{und*}$ spermatogonia remained constant. The authors [22] suggested that type $A_{und}$ constitute an "active" population with rapid proliferation and differentiation, as indicated by the more rapid loss of BrdU; $A_{und*}$ are the "reserve" population with slow self-renewal proliferation, as indicated by relatively stable BrdU labeling. Two types of single A spermatogonia in humans display similar characteristics: a "pale" type acting as reserve, and a "dark," active type [42,43]. In medaka *Oryzias latipes*, Nakamura and collaborators [44], using a BrdU pulse-chase experiment, also revealed distinct rapid and slow-dividing populations of oogonial stem cells.

With respect to whether spermatogonia display a preferential distribution within the fish testes, studies of zebrafish [22] and *Astyanax altiparane* [45] have shown that both $A_{und}$* and $A_{und}$ spermatogonia are located near the interstitial compartment, in contact with androgen-producing Leydig cells and blood vessels (Figure 4A–C). These observations suggest that the androgens; growth factors; and vascular supplies of oxygen, nutrients, and hormones may play essential roles in SSC maintenance and self-renewal vs. differentiation in the fish testis niche [10,21].

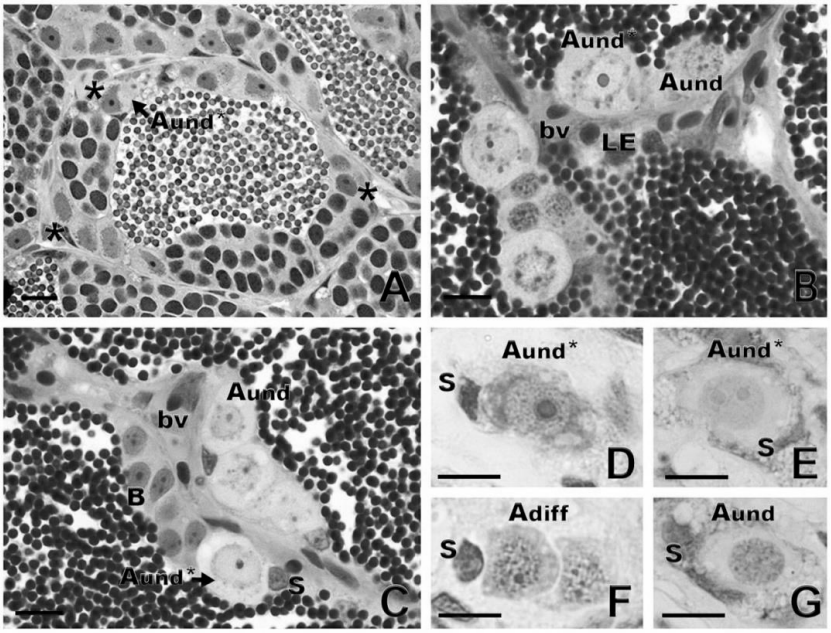


**Figure 4.** Spermatogonial niche and type A undifferentiated ($A_{und}$) spermatogonia. (**A**) Asterisks show the most undifferentiated type A spermatogonia ($A_{und*}$), preferentially located near the interstitial compartment (delimited in yellow) in zebrafish *Danio rerio*. (**B**,**C**) In common carp *Cyprinus carpio*, both $A_{und*}$ and $A_{und}$ are located near the interstitium, close to Leydig cells (LE) and blood vessels (bv). Sertoli cell (S) and type B spermatogonia are shown. (**D**–**G**): Generations of sterlet *Acipenser ruthenus* spermatogonia: $A_{und*}$ and $A_{und}$ and type A differentiated ($A_{diff}$) spermatogonia. Staining: periodic acid–Schiff (**A**,**E**,**G**) and toluidine blue (**B**,**C**,**D**,**F**). Scale bar: 10 μm.

Functional assays have been reported that consist of transplanting SSCs from a donor into a recipient testis in which endogenous spermatogenesis has been blocked [46]. Depending on self-renewal and differentiation capacities, transplanted spermatogonia are able to colonize the recipient testis and differentiate into functional gametes [46]. Studies have demonstrated donor-derived spermatogenesis following type $A_{und}$ spermatogonia transplantation into recipient testes in several species of fish [22,41,47–49]. Type A undifferentiated spermatogonia have also been shown to exhibit sexual plasticity and the ability to de-differentiate and differentiate into oocytes when transplanted into zebrafish ovaries [22]. In addition, $A_{und}$ spermatogonia transplanted into sexually undifferentiated larvae were able to differentiate into functional spermatozoa or eggs, depending on the genetic sex of the recipient [50,51]. Taken together, these findings provide evidence that SSCs represent a subset of type $A_{und}$ spermatogonia.

Although available information on fish SSCs has expanded in recent decades, markers for SSCs have been identified in only a few species [52], presenting limitations to detecting and isolating SSCs. Similar to the situation in mammals, in the past decade, spermatogonium transplant was the only means of assessing the "stemness" of putative SSCs in fish [24,48,53–55].

There is evidence that promyelocytic leukemia zinc finger protein (PLZF), a transcription repressor essential for the maintenance of mammalian SSCs [56], can be a marker of SSCs in fish, as demonstrated in the rohu *Labeo rohita* [57], zebrafish [58,59], dogfish *Scyliorhinus canicula* [60], rainbow trout *Oncorhynchus mykiss* [61], and several species of catfish [9,62,63]. In the neotropical catfish *Rhamdia quelen*, in situ hybridization showed that *plzf* is strongly expressed in type $A_{und}$ spermatogonia, but was also detected in type $A_{diff}$ spermatogonia, although at less intensity [63].

Glial cell-derived neurotrophic factor (GDNF) is a Sertoli cell growth factor involved in mammalian SSC maintenance [64]. The GDNF-binding receptor GDNF family receptor alpha1 (GFRα1) attaches to the membrane of SSCs, and is considered a SSC marker in several mammalian species [64–67]. Recently, GFRα1 has been detected in type $A_{und}$ of Nile tilapia *Oreochromis niloticus* [68], rainbow trout [69], dogfish [60], and common carp (Figure 5A). In rainbow trout, *gdnf* is expressed in germ cells from spermatogonia to spermatocytes but not in Sertoli cells, indicating that it is not secreted as an autocrine SSC niche factor in rainbow trout testes, unlike in mammals [69].

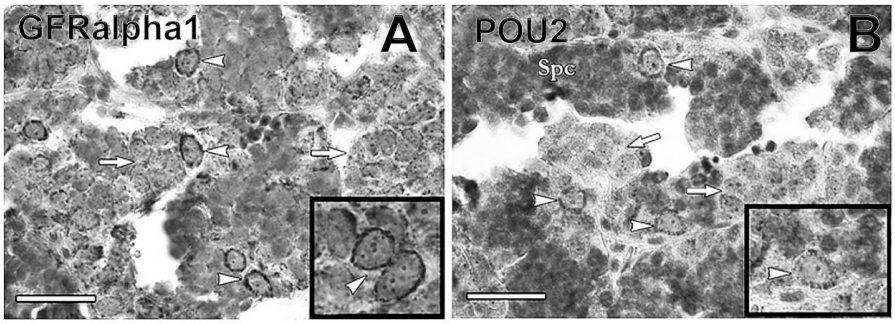


**Figure 5.** (**A**) Immunoreactivity of GFRα1 was found preferentially in type A undifferentiated spermatogonia (arrowheads) of common carp. In contrast, GFRα1 immunoreactivity decreased in differentiated spermatogonia (arrows). The inset shows GFRα1-positive undifferentiated spermatogonia (arrowheads). (**B**) POU2 was detected in common carp type A undifferentiated spermatogonia (arrowheads), whereas its immunoreactivity decreased in differentiated spermatogonia (arrows). Inset shows POU2-positive undifferentiated spermatogonia. Spc = spermatocyte. Scale bar: 25 μm.

Transcription factors NANOG and Pou5f3 (POU family/Oct4) are expressed in SSCs of mammals, and in some fish, including medaka, zebrafish, Nile tilapia, and rainbow trout [61,68,70–72]. Both NANOG and Pou5f3 play important roles in the maintenance and self-renewal of undifferentiated and pluripotent cells [73,74]. In medaka gametes, *nanog* is expressed only in spermatogonia, being absent in somatic cells of ovary and testis [72]. In common carp, POU2 was detected in $A_{und}$ spermatogonia, decreasing in expression as spermatogonia differentiated (Nobrega et al.—unpublished observations) (Figure 5B).

Recently, an approach to establishing markers for SSC in fish has been developed [75]. The method consisted of generating monoclonal antibodies (mAb) to cell-surface molecules of rainbow trout type $A_{und}$ spermatogonia by inoculating enriched live $A_{und}$ spermatogonia into mice and screening with a combination of cell enzyme-linked immunosorbent assay, live-cell staining, and flow cytometry (FCM) [75]. Among the obtained antibodies, two (numbers 80 and 95) were capable of specifically labelling $A_{und}$ spermatogonia of rainbow trout and zebrafish [75], while other antibodies (numbers 172 and 189) showed strong signals for type A spermatogonia and the oogonia of several species of salmonid [76]. By using these antibodies with fluorescence-activated cell sorting (FACS) [75] or magnetic-activated cell sorting (MACS) [77], it was possible to enrich $A_{und}$ spermatogonia and increase transplant success rate in selected teleosts. This method presents the potential to identify molecular markers of SSCs in fish, and the potential to isolate and enrich SSCs for downstream studies, such as single-cell RNA-seq or in vitro experiments. Table 1 presents the major SSC markers currently reported in fish.

**Table 1.** Spermatogonial stem cell markers in fish.

| Marker | Specification | Species | Reference |
|---|---|---|---|
| SGSA-1 | Spermatogonia specific-antigen-1 | Japanese eel | [78] |
| Notch1 | Notch homolog protein 1 | Rainbow trout | [79] |
| Pou5/2 (Oct-4) | POU domain, class 5/2 | Medaka<br>Common carp | [71]<br>Nobrega et al. unpublished observations |
| Ly75 (CD205) | Lymphocyte antigen 75 | Rainbow trout<br>Pacific bluefin tuna | [80]<br>[81] |
| PLZF | Promyelocytic leukemia zinc finger | Zebrafish<br>Carpa rohu<br>Dogfish<br>Rainbow trout<br>Catfish (several species) | [58]<br>[57]<br>[60]<br>[61]<br>[9,62,63] |
| GFRα1 | GDNF-family receptor α1 | Nile tilapia<br>Dogfish<br>Rainbow trout<br>Common carp | [68]<br>[60]<br>[69]<br>Nobrega et al. unpublished observations |
| NANOG | NANOG homeobox | Medaka<br>Nile tilapia | [72]<br>[68] |
| NANOS2 | NANOS homolog 2 | Medaka<br>Nile tilapia<br>Rainbow trout | [82]<br>[68]<br>[61] |
| NANOS3 | NANOS homolog 3 | Rainbow trout | [61] |
| Antibodies numbers 80 and 95 | Not identified | Rainbow trout<br>Also applied in<br>Zebrafish<br>Salmonids | [75]<br><br>[75]<br>[77] |

### *1.3. Endocrine and Paracrine Regulation*

In vertebrates, pituitary gonadotropin follicle stimulating hormone (FSH) and luteinizing hormone (LH) control testicular development, and function by regulating the activity of local signaling systems involving sex steroids and growth factors [83,84], small RNAs [85], and epigenetic switches [86].

In rodents, FSH can modulate the production of Sertoli cell growth factors that are relevant for SSC self-renewal or differentiation [38]. Among the growth factors, the GDNF secreted by Sertoli cells plays an important role in SSC self-renewal [64], while activin A and bone morphogenetic protein 4 (BMP4), also produced by Sertoli cells, promote differentiation [87]. Growth factors produced by Leydig and peritubular myoid cells can also modulate SSC self-renewal or differentiation [32]. For example, colony-stimulating factor 1 secreted by Leydig and some peritubular myoid cells [32] stimulates SSC self-renewal.

The gonadotropic hormones FSH and LH are important for testis development and spermatogenesis in fish [10]. Despite their similar roles in vertebrates, evolution has taken a different path for the gonadotropic hormones and their biological activities in teleost fish when compared to the other vertebrates. Leydig cells express not only the receptor for LH, typically seen in all vertebrates, but also the receptor for FSH [88–91]. Therefore, FSH can regulate Leydig cell functions, including stimulation of androgen [88–90] and production of growth factors acting on spermatogonial self-renewal and differentiation, such as Wnt5a [92] and insulin-like peptide 3 (Insl3) [93], respectively (Figure 6). Studies have reported elevated levels of circulating androgens and FSH coinciding with active spermatogonial proliferation in male Chinook salmon *Oncorhynchus tshawytscha*, and FSH stimulates spermatogonium proliferation in juvenile Japanese eel *Anguilla japonica* [88] as well as androgen production in several fish species [88–90,94]. Research in immature Japanese eels has shown FSH-induced spermatogenesis to be blocked by trilostane, a steroid hormone synthesis inhibitor, suggesting that FSH effects were mediated by androgens [88]. On the other hand, studies in zebrafish have demonstrated the impact of FSH on germ cell development independent of androgens [20]. This is supported by evidence that hundreds of testicular transcripts respond to FSH but not to sex steroids in the testes of zebrafish [95] and rainbow trout [96]. Most of these genes belong to cellular pathways known to regulate cell proliferation and differentiation, such as insulin-like growth factor (Igf3), Insl3, transforming-growth factor members (Tgf-β), Wnt, Notch, and Hedgehog signaling [95]. Since Sertoli

cells express FSH receptor [88–90], it is likely that Sertoli cells act as a paracrine relay station for FSH signal in the testes [15].

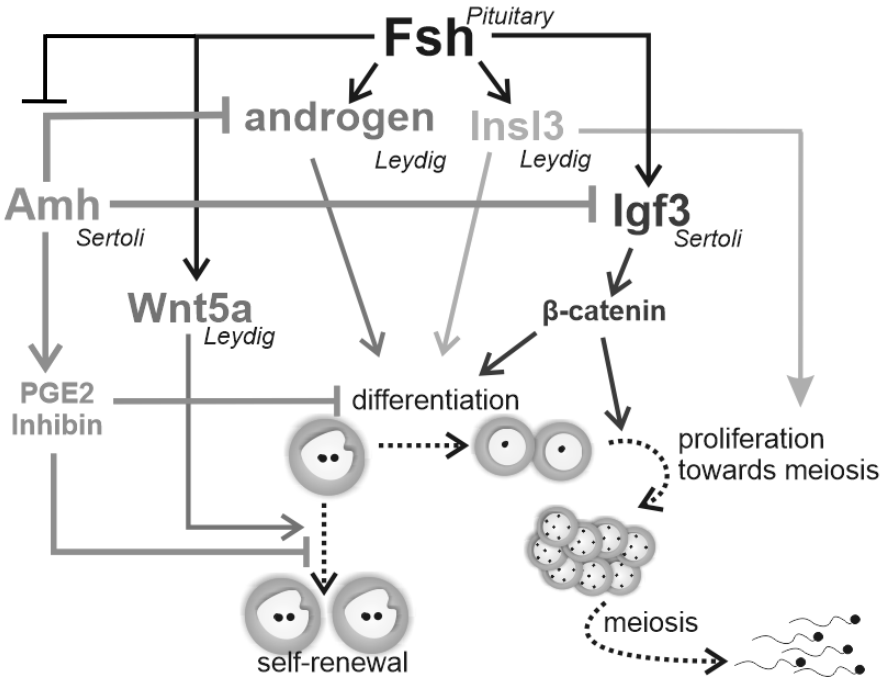


**Figure 6.** Schematic representation to summarize the regulation of zebrafish spermatogonial phase. Fsh exerts a central role in the zebrafish's spermatogonial phase by triggering and balancing steroid and growth factor production in testicular somatic cells (Leydig and Sertoli cells). In Leydig cells, Fsh stimulates the production of androgens and Insl3, which are both involved in the spermatogonial differentiation and proliferation. Moreover, Fsh promotes spermatogonial self-renewal by increasing Wnt5a release by Leydig cells. In Sertoli cells, Fsh increases Igf3, which in turn promotes differentiation and proliferation by activating β-catenin signaling in the germ cells. Fsh also down-regulates Amh, a member of the TGF-β (transforming growth factor-beta) superfamily produced by Sertoli cells. Amh, through PGE2 or inhibin, exerts an inhibitory role on spermatogonial self-renewal and germ cell differentiation in the zebrafish testes. To sustain its inhibitory role, Amh also decreases androgen and Igf3 production in Leydig and Sertoli cell, respectively.

Most of the accumulated knowledge regarding the role of growth factors in spermatogonial self-renewal vs. differentiation was derived from zebrafish studies (Figure 6) [20,21,89,95,97,98]. The Tgf-β Amh, expressed in Sertoli cells [97–99], has been shown to exert a role in adult teleost gonad development in both males and females, particularly at germ cell early stages [100]. In zebrafish and Japanese eels, Amh is characterized as a "spermatogenesis-preventing substance" [97,101]. Studies of zebrafish have demonstrated that Amh counteracts gonadotropin-induced effects on Leydig cell steroidogenesis [97], inhibits Sertoli cell pro-differentiation Igf3, and stimulates inhibitory factors such as inhibin-α and prostaglandin E2 (Figure 6) [98]. These data clearly demonstrate that Amh inhibits spermatogonium differentiation through modulation of growth factor production and suppression of Leydig cell function [97,98], maintaining spermatogonia in an undifferentiated state (Figure 6).

The other well-known studied growth factor, Igf3, has been shown to be expressed exclusively in the gonad tissue in several teleost species [21]. In the testes, Igf3 has been detected in Sertoli cells [20,102], as well as in undifferentiated and differentiated spermatogonia, spermatocytes, and spermatids of

tilapia [102]. There is evidence that FSH stimulates Igf3, which, in turn, promotes spermatogonial proliferation and differentiation in zebrafish testes (Figure 6) [20]. A recent study has shown that FSH-stimulated Igf3 release activates β-cateninc signaling in type A spermatogonia to stimulate their differentiation (Figure 6) [103].

Accumulated evidence in zebrafish demonstrates that FSH stimulates spermatogonial differentiation by down-regulating Amh and activating β-catenin signaling via Igf3. In addition, FSH promotes spermatogonial self-renewal through Wnt5a and the non-canonical Wnt pathways [21]. This balanced regulation could counteract depletion of $A_{und}$ spermatogonia while promoting spermatogonial differentiation (Figure 6).

## 2. Isolation and Enrichment of Germ Cells in Fish

### *2.1. Enzymatic Digestion*

Isolation of germ cells by enzymatic digestion has been widely applied in both mammalian and teleost species [104–107]. The technique involves decapsulating and mechanically mincing the gonads, followed by dissociation via incubation with enzymes. Combinations of collagenase, trypsin-EDTA, dispase, and DNase I are commonly used.

In fish, trypsin and collagenase are the most common enzymes employed in gonad dissociation [108–112]. Okutsu et al. [51] reported use of 0.5% trypsin to digest testes of rainbow trout, and the obtained spermatogonia produced viable offspring when transplanted into masu salmon *Oncorhynchus masou* larvae. Lacerda et al. [48,79] dissociated testes of tilapia using collagenase, trypsin, and DNAse I. For ovaries, enzymatic dissociation has been performed with collagenase in rainbow trout [113] and zebrafish [114].

Variables to be considered for enzymatic dissociation include species, sex, enzyme selection and concentration [115], and exposure time [111]. Insufficient digestion may cause low yield of single cells, while over-digestion may lead to cell damage. Disruption of epitopes from the cell membrane surface, which may impair germ cell function, has been observed during trypsin digestion [76,116,117]. Dispase is reported to be a relatively mild enzyme with which to minimize damaged effects on cells [118]. In sterlet, trypsin appeared to be more effective at dissociating germ cells from gonadal tissue when compared to collagenase [111]. More studies to address the effects of trypsin on fish germ cells are needed.

### *2.2. Germ Cell Purification*

Enrichment and purification are required to counteract gonad cell heterogeneity and low quantities of spermatogonia/oogonia. Cell isolation techniques are used to separate cell populations with respect to physiochemical and biochemical properties, including size, density, electrostatic characteristics, and differential expressions of specific cell surface markers [119]. Germ cell purification methods have been established to isolate homogenous populations using centrifugal elutriation [120], density gradient centrifugation [48,121,122], differential plating [123], FACS [124,125], MACS [76], and combinations of those methods [57,68].

#### 2.2.1. Density Gradient Centrifugation

Discontinuous density gradients, such as Percoll and Ficoll, to separate cell populations based on density, are commonly used for germ cell purification, and are appropriate for spermatogonia of several fish species [10,126]. This technique has been widely applied to enrich spermatogonia before culture or transplant in mice [48], sheep [127], primates [128], and humans [129].

In Nile-tilapia, Lacerda et al. utilized Percoll to isolate the most immature spermatogonia before transplantation [48,79]. In loach *Misgurnus anguillicaudatus* [121], 60% of type A and early type B spermatogonia were collected in the 30–36% Percoll fractions, while late type B spermatogonia, spermatocytes, and spermatids were distributed in the 40% fraction. Wong et al. found the majority

of zebrafish spermatogonia in the 25% and 30% Percoll fractions [122]. Percoll is currently the most commonly used method to isolate and purify fish spermatogonia [112,130,131]. However, the technique has low resolution capacity, since it may not distinguish spermatogonia from Sertoli cells, peritubular myoid cells, and Leydig cells. This contamination has been reported in rainbow trout [132], sterlets [111], channel catfish *Ictalurus punctatus,* and blue catfish *Ictalurus furcatus* [62].

In contrast to what is seen during spermatogenesis, germ cells increase in size during oogenesis, with oogonia being slightly smaller than early-stage primary oocytes [133]. The ovary also contains a larger quantity of somatic cells. Since oogonia exhibit an intermediate size among ovarian germ cells, their isolation is more challenging compared to that of spermatogonia. Wong et al. [114] detected germ cell distribution in Percoll fractions in transgenic zebrafish (*vasa:DsRed2-vasa*). The majority of ovarian germ cells were collected in the 25–35% Percoll interface, which provided approximately 20-fold enrichment of the initial cell suspension. The enriched cells were able to colonize the transplant recipient gonad and produce offspring. Wong et al. [122,134] also showed that Percoll-enriched oogonia were able to proliferate in vitro.

#### 2.2.2. Differential Plating

Differential plating is a classic method in cell culture based on the adhesive properties of the cells. Unlike somatic cells, SSCs will adhere to laminin or feeder cells rather than directly to a culture plate in in vitro conditions [135]. Spermatogonial stem cells are more likely to adhere to a feeder layer after somatic cell attachment to culture plates. Based on this attachment, differential plating can eliminate the somatic cells. However, it is possible to lose a substantial quantity of adherent germ cells if they are harvested at an inappropriate time. Currently, differential plating is usually combined with other separation methods.

As an example in mammals, based on the velocity of sedimentation and differential attachment, cell populations containing up to 70% swine SSCs were obtained, 80% of which were viable [136]. Differential plating applied in cattle can eliminate most of the somatic cells in culture [137]. The purity of rainbow trout spermatogonia can reach >95% using differential plating [110,115]. Lacerda et al. [68,79] combined Percoll and differential plating to enrich tilapia spermatogonia. Although differential plating is an effective method of obtaining high purity in germ cells without causing damage, enrichment usually takes 5–7 days, and cell properties might change during the in vitro culture period [110].

#### 2.2.3. Flow Cytometric and Magnetically-Activated Cell-Sorting

Flow cytometry, as well as FACS, has been employed for cell sorting for more than four decades [138]. Fluorescence-activated cell sorting is a rapid and quantitative technique with which to examine individual cells using a range of fluorescent and light scattering signals related to cell size, shape, granularity, surface, intracellular protein, and gene expression. When a heterogeneous group of cells is passed through a laser system, characteristics such as morphology, viability, and surface markers indicated by light scattering and fluorescence are captured and analyzed, and cells with defined signals can be collected [139–141]. For some transgenic fish, target cells can be sorted by FACS according to their self-fluorescence properties. In some endangered and cultured species in which transgenic techniques are not suitable or available, high resolution immunoaffinity techniques are generally conducted, based on the number and type of molecules present on the cell surface that can be targeted by specific monoclonal antibodies conjugated with florescence or magnetic microbeads.

In mammals, FACS has been used for sorting SSCs from mouse testicular cell suspension [142]. Spermatogonial germ cells are highly enriched with characteristics such as α6-integrin positivity, c-kit expression, low side scatter, and negative or low av-integrin expression [142]. Other SSC surface markers that have been identified and used to sort and enrich mammalian SSCs include CD9, ITGB1, ITGA6, GFRA1, EPCAM, NCAM1, THY1, CDH1, SSEA-4, and MCAM [143–147]. Recently, FACS has been employed for separating spermatogonia from cancer cells in monkey testis [148], indicating a potential application in cell therapy.

In tilapia, cell populations differentially stained with propidium iodide and carboxyfluorescein succinimidyl ester were identified and quantified by FACS [149]. However, there are few studies of germ stem cell surface markers. Nagasawa and colleagues [80,81,150] identified lymphocyte antigen 75 (Ly75/CD205) as a surface marker of primordial germ cells and mitotic germ cells in rainbow trout and Pacific bluefin tuna *Thunnus orientalis*. However, there is still no evidence that Ly75/CD205 is capable of labeling and fractionating live type A spermatogonia. To isolate and enrich type A spermatogonia, Yano et al. used pvasa-GFP transgenic rainbow trout and sorted cells based on diameter and green fluorescent protein (GFP) intensity [54,151]. Further study has shown that physiochemical characteristics such as high forward scatter and low side scatter could be used for sorting type A spermatogonia from GFP-transgenic rainbow trout [124]. These studies showed the possibility that spermatogonia can be dramatically enriched in a population of large cells with a simple intracellular structure. Subsequently, Ichida et al. [125] established a method of purifying type A spermatogonia of immature, maturing, and spermiogenic testes of Pacific bluefin tuna according to light scattering properties using FCM. In this study, spermatogonia were enriched 15-fold compared to the unsorted cell fraction. These findings indicate that light scattering properties are applicable to enriching type A spermatogonia without cell-labeling systems such as transgenes and cell surface antibodies. Monoclonal antibodies (numbers 172 and 189) have been produced by inoculating Pacific bluefin tuna-enriched live type A spermatogonia into mice, and screened using cell-based enzyme-linked immunosorbent assay (ELISA), immunocytochemistry, FCM, and immunohistochemistry [77]. These antibodies were able to recognize cell surface antigens of Pacific bluefin tuna type A spermatogonia and be used to identify live spermatogonia in a recipient following transplanattion when conjugated with fluorescent dye [77]. This represents an important advantage in applications to commercially valuable or endangered species that lack transgenic strains or specific molecular markers for identifying cell lineages.

Flow cytometry is an automated, multiparametric, and sophisticated sorting tool requiring skill and costly equipment, and it is not affordable for many laboratories. Moreover, it can require cell labelling with fluorescent antibodies, which might alter cells and their functions.

In contrast, MACS does not require a flow cytometer and can be performed in a short period of time through relatively simple methodology. It uses magnetic beads that bind specifically to target cells with mAbs [152]. The dissociated testicular or ovarian cell suspension is incubated with magnetic nanoparticles that are directly or indirectly conjugated with mAbs against a particular surface antigen. Once bound to the intended target, beads are subjected to magnetic forces that allow immobilization of the bound cell type and concurrent separation from other components in the suspension. Additional washing and elution steps complete the purification cycle, resulting in an enriched preparation of a specific cell type [152]. This technique can also be used for negative selection to eliminate undesired cells. Compared with methods requiring FCM, MACS is a simple technique and requires no special equipment except the magnetic beads and a magnet stand. The technique is effective for large quantities of cells and more rapid than FCM at collecting a high number of cells [113].

Magnetically-activated cell sorting is commonly used in mammalian germ cell research [153–156], with several surface markers employed to differentially sort spermatogonia. The c-Kit protein has been used to separate types of spermatogonia in hamsters, mice, and monkeys [157]; GFRα1 [158], α6-integrin [159,160], CD9 [161,162], and Thy-1 [153,163,164] have been used to isolate SSCs. Most of these surface markers have also been applied to sorting fish spermatogonia. Panda et al. [57] enriched highly pure type A spermatogonia from carp testis using Thy1.2 (CD90.2) antibody from mouse. The same marker has been employed in channel catfish *I. punctatus* and blue catfish *I. furcatus* spermatogonia [9].

It is believed that cell surface markers should exhibit low species-specificity, but mammalian antibodies are not always suitable for work with fish spermatogonia. For this reason, Yoshizaki and colleagues produced cell surface mAbs by inoculating enriched and live type A spermatogonia from vasa::GFP rainbow trout into mice [75]. Using these antibodies, it was possible to isolate

undifferentiated germ cells from vasa::GFP rainbow trout with MACS [75]. The MACS-enriched cells showed a strong GFP signal and significantly higher transplantability than the unsorted cells, especially the ovarian cells [76]. However, like in FACS, antibodies bound to the cell surface may inhibit some cell functions, since cell surface proteins or sugar chains could be masked by the antibodies [77]. In addition, the surface markers/antibodies developed for MACS may not be effective for all species. It is also lower resolution than FACS, since some antibodies identified by FACS are not recognized by MACS [147]. Therefore, more studies regarding antibody identification in fish are required.

### *2.3. Future Trends*

Recently, microbeads and nanobeads have been used to isolate human stem cells and cancer cells [9,165] and provide new possibilities for sorting fish germ stem cells. Microbeads and nanobeads enable rapid binding and short labeling procedures [9,165]. Due to their small sizes, these particles do not saturate cell epitopes and do not interfere with downstream applications. Other novel separation methods in the stem cell field, such as aptamer-based separation [166] and affinity chromatography, can be considered potential tools for sorting germ stem cells.

## 3. Germ Cell Cultures

### *3.1. Serum in Germ Cell Cultures*

Serum is used to supply essential nutrients for in vitro cell growth [167]. Serum is a mixture containing plasma proteins, polypeptides, growth factors, hormones, and binding proteins along with contact and extension factors that protect cells from damage when they adhere to culture plates [168]. It may also contain unknown components that affect cell growth. The most commonly used serum in cell culture is fetal bovine serum (FBS). Commercial media, such as Leibovitz's L-15 medium and Dulbecco's modified eagle's medium, supplemented with FBS, have become widespread in vertebrate cell culture. In early fish germ cell culture, FBS was used to support germ cell proliferation [167]. There is evidence that rainbow trout type A spermatogonia proliferate in culture conditions that include a high FBS concentration in the medium, but propagation eventually ceases due to the overgrowth of somatic cells [115]. Because rainbow trout type A spermatogonia have an extremely slow cell cycle, somatic cells gradually occupy space and nutrition in the culture, especially at higher serum concentrations [115]. In mice, FBS concentrations of 0.3% to 2% in media allow initial somatic cell attachment and proliferation on flasks and subsequent SSC attachment to somatic cells with colony formation after 5 to 7 days of culture [115]. However, when a higher concentration (5–15%) of serum was used, SSCs propagated and formed colonies, but eventually ceased growth and detached, due to the extensive growth of fibroblasts [169]. Thus, high serum concentrations may stimulate germ cell proliferation, while simultaneously causing extensive growth of somatic cells, inhibiting continued germ cell proliferation. Reports have shown that germ cells cultured with FBS were not able to achieve long-term proliferation and maintenance of original characteristics in zebrafish testicular cell culture [105,170]. McClusky [171] reported that FBS simultaneously stimulated both in vitro apoptosis and [3H]thymidine incorporation in immature spermatogonia in a concentration-dependent manner. Fetal bovine serum contains a wide range of both inhibitory and stimulatory factors [172,173]. It is possible that germ cells and/or their associated Sertoli cells are responsive to both inhibitory and stimulatory signals, resulting conflicting signals, and ultimately, cell death.

Bone morphogenic protein 4 (BMP-4), a stimulatory factor present in FBS [174], can induce differentiation in cultures of zebrafish [175] and mouse spermatogonia [87,176,177]. To overcome this, substitutes for serum are being explored. A serum-free culture of mouse SSCs was first developed by Kutoba [163]. Mouse SSCs could maintain proliferation more than six months on minimum essential medium-alpha supplemented with 0.2% bovine serum albumin (BSA). Recently, Aoshima et al. demonstrated that "knockout serum replacement" medium maintained long-term growth of SSCs in the absence of BSA and serum [178]. In fish, to suppress the overgrowth of testicular somatic

cells, Shikina and Yoshizaki replaced FBS with soluble factors, such as BSA, adenosine, and salmonid serum [110]. The new culture extended the duration of type A spermatogonia culture, maintaining their original morphology and GFP intensity similarly to that in *vas::GFP* trout spermatogonia, without overgrowth of somatic cells [110]. In zebrafish, spermatogonia were reported to show effective propagation for up to three months in medium with 3% FBS [179]. Wong et al. established a female germline stem cell (FGSC) culture based on a serum-supplemented, proprietary, StemPro-34-based (Gibco) medium, which contained the original StemPro-34 supplement plus 16 individual compounds along with basic nutrients, including 1% FBS [122]. The proliferation of FGSCs continued for more than 6 weeks in vitro with unchanged germ cell markers, and cells generated normal offspring after transplantation. Multiple germ cell cultures with decreased, or no, FBS for long-term maintenance have been reported [60,122,134]. Research should focus on development of a well-defined medium for mixed cell populations that inhibits growth of nontarget cells while stimulating proliferation of target cells.

### *3.2. Feeder Cells and Growth Factors*

Fish germ stem cell survival, self-renewal, and differentiation are regulated by a combination of intrinsic and extrinsic factors [10,97,180]. Germ cells are capable of autonomous control of the differentiation pattern, and somatic cells, especially Sertoli cells, support germ cell development and provide growth factors that modulate germ cell proliferation and fate. The growth factors released by Sertoli cells are required for germ stem cell proliferation and differentiation [181], as well as initiation of meiosis [182].

In 1994, Loir [183] cultured rainbow trout spermatogonia with L-15 supplemented with Ultroser$^{TM}$ serum substitute medium and 5% rainbow trout serum. In the presence of Sertoli cells, spermatogonia survived for two weeks [183]. A co-culture system of germ cells and somatic cells was established for the Japanese eel in 1996 [184], in which the aggregated cells undergo spermatogenesis in the presence of 11-Ketotestosterone. In 2002, using testis-derived tumor-like cells, designated ZtA6—expressing *sox9a* and phagocytotic activity (Sertoli cell features)—as feeder cells, Sakai et al. reported that male zebrafish germ cells went through mitosis and meiosis and developed into functional spermatozoa in vitro [105]. Prolongation of vasa expression in the culture suggested that ZtA6 cells promoted survival and propagation of spermatogonia [105]. In addition, ZtA6 cells contributed to the attachment and survival of spermatogonia at the beginning of culturing [105]. The formation of flagellated spermatozoa was observed on day nine of culture, and clusters of spermatogonia disappeared on day 20. In this culture system, all types of germ cells, including type A and B spermatogonia and primary and secondary spermatocytes were cultured, precluding accurate identification of the type of germ cell stimulated by the feeder cells. Feeder cells were heterogeneous, suggesting that somatic cells other than Sertoli cells could be responsible for germ cell proliferation and spermatogenesis. Kurita and Sakai further isolated and established two Sertoli cell lines (ZtA6-2 and ZtA6-12) from testis-derived tumor-like ZtA6 cells [185]. The ZtA6-2 cell line was able to support germ cell growth, and ZtA6-12 promoted germ cell differentiation into spermatozoa [185]. The feeder cells were successfully employed in production of spermatozoa from cultured spermatogonia [170]. The transgenic spermatozoa were differentiated from premeiotic germ cells which were transfected with a pseudotype retrovirus in vitro. In medaka, primary culture of testicular cells has shown formation of spermatozoa after 24 h [186]. The culture generated both adherent and suspended cells. Proliferating cells and differentiation of spermatocytes into spermatids or spermatozoa were observed mainly among the suspended cells. Interestingly, the primary cells showed a dynamic equilibrium between adherent and suspended cells and could not be separated into their respective cell populations. This study suggested that medaka germ cells are more likely to spontaneously initiate spermatogenesis than to remain undifferentiated [186].

A normal spermatogonial cell line (SG3) derived from a mature medaka testis was established in 2004 [108]. After 140 passages, the SG3 cell line remained stable with respect to proliferation, karyotype, and phenotype when cultured with basic fibroblast growth factor (bFGF) and medaka

embryo extract. The SG3 cell line expressed spermatogonial stem cell genes rather than Sertoli cell markers. In this culture system, spermatogonial cells proliferated in the absence of feeder cells, but ceased rapid growth upon depletion of growth factors in the culture medium. The SG3 cell line was observed to undergo meiosis and spermiogenesis to generate motile spermatozoa in vitro [108]. Similar to a previous study [186], spermatogenesis occurred in suspended aggregated germ cells. Spermatogenesis was automatically induced by high cell confluence without subculture. It has been suggested that bFGF may inhibit differentiation or promote self-renewal in medaka [108], indicating that, although spermatogonia exhibit potential for continuous proliferation, their long-term culturing depends strongly on culture conditions.

In mammalian cultures, various feeder layers have been tested. The effects of the Sandos inbred line, mouse embryo-derived, thioguanine-resistant, ouabain resistant cells; mouse embryonic fibroblasts; and mouse testicular stromal cells on SSC proliferation and maintenance differ between species [87,187,188]. All these substances interfere with stability of feeder cells and make the culture process more difficult to control. Mouse SSCs have been successfully cultivated without feeder cells on a laminin-coated plate in the presence of GDNF [169].

As germ-cell xenotransplantation has been established for rainbow trout [55,189,190], the high demand for germ stem cells requires an in vitro culture system to propagate and maintain spermatogonia for long periods before transplant. Using immature rainbow trout, Shikina and Yoshizaki reported optimized spermatogonium survival and proliferation in type A undifferentiated spermatogonia with culture in L-15 supplemented with 10% FBS [115]. Spermatogonial proliferation was maintained for more than one month in vitro by addition of insulin, trout embryonic extract, and bFGF to the culture medium [115]. However, the overgrowth of testicular somatic cells restricted proliferation of spermatogonia after one month of culture. The cultured spermatogonia colonized and proliferated in the recipient gonads following transplantation, although at a lower rate than observed for fresh disassociated cells [115].

To further suppress the overgrowth of testicular somatic cells and enhance the survival, proliferation, and transplantability of spermatogonia, Shikina and Yoshizaki replaced FBS with adenosine and salmonid serum [110]. These conditions enhanced the transplantability of cultured spermatogonia, indicating that adenosine and salmonid serum were able to stimulate SSC survival and self-renewal in vitro.

The development of culture media has increasingly been focused on improving germ cell expansion while maintaining "stemness." Initial studies expressed uncertainty as to whether germ cells in clumps grow during in vitro culturing [105,108,110]. Several studies observed spermatogenesis after clump formation, suggesting that differentiation may have occurred [108,186]. However, SSC proliferation may also take the form of clumps. In *L. rohita*, enriched SSCs sorted by MACS with Thy 1+ were capable of proliferation and remaining undifferentiated for more than 60 days [57]. In this feeder-free culture, cells proliferated to form colonies with unique morphological compactness features. The induction of proliferation was not observed in the presence of either mouse or human recombinant GDNF in the culture medium [57]. In zebrafish, most clumps of spermatogonia were positive for BrdU, indicating active proliferation [179]. Lacerda [68] showed that Nile tilapia spermatogonia remained undifferentiated and able to proliferate for at least one month of culture. When purified spermatogonia were kept at high confluence without subculture, large colonies expressing *vasa*, *sox2*, and the *gfra1* were generated, without expression of meiotic marker (*dmc1*). Thus, it could be assumed that clumps were likely derived from a few aggregated SSCs and expanded during proliferation [68].

Spontaneous germ cell differentiation has been observed, and probably can be attributed to cell-cell communication [2–4]. Fish embryo stem cells and spermatogonia have been demonstrated to differentiate in vitro via enhanced cell–cell interactions [191]. Nevertheless, it is possible that, in contrast to in vivo, the surface properties of the cells may change in vitro and the adhesiveness reflect specific developmental and/or cell cycle phases.

In 2012, Kawasaki et al. showed that zebrafish spermatogonia proliferate continually for longer than one month in a culture medium supplemented with a cocktail of recombinant mammalian growth factors, including GDNF, IGF-1, and bFGF. The cultured spermatogonia produced functional spermatozoa after transplantation [179]. In contrast to previous studies in which GDNF showed no effect on germ cell proliferation [57], in the zebrafish culture, Kawasaki and collaborators showed that recombinant human GDNF enhanced spermatogonial proliferation [179]. However, compared to mouse SSC culture, a 5 to 10-fold concentration of GDNF was needed to promote proliferation of zebrafish spermatogonia [163,192]. In 2013, Wong et al. further studied the role of homologous growth factors [122] in homogeneous cultures of zebrafish FGSCs. The FGSCs remained undifferentiated for more than six weeks in vitro and showed high transplantability [122]. The FGSC proliferation was enhanced when cultured on ovarian-somatic feeder cells (OFCs) engineered to express zebrafish leukemia inhibitory factor (LIF), bFGF, and GDNF in a serum-free culture [122]. These conditions were also applied to a zebrafish SSC culture [134,175] with the opposite effect observed: the number and proliferation of spermatogonia decreased, and differentiation was stimulated after three weeks [175]. The OFCs used in this culture were assumed to express Bmp and induce spermatogonium differentiation, since the expression of Bmp was increased in the latter part of the culture period. When blocking Bmp signaling with the specific inhibitor dorsomorphin, spermatogonium growth could be prolonged to 6 weeks. Thus, the effect of Bmp was shown to differ in culture of zebrafish male and female germline stem cells.

In culture of dogfish spermatogonia, GDNF has been shown to induce spermatogonium proliferation by reducing apoptosis, and cells remained pluripotent and capable of self-renewing for more than five months [60]. Similarly, recombinant medaka GDNFa/b enhanced the proliferative activity of SG3 cells, which retained the spermatogonial gene expression pattern and showed alkaline phosphatase activity [193]. There is also evidence that a high concentration of recombinant human GDNF and LIF in culture medium was responsible for maintaining spermatogonia of sturgeon in vitro [194]. Satoh et al. examined the in vitro effects of baculovirus-produced recombinant medaka LIF proteins on medaka spermatogenesis [195], and found that LIF protein in the culture medium and in co-culture with LIF-overexpressing medaka testicular somatic cells promoted spermatogonial proliferation [195]. The influences of physiological and biochemical factors on germ cell culture have been investigated [196,197]. Kawasaki et al. found high oxygen (20%) concentration to stimulate spermatogenesis, while heparin contributed to propagation of spermatogonia [196].

In summary, supplementation of culture media with appropriate soluble growth factors is essential for germ cell survival and proliferation for long-term incubation. However, in co-culture with somatic cells, germ cells are likely to attach to somatic cells, form clumps, expand in number, and initiate spermatogenesis. This indicates that germ cell in vitro activity may occur partly through intercellular junctions and not only through paracrine signals from Sertoli cells. The germ cell attaches to feeder cells prior to initiating proliferation. Some studies have demonstrated that Sertoli cell-germ cell and germ cell-germ cell adhesive and gap junctions are involved in Sertoli cell functions to support germ cell development within the cyst [198,199]. Thus, the role of intercellular junctions on germ cell development, and whether there is synergism/antagonism between soluble growth factors and the cell junctions need to be investigated.

### *3.3. Organ Cultures*

In general, cell culture systems are not suitable for evaluating complex signal pathways among cells and the extracellular matrix [200]. In a germ cell culture, it is difficult to control the re-association of somatic and germ cells, and feeder cell layers may be required. In order to study gametogenesis and its endocrine and paracrine control ex vivo, an effective organ culture system that allows germ cells to maintain their spatial arrangement and their normal cellular and microenvironmental composition when grown in vitro must be established. Organ culture must also maintain the integrity of the complex regulation existing in the gonads.

The effort to develop a testis tissue culture system began in the 1960s in mammals [201]. Trowell [202] developed an organ culture system, and Steinberger et al. [203] adapted it to rats. It is a method in which tissue fragments are placed on a thin layer of agarose covering a perforated metal grid (Figure 7A). Calf serum (10%) was added to maintain the viability of the seminiferous epithelium [203]. This agarose gel organ culture became a reliable standard method to study in vitro spermatogenesis in mammalian species including rabbits and humans [204–206].

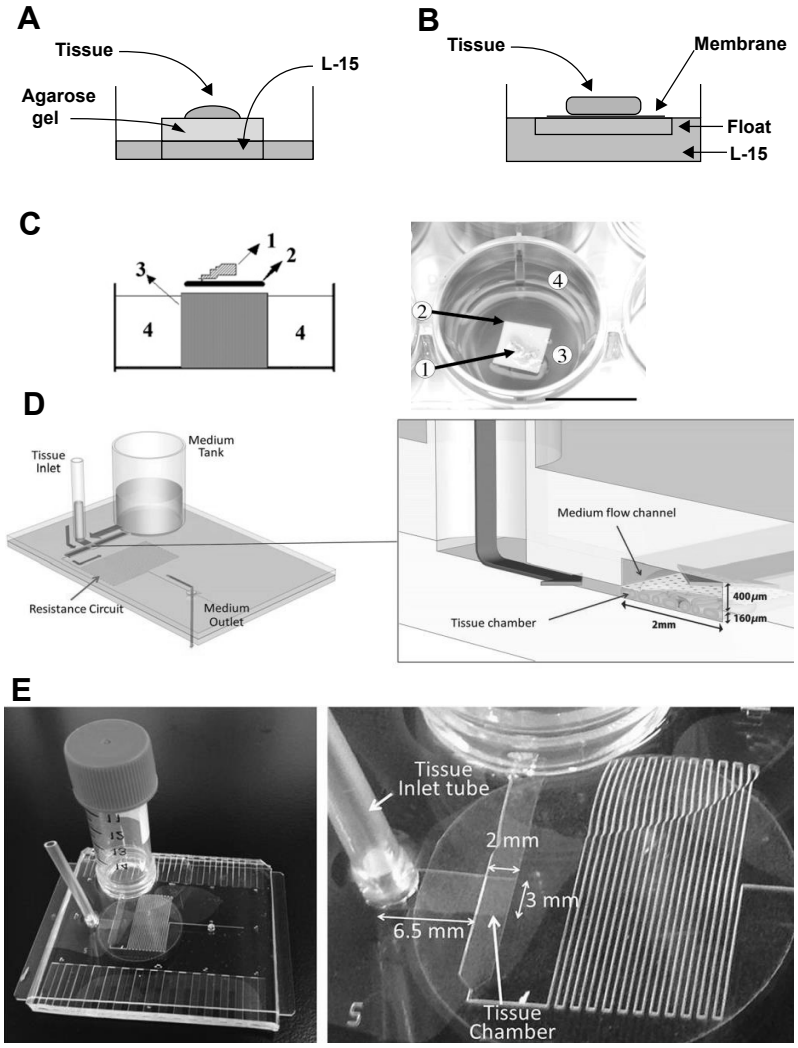


**Figure 7.** Organ culture systems. (**A**) The classical agarose gel model in mammals [203]; (**B**) the Japanese eel testis culture model [207]; (**C**) the zebrafish testis primary culture model [208] (1, testis tissue; 2, nitrocellulose membrane; 3, agarose cylinder; 4, medium (1 mL); scale bar: 1 cm); (**D**) Microfluid culture model [209]. (**E**) Pumpless microfluid culture [209].

Organ culturing for spermatogenesis was reported in immature Japanese eels in the 1990s [207]. Similarly, to techniques use in mammals, freshly minced eel testes were placed on floats of elder pith covered with a nitrocellulose membrane in tissue culture dishes (Figure 7B). The basal culture medium

consisted of L-15 supplemented with BSA, a cocktail of amino acids, and other substances necessary to maintain cell activity. This culture system has been employed in analyzing effects of several steroid hormones on eel spermatogenesis, and, with some modifications, in studies of Japanese Huchen Hucho perryi testes and ovaries [210,211]. In 2009, a zebrafish testes culture system was developed, based on the Japanese eel model [208]: Following disinfection, testes were placed on a 0.25 $cm^2$ nitrocellulose membrane in a 750 μm agarose cylinder and kept in 1 mL medium in each of 24-well flat-bottom plates (Figure 7C). The basal culture medium was composed of L-15 supplemented with HEPES, BSA, retinoic acid, and antibiotics [208]. The agarose cylinder was prepared using 48-well plates as molds [208]. The zebrafish testis culture model has been widely used to study endocrine and paracrine regulation of zebrafish spermatogenesis [20,22,89,212].

A Nile tilapia testis culture model similar to the eel system was developed to study sex differentiation in vitro [213]. A testis tissue culture system has also been established to examine effects of FSH and LH on gene expression in rainbow trout testis [96,214]. A novel culture system capable of effectively replicating the microcirculatory system of the mouse body was developed in 2016 [215]. The device consisted of a chamber for tissue and a channel for medium flow. The testis tissue was separated from the flowing medium by a thin porous membrane. Nutrients and waste products were exchanged by molecular diffusion as in vivo (Figure 7D). Using this microfluidic culture method, mouse testis tissue could be maintained six months in vitro and could undergo spermatogenesis [215]. With the addition of appropriate hormones to the culture medium, the system can mimic in vivo conditions. In 2018, a simpler, user-friendly system [201] using hydrostatic pressure of the medium in a reserve tank, along with a resistance circuit, was developed to control the flow rate without the use of a pump. The pumpless device can create culture conditions for testis tissue in a tissue chamber (Figure 7E). These microfluidic devices have successfully created a novel organ culture system that differs from the classic agarose gel system and has the potential to revolutionize organ culture.

The current organ culture systems described for fish can also support culture conditions suitable for ex vivo study of testis function. Organ culture can maintain the integrity of gonadal processes and, hence, is a powerful tool to evaluate the effects of a wide range of substances involved in fish spermatogenesis, among them pituitary hormones, steroids, and growth factors, as well as suspected endocrine disruptors.

### *3.4. Three-Dimensional Cultures*

The ability to view the gonad as a three-dimensional structure, as it is in vivo, could be valuable for study of testis development and spermatogenesis. Three-dimensional (3D) models currently represent an optimal tool to provide a tissue-like context for cell culture. In 3D cell cultures, the communication between cells and the scaffold is regulated by the material characteristics and scaffold properties [216]. In order to enhance cell adhesion, proliferation, and activation, materials employed in the fabrication of scaffolds must possess characteristics involved in intrinsic biocompatibility along with the appropriate chemistry to induce molecular recognition from cells. Three-dimensional culture systems were initially established for clonogenic assays to explore the complex mechanisms of multipotent hematopoietic cell proliferation and differentiation [217]. Later they were widely applied to stem cell studies, including exploring the impact of different scaffolds on mammalian SSC culture. Lee et al. first reported the reconstruction of the tubular structure of rat testis using collagen gel and Matrigel (BD Biosciences) [218], obtaining germ cell differentiation with a mixture of germ cells and testicular somatic cells cultured in a 3D system. In humans, spermatocytes have been induced to differentiate into presumptive spermatids in a collagen gel matrix culture [219]. A novel method using a soft agar culture system has become a common method for culture of stem cells and has also been applied on mouse SSCs [220–223]. Another 3D culture model using a bicameral chamber was developed by Legendre [224]. In fish, there are few studies of 3D culture, but fish collagen (FC) (Figure 8) is considered an ideal scaffold material due to its biocompatibility, low immunogenicity, low cytotoxic effects, high cell viability, and high biodegradability [225]. Studies have evaluated the FC of several species, including bester (beluga *huso*

*huso* × sterlet) and tilapia, as 3D culture scaffold materials [225–229]. To best of our knowledge, a 3D culture of fish germ cells has not been reported. This system is considered a novel technique for study of fish gametogenesis, with methods and protocols to be standardized.

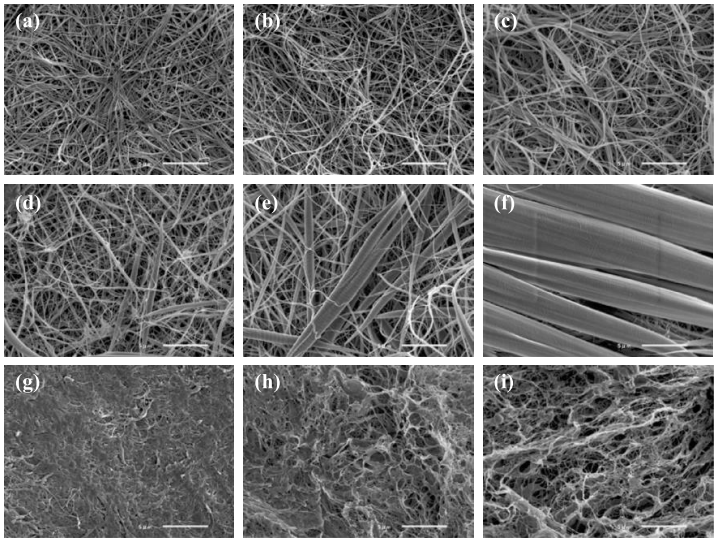


**Figure 8.** Scanning electron micrographs of skin collagen, swim bladder collagen, and porcine tendon collagen fibrils formed at 21 ± 1 °C for 24 h. (**a**–**c**) bester skin with NaCl concentrations of 0, 70, and 140 mM; (**d**–**f**) bester swim bladder with NaCl concentrations of 0, 70, and 140 mM; (**g**–**i**) porcine collagen with NaCl concentrations of 0, 70, and 140 mM. Scale bars, 5 μm [227].

A three-dimensional culture creates a complete microenvironment resembling the in vivo conditions. Although such systems have been shown to successfully induce spermatogenesis, conditions differ from those *in vivo*, and comparisons may be inaccurate. Using an appropriate scaffold, 3D culture can avoid the ischemia that develops in long-term organ tissue culture and mimic the structure of germ cells within the cyst.

## 4. Conclusions

This review summarizes the most relevant aspects of teleost germ cell biology, including spermatogonium characterization, and novel techniques for isolation and culture. Combined with such procedures as cryopreservation and transplantation, stem germ cell propagation can be a powerful tool for research, aquaculture, and conservation of endangered species (Figure 9). Stem germ cell isolation, enrichment, and culture conditions to support their proliferation without differentiation will help to overcome the limitations of low germ cell numbers for cryopreservation and transplantation (Figure 9). Germ cell culturing is valuable for studying the mechanisms underlying mitotic proliferation, differentiation of stem cells, meiosis, and stem cell regulation *in vitro*. Of particular interest is the potential to combine germ cell culture with gene editing techniques such as CRISPR/Cas for germ-line transmission by cell transplantation, nuclear transfer, and/or in vitro spermatozoon production for artificial insemination. Knowledge in this area has evolved rapidly, and has revealed novel and promising approaches to germ stem cell manipulation.

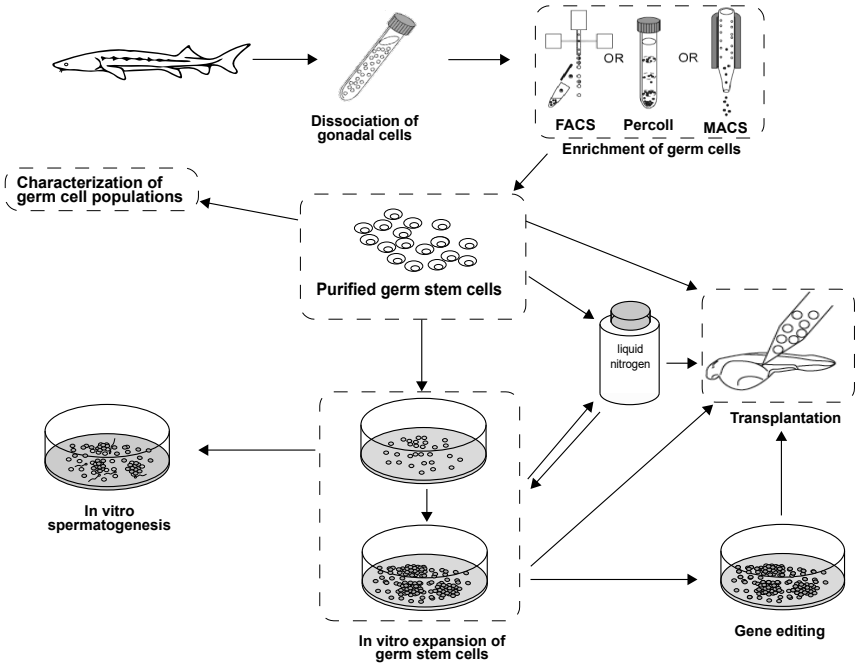


**Figure 9.** Potential applications of germ cell isolation and culture. Testes are removed from the fish body and dissociated, providing a cell suspension that can be sorted using fluorescence-activated cell sorting or centrifugation in a density gradient; e.g., in Percoll solution or by magnetic-activated cell sorting. The purified germ cell suspension can be characterized with respect to molecular characteristics and morphology. Purified cells may be injected into a recipient to generate germ-line chimera or amplified by in vitro culture. Cultured cells can be employed in gene editing or subjected to cryopreservation.

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## CHAPTER 3

# OPTIMIZATION OF *IN VITRO* CULTURE CONDITIONS OF STURGEON GERM CELLS FOR PURPOSE OF SURROGATE PRODUCTION

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My share on this work was about 40%.

*animals*

MDPI

*Article*

# Optimization of In Vitro Culture Conditions of Sturgeon Germ Cells for Purpose of Surrogate Production

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**Simple Summary:** The sturgeon is among the most ancient of actinopterygian fishes. Most species of sturgeon are listed as critically endangered due to habitat alteration caused by damming of rivers, pollution and overharvesting. Germ cell transplant is a useful tool to save these endangered species. To expand germ cell populations and sustain the supply for long periods for transplant, we established basal culture conditions for sturgeon germ cells. Germ cell mitotic activity has been enhanced by eliminating gonad somatic cells, supplementing with growth factor and using an alternative to fetal bovine serum. The optimal condition identified was purified germ cells cultured in serum-free medium supplemented with leukemia inhibitory factor (LIF) and glial cell line-derived neurotrophic factor (GDNF) at 21 °C. Cultured sterlet germ cells showed development after transplant into Russian sturgeon. The study provided useful information for sturgeon germ cell culture.

**Abstract:** To expand germ cell populations and provide a consistent supply for transplantation, we established basal culture conditions for sturgeon germ cells and subsequently increased their mitotic activity by eliminating gonad somatic cells, supplementing with growth factor, and replacing fetal bovine serum (FBS). The initial basal culture conditions were Leibovitz's L-15 medium (pH 8.0) supplemented with 5% FBS ($p < 0.001$) at 21 °C. Proliferation of germ cells was significantly enhanced and maintained for longer periods by elimination of gonad somatic cells and culture under feeder-cell free conditions, with addition of leukemia inhibitory factor and glial-cell-derived neurotrophic factor ($p < 0.001$). A serum-free culture medium improved germ cell proliferation compared to the L-15 with FBS ($p < 0.05$). Morphology remained similar to that of fresh germ cells for at least 40 d culture. Germline-specific gene expression analysis revealed no significant changes to germ cells before and after culture. Sterlet *Acipenser ruthenus* germ cells cultured more than 40 days showed development after transplant into Russian sturgeon *Acipenser gueldenstaedtii*. Polymerase chain reaction showed 33.3% of recipient gonads to contain sterlet cells after four months. This study developed optimal culture condition for sturgeon germ cells. Germ cells after 40 d culture developed in recipient gonads. This study provided useful information for culture of sturgeon germ cells.

**Keywords:** feeder cells; germ cell culture; glial-cell-derived neurotrophic factor; sturgeon; transplantation

## 1. Introduction

Germ stem cells have the ability to self-renew as well as to differentiate into other germ cell stages. Xenotransplantation of germ cells has been conducted in fish [1]. In contrast to mammals, in fish, both type A spermatogonia and oogonia (probably germ stem cells) show high sex plasticity even after sexual maturation [2]. Germ cells cultured in vitro could be transplanted in cases of limited numbers of an individual's own germ cells, low stem cell purification efficacy, and cells damaged by enzymatic dissociation [3]. In recent years, in vitro culture of germ cells was established in Medaka *Oryzias latipes* [4], zebrafish *Danio rerio* [5], Nile tilapia *Oreochromis niloticus* [6] and rainbow trout *Oncorhynchus mykiss* [7].

Sturgeons belong to the order Acipenseriformes, which are among the most ancient of actinopterygian fishes [8]. According to the International Union for Conservation of Nature and Natural Resources' Red List, 64% of sturgeon species are critically endangered due to habitat alteration caused by damming of rivers, pollutio, and overharvesting [9–11]. Most sturgeon species are late maturing, making culture and conservation costly and time consuming [12,13]. Germ cell culture and transplant could be an available and rapid method for surrogate production of endangered fishes with large bodies and a long life-cycle. To establish optimal culture conditions for sturgeon germ cells and improve their mitotic activity, we investigated the basal culture conditions for gonad cells and examined the effect of somatic cells on germ cell proliferation and assessed the influence of growth factor on germ cell mitotic activity. The L-15 modified culture medium with fetal bovine serum (FBS) was replaced with a serum-free medium. The identity of cultured germ cells was confirmed by RT-qPCR (Quantitative real-time PCR) targeting germ cell specific genes, and the cells were transplanted into sturgeon larvae to assess their transplantability and proliferation.

## 2. Materials and Methods

### *2.1. Animal Ethics Statement*

Animal handling and experimentation were approved by the Ethics Committee on Animal Care of Chinese Academy of Fishery Science and the Ministry of Agriculture of the Czech Republic (reference number: 53100/2013-MZE-17214).

### *2.2. Fish Selection and Sampling*

Dabry's sturgeon *Acipenser dabryanus*, used for optimization of germ cell culture conditions, were reared in the Taihu Station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Sterlet *Acipenser ruthenus* used for germ cell transplantation were cultivated at the Faculty of Fisheries and Protection of Waters, University of South Bohemia. Gonads were collected from 22–26-month-old Dabry's sturgeon (length ~92 cm; weight ~3.5 kg). Sterlet gonads were collected from 10–13-month-old specimens (~52 cm; ~520 g). The gonads were at maturity stage II: containing mostly spermatogonia or oogonia and previtellogenic oocytes. Deep anesthesia was induced by 0.05% 3-aminobenzoic acid ethyl ester methanesulfonate-222 (MS-222) (Sigma, St. Louis, MO, USA). Russian Sturgeon *Acipenser gueldenstaedtii* larvae obtained from combined eggs and sperm of three females and three males were used as recipients for cultured germ cells.

### *2.3. Dissociation and Culture of Gonad Cells*

Gonads of Dabry's sturgeon were washed in phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA) containing 50 μg/mL ampicillin, 200 U/mL penicillin, and 20 μg/mL streptomycin

(Sigma) (pH 8.0) and minced into 1-$mm^3$ pieces. Fragments were dissociated using various proteinases with gentle pipetting. For all experiments, cells were seeded at a concentration of $1.6 \times 10^4$–$2 \times 10^4$ cells/$cm^2$ in 25-$cm^2$ culture flasks containing 5 mL culture medium.

*2.4. Optimization of Basal Culture Conditions*

To assess the effect of enzymes on germ cell mitotic activity, gonads were dissociated with one of three enzyme treatments: (1) 0.47% trypsin–Ethylenediaminetetraacetic acid (trypsin–EDTA; Gibco, Grand Island, NY, USA) digestion for 15 min with gentle pipetting; (2) 0.25% trypsin (Worthington Biochemical Corporation, Lakewood, NJ, USA) digestion for 3 h; or (3) a combination of 2 mg/mL collagenase H (Roche Diagnostics, Mannheim, Germany) and 500 U/mL Dispase II (Sigma-Aldrich, St Louis, MO, USA) digestion for 3.5 h, as previously described [14]. The digestion was stopped by Leibovitz's L-15 medium (Sigma-Aldrich, St Louis, MO, USA) with 20% FBS, filtered through a 40-μm pore-size nylon screen, and centrifuged at 200× *g*. The dissociated cells were cultured in L-15 medium supplemented with 15% (*v/v*) FBS, 25 mM HEPES (Sigma-Aldrich, St Louis, MO, USA), and antibiotics at 25 °C. Cells were cultured for 10 d, with 5-Bromo-2-deoxyUridine (BrdU, Sigma-Aldrich, St Louis, MO, USA) and immunocytochemical assays conducted on the final day. To investigate the influence of temperature, the dissociated cells were cultured in L-15 medium supplemented with 15% FBS, and cultured at 17, 21, and 25 °C. The BrdU and immunocytochemical assays were performed on day 10.

Based on these investigations, the optimal concentration of FBS was determined. The selected culture medium was L-15 supplemented with 5, 10, or 15% (*v/v*) FBS. Cells were cultured at 21 °C. BrdU and immunocytochemical assays were performed on days 5, 10, 15, and 20.

*2.5. Elimination of Gonad Somatic Cells and Effect of Feeder Layer on Germ Cell Mitotic Activity*

The survival and proliferation of germ cells was inhibited by over-growth of gonad somatic cells. We attempted to eliminate somatic cells and assess the influence of the addition of a feeder layer to germ cells. The dissociated gonad cells were cultured for 36 h, and floating cells were gently pipetted and passaged two or three times to obtain purified germ cells.

The feeder cells were fibroblastoid somatic cells derived from sturgeon gonad: Dissociated gonad cells were cultured with L-15 supplemented with 10% (*v/v*) FBS. After three or four passages, the cells were treated with a 10 μg/mL mitomycin C solution for 3 h to arrest growth and then washed three times with L-15. Purified germ cells were transferred onto feeder cells. For feeder-free culture, purified germ cells were transferred directly into a new culture flask. Dissociated non-purified gonad cells were used as controls. The three groups were cultured in L-15 and 5% FBS at 21 °C. Germ cell and somatic cell proliferation was analyzed by BrdU and immunocytochemical assays after 7, 14 and 21 d.

*2.6. Effects of Growth Factor on Germ Cell Proliferation*

Selected growth factors were added at the following concentrations: 25 ng/mL epidermal growth factor (EGF, Pufei, Shanghai, China); 25 ng/mL basic fibroblast growth factor (bFGF, Pufei, Shanghai, China); 5 IU/mL human chorionic gonadotropin (hCG, Sigma-Aldrich, St Louis, MO, USA); 2 IU/mL pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich, St Louis, MO, USA); 25 ng/mL glial cell line-derived neurotrophic factor (GDNF, Thermo Scientific, Waltham, MA, USA); and 25 ng/mL leukemia inhibitory factor (LIF, Peprotech, Rocky Hill, NJ, USA). As Sakai et al. [5] reported that 7% carp serum enhanced the proliferation of germ cells derived from zebrafish, we also evaluated the effect of sturgeon serum. The serum was derived from blood of an 18-month-old male sturgeon, centrifuged at 1000× *g* and filtered through a 0.2-μm pore nylon screen. Serum (7% *v/v*) was added to the L-15 and 5% FBS culture medium. Growth factor was added to the culture medium as follows: (1) EGF and bFGF; (2) hCG and PMSG; (3) EGF, bFGF, hCG and PMSG; (4) Sturgeon serum; (5) GDNF and LIF.

Based on results of the previous step, purified germ cells were seeded in culture flasks and cultured at 21 °C. The mitotic activity of germ cells and somatic cells was investigated by BrdU and immunocytochemical assay on day 10 of culture.

### *2.7. Replacement of FBS*

We attempted to establish a serum-free medium to assess the effect of FBS and determine a potential alternative. The serum-free medium comprised StemPro-34 SFM (Invitrogen, Carlsbad, CA, USA) supplemented with StemPro Supplement (Invitrogen, USA). The FBS was replaced with 0.5% (*w/v*) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), 0.5% (*v/v*) B27 Supplement (Gibco, USA), 20 μg/mL L-Lys, 20 μg/mL L-Pro, 20 μg/mL L-Asp, and 10 mM/L sodium pyruvate (Sigma-Aldrich, St Louis, MO, USA). We also added 25 mM HEPES, 50 μg/mL ampicillin, 200 U/mL penicillin and 20 μg/mL streptomycin (pH 8.0). Purified germ cells were cultured at 21 °C with serum-free medium and optimal growth factors. The mitotic activity of germ cells and somatic cells was investigated by BrdU and immunocytochemical assay after 10 d culture.

### *2.8. Analysis of Germ Cell Mitotic Activity*

To detect germ cell mitotic activity, a BrdU incorporation assay was performed by adding 25 μM BrdU (Sigma-Aldrich, USA) to the culture medium during the final 24 h of culture. The cells were fixed by 4% paraformaldehyde for 30 min. Immunocytochemical detection of BrdU was performed with mouse anti-BrdU antibody (ab8039; Abcam, Cambridge, MA, USA) and Alexa-Fluor-488-labeled Goat Anti-Mouse IgG (H+L) (Beyotime Biotechnology, Hangzhou, China). The germ cells were identified with anti-Vasa antibody from sturgeon [15] (diluted 1:500) and exposed for 1 h to fluorescein-conjugated Alexa-Fluor-647-labeled Goat Anti-Rabbit IgG (H+L) (diluted 1:500). The cells were observed with an inverted fluorescent microscope (Leica, Wetzlar Germany). The percentage of BrdU-labeled germ cells was calculated from randomly selected 500 BrdU-labeled cells.

### *2.9. Expression Analysis*

To analyze the fate of the cultured cells, we investigated the presence of germ-line-specific gene transcripts *dead end*, *gfra1a*, *grip2*, *plk3*, and *ednrba*. Total RNA of each sample was reverse transcribed with the TATAA Biocenter Kit. Primers (Table 1) were designed based on the sturgeon gonad transcriptome. Cell expression of selected genes before and after culture was analyzed using quantitative real-time polymerase chain reaction (PCR) performed with SYBR Green Real-time PCR Master Mix according to the manufacturer's protocol. PCR amplification was conducted with an initial activation of 3 min at 95 °C followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 70 °C for 30 s. The fluorescent SYBR Green signal was detected immediately after the extension step of each cycle. Gene expression was compared by $2^{-\Delta\Delta Cq}$.

**Table 1.** Quantitative polymerase chain reaction (PCR) primer sequences for germ-line specific genes (*dead end*, *gfra1a*, *grip2*, *plk3* and *ednrba*).

| Name | Sequence (5′-3′) | Orientation | Length bp |
|---|---|---|---|
| Ardnd1 | AAACGTGAGGCACGGGTATT<br>CCTGGATCGGTATCCACAGC | Forward<br>Reverse | 705 |
| cds(a)_gfra1a | GGAAGTGGGAACAGGGAAGA<br>GGGTTTGGGTGCTAGATTTGT | Forward<br>Reverse | 123 |
| grip2 | TGCTGAAGAATGTGGGCGA<br>CCCTCTCAACACGAAGCCA | Forward<br>Reverse | 149 |
| plk3 | ACCCGAGTCAGATGTGTGGT<br>AGCAGGAAGGGAGAGGAAGT | Forward<br>Reverse | 148 |
| ednrba-201_CDS | TTAGGCGCTTCCGAGACTAC<br>CCGGGTTCATGGTTTTGGG | Forward<br>Reverse | 81 |

### *2.10. Cultured Germ Cell Transplantation*

To examine the proliferation of cultured germ cells in recipient gonads, xenotransplantation was performed. Russian sturgeon larvae two-weeks post-hatching with fluorescein isothiocyanate (FITC)-labeled endogenous primordial germ cells were used as recipients, as described by Pšenička [16]. Culture medium as described (Section 2.7) was used in sterlet germ cell culture for more than 40 d. Paraffin sections of testes and ovaries used are shown in Figure 1A, B. Before transplant, cultured cells were digested by trypsin and resuspended. The germ cell ratio was calculated by immunolabeling. Membranes of both cell types were labeled by the PKH26 Red Fluorescent Cell Linker Kit according to the manufacturer's protocol (final dye concentration 2 µL/mL). The labeled cells were injected into the body cavity of the host using a micromanipulator M-152 (Narishige, Japan) and a microinjector Cell Tram Vario (Eppendorf, Germany) at ~2.5 × $10^3$ cells per recipient. The larvae were maintained at 18 °C and fed on *Tubifex*. Larvae were examined by fluorescence stereomicroscope at 40 days post-transplant (dpt).

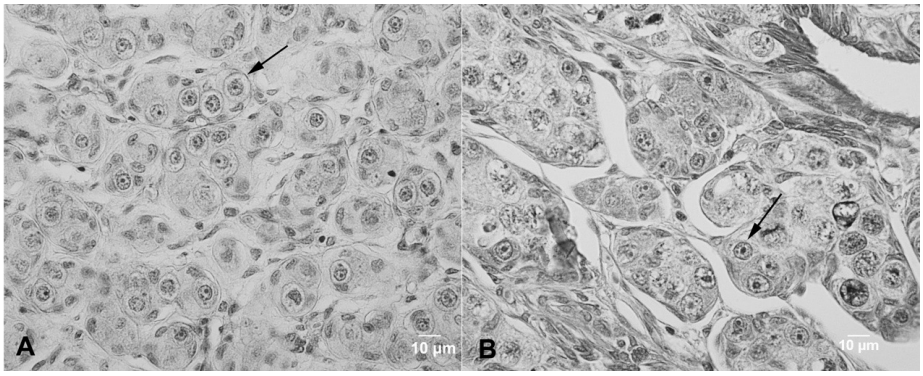


**Figure 1.** Sterlet *Acipenser ruthenus* gonads. (**A**) Sterlet testis at maturity stage II (10-month-old); (**A**) showing spermatogonia (black arrow) and Sertoli cells; (**B**) Sterlet ovary in maturity stage II (10-month-old); (**B**) showing oogonia (black arrow) and early oocytes.

After four months, fish were dissected under stereomicroscope. DNA was extracted from gonads using Exgene$^{TM}$ Genomic DNA micro kit according to manufacturer's instructions (Gene All Co. Exgene$^{TM}$, South Korea). Sterlet-specific primers (Table 2) were applied for identification of sterlet cells as described by Havelka et al. [17]. The PCR conditions were initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 15 s, and extension at 72 °C for 30 s.

**Table 2.** Primers used for the DNA amplification of sterlet and Russian sturgeon.

| NAME | SEQUENCE (5′-3′) |
|---|---|
| 247_ARp | TAAGGGTCCATGCATGCAG |
| 247_ARn | TAAGGGTCCATGCATGCCT |
| 247_uni | TTTTAGCTGCACCGTGGC |

### *2.11. Statistical Analysis*

The results were expressed as a ratio against control as mean ± Standard Error of Mean (SEM). Significance was determined with one-way and two-way analysis of variance (ANOVA) followed by Turkey tests (see Sections 3.1, 3.3 and 3.4). A paired T-test was used to compare two groups (see Section 3.5). Probability values of $p < 0.05$ were considered significant.

## 3. Results

### *3.1. Optimal Basal Culture Conditions*

Results of BrdU assays are shown in Figure 2. Germ cells digested with 0.47% trypsin-EDTA showed significantly lower proliferation rate than the other two groups ($p < 0.05$, Figure 3A). The mitotic activity of gonad somatic cells in the 0.25% trypsin-treated group was significantly higher than that seen in the other two groups ($p < 0.05$, Figure 3B). Although statistical analysis did not reveal significant differences in proliferation rates for germ cells with dissociation method, the mean value for 0.25% trypsin was higher; therefore, we used it in subsequent trials. The mean value of germ cell proliferation was higher at 21 °C than at 17 and 25 °C, but somatic cells cultured at 25 °C showed a significantly higher proliferation rate ($p < 0.05$, Figure 3C,D). The mitotic activity of germ cells with different concentrations of FBS were analyzed as proportion relative to controls, using a two-way ANOVA with concentration and cultured days as the dependent variables. Significant differences in mitotic activity were seen among germ cell groups treated with 5%, 10%, and 15% FBS ($p < 0.001$). The proportion of germ cells with BrdU incorporated was highest when cultured in L-15 medium containing 5% FBS (Figure 3E). Proliferation of germ cells differed significantly at 5 d, 10 d, 15 d, and 20 d ($p < 0.001$) with highest proliferation observed at 10 d culture. The gonad somatic cells showed an opposite trend to that of germ cell proliferation (Figure 3F). Immunolabeling after digestion indicated 44.4% ± 9.81% of cells in testes to be germ cells and 37.5% ± 9.66% of those in ovaries to be germ cells.

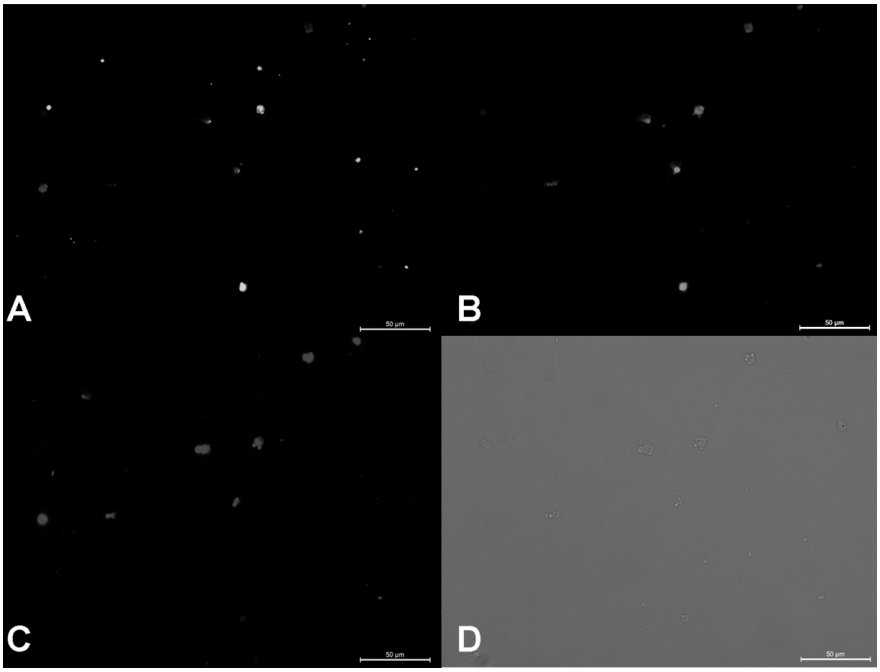


**Figure 2.** Immunofluorescence showing co-localization of neonatal cells at 10-days culture. Gonads were digested with trypsin and cultured at 21 °C in L-15 medium and 10% fetal bovine serum (FBS) (*v*/*v*). Cells were stained green for BrdU protein (**A**), red for Vasa protein (**B**) and blue for 4′,6-diamidino-2-phenylindole (DAPI) (**C**). (**D**) White light.

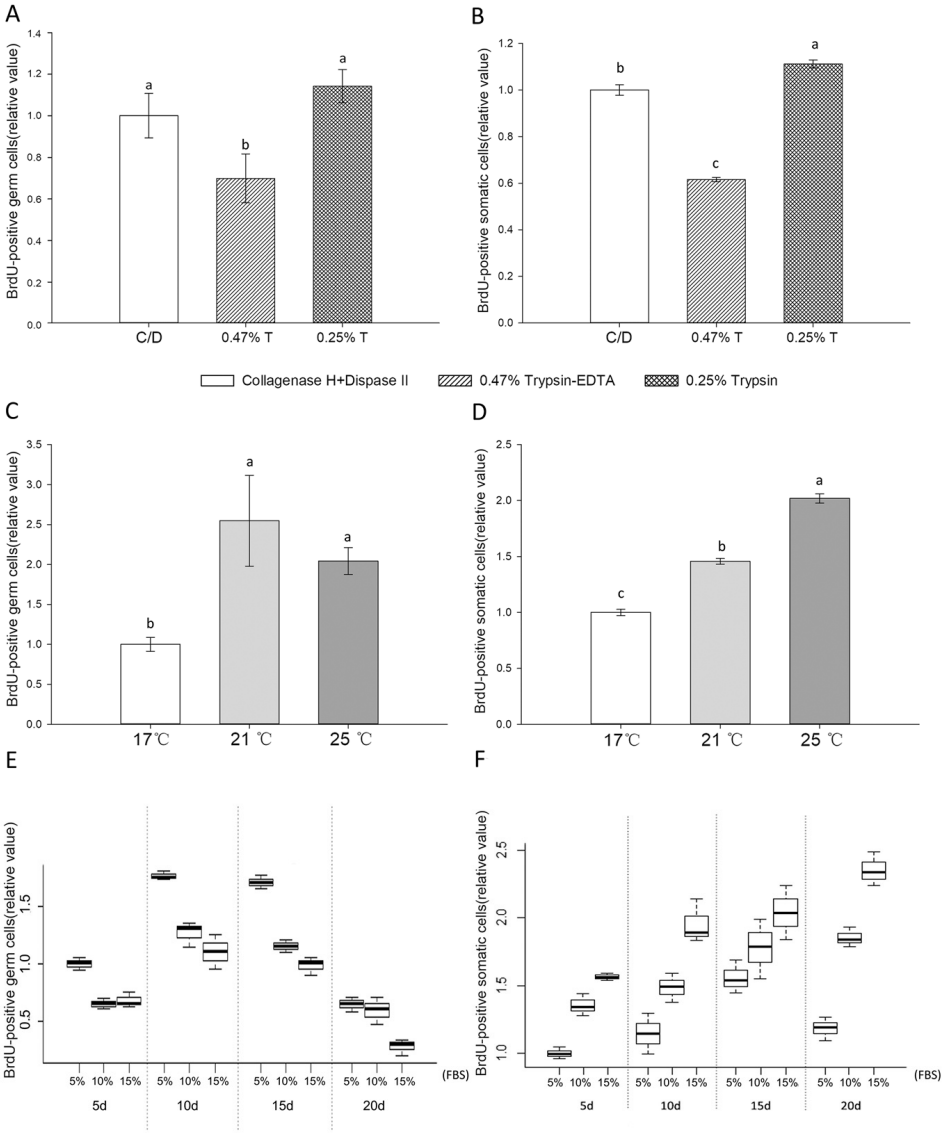


**Figure 3.** Influence of enzymatic dissociation (**A**,**B**), culture temperature (**C**,**D**), and FBS (5–15%) (**E**,**F**) on BrdU incorporation into Dabry's sturgeon germ cells. (**A**,**B**): Gonads were dissociated with 2 mg/mL collagenase H and 500 U/mL Dispase II (C/D); 0.25% trypsin (0.25% T), or 0.47% trypsin–Ethylenediaminetetraacetic acid (EDTA, 0.47% T-EDTA). Cells were cultured in L-15 medium (pH 8.0) supplemented with 15% FBS for 10 d at 25 °C. (**C**,**D**) Gonad was dissociated with 0.25% trypsin and cultured in L-15 medium supplemented with 15% FBS (pH 8.0) for 10 d at 17, 21, and 25 °C. (**E**,**F**) Gonad was dissociated with 0.25% trypsin and cultured in L-15 medium (pH 8.0) under selected concentrations of FBS (5–15%) at 21 °C. BrdU assays were performed every 5 d. Data are shown as mean ± SEM ($n$ = 4) of relative values for germ cells and somatic cells by immunofluorescent co-localization of BrdU and Vasa. The values of collagenase H, Dispase II (**A**,**B**), 17 °C (**C**,**D**), and 5% FBS (5 d) (**E**,**F**) were defined as the relative value 1.0. Values with different lowercase letters are significantly different ($p < 0.05$).

### *3.2. Effect of Feeder Layer on Germ Cell Mitotic Activity*

Two-way ANOVA, using quantity of germ cells with BrdU incorporated as the dependent variable, and *culture day* and *treatment* as factors, revealed significant variation ($p < 0.001$) in the proliferation of germ cells with both factors (Figure 4). Day and treatment interaction was also significant ($p < 0.001$). Of the groups with and without feeder cells and control, significantly greater proliferation was observed in those cultured without the feeder layer. In control group, germ cell proliferation was decreased after 21 d culture compared to that at 14 d, while somatic cells maintained stable mitotic activity during the same period (Figure 5A,B). In the feeder-free culture, the suspended germ cells tended to form clumps at 3 d culture after purification. The clumps became larger during 3–14 d of culture (Figure 5C,D). Germ cell propagation was reduced after 21 d culture compared to that at 14 d, and the clumps gradually ceased expanding. In the feeder layer group, some cells attached to the feeder cells and were observed to proliferate. There was no further cell attachment to the feeder cells after 2 d. Germ cell growth took place during 3–14 d of culture (Figure 5E,F). Cells were shed from the feeder layer, and the number of germ cells after 21 d culture was lower than that seen at 14 d.

With and without feeder cells, BrdU incorporation showed that mitotic activity of germ cells was highest at 14 d culture and decreased thereafter. The residual somatic cells showed stable growth after 14 d culture. During the same period, the total number of germ cells decreased gradually, although though mitosis was confirmed by BrdU assay.

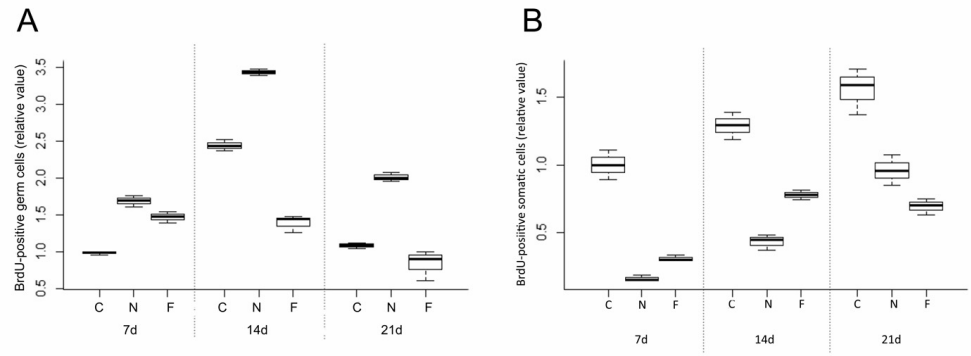


**Figure 4.** Effects of somatic cells on mitotic activity of Dabry's sturgeon germ cells (**A**) and somatic cells (**B**). The figure shows mean ± SEM ($n = 3$) of total mitotic activity under control (C), feeder-free (N), and feeder layer conditions (F). The BrdU assay was performed on days 7, 14 and 21 of culture. Data are shown as the mean ± SEM ($n = 3$). The values for 7-d control were defined as the value relative to 1.0. Values with different lowercase letters were significantly different according to two-way ANOVA ($p < 0.05$).

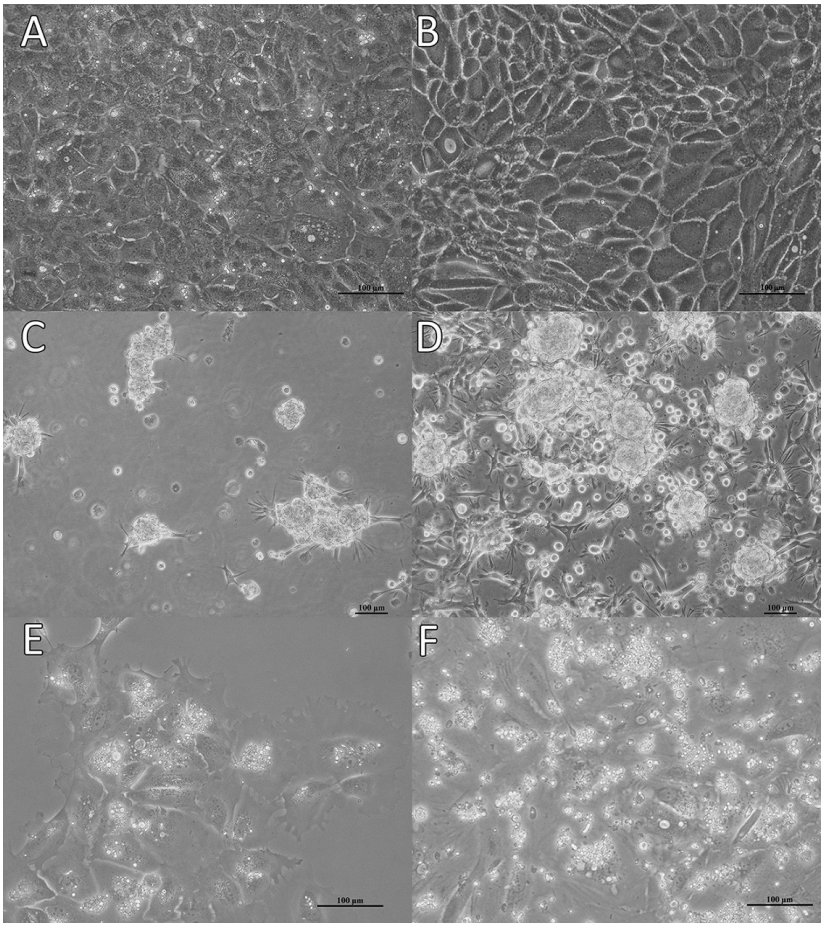


**Figure 5.** Morphology of Dabry's sturgeon gonad cells (**A**,**B**) and purified germ cells cultured without (**C**, **D**) or with feeder cells (**E**,**F**), after 3 d (**A**,**C**,**E**) and 14 d (**B**,**D**,**F**).

### *3.3. Effect of Growth Factors on Germ Cell Mitotic Activity*

There were significant differences in mitotic activity of germ cells treated with different growth factors ($p < 0.05$, Figure 6A). Addition of LIF and GDNF to the culture medium significantly increased germ cell mitotic activity, with BrdU incorporation in germ cells 1.8-fold that of cells cultured without growth factor after 10 days culture. The cultured germ cells proliferated and formed large clumps over a period of 28 days. Sturgeon serum (7%) was associated with germ cell mitotic activity 6-fold that seen in control conditions. With addition of hCG and PMSG, BrdU incorporation in germ cells was similar to growth-factor-free conditions. Addition of LIF, GDNF, or sturgeon serum was not shown to contribute to somatic cell proliferation ($p < 0.05$, Figure 6B).

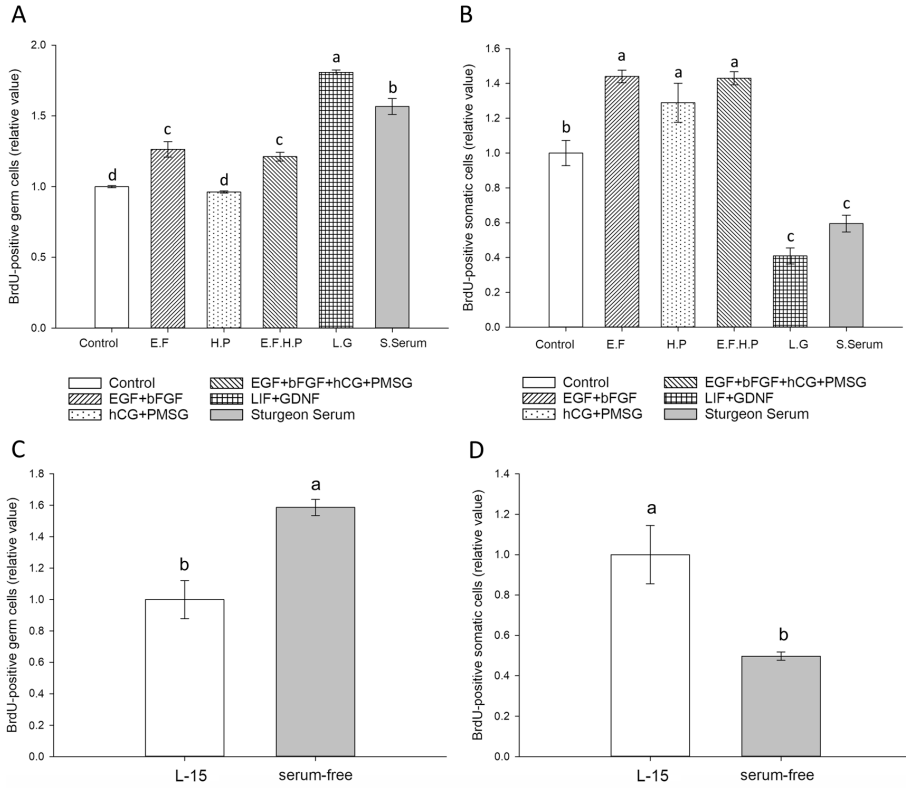


**Figure 6.** Effects of selected soluble growth factors on mitotic activity of Dabry's sturgeon germ cells (**A**) and somatic cells (**B**). The number of germ cells was counted on Day 10. The somatic cell mitotic activity after addition of EGF and bFGF (E.F), hCG and PMSG (H.P), EGF/bFGF/hCG and PMSG (E.F.H.P.), LIF and GDNF (L.G.), and sturgeon serum (S. Serum) is shown. Data are shown as the mean ± SEM ($n$ = 3). The control values (**A**,**B**) were defined as the relative value 1.0. Values with different lowercase letters are significantly different ($p < 0.05$) according to one-way ANOVA. Effect of replacement of FBS on mitotic activity of germ cells (**C**) and somatic cells (**D**). The number of germ cells were counted on Day 10. Cells were cultured at 21 °C in L-15 medium (pH 8.0) supplemented with 25 mM HEPES, antibiotics, 5% FBS, and 25 ng/mL LIF and GDNF (L-15, white bar) and StemPro-34 Serum-Free Medium with supplement (serum-free, gray bar). Data are shown as mean ± SEM ($n$ = 3). The values of L-15 medium were defined as the relative value 1.0. Values with different lowercase letters are significantly different ($p < 0.05$) according to $t$-test.

### *3.4. Replacement of FBS*

BrdU incorporation under serum-free conditions showed significantly higher germ cell mitotic activity than seen with L-15 ($p < 0.05$, Figure 6C,D). After 34 days, the germ cells cultured in serum-free medium were proliferated and tended to form large clumps. Their morphology after at least 38 d culture remained similar to that of fresh germ cells. The number of cells after 40 d culture was 9.6-fold the initial number.

### *3.5. Expression and Transplant Effectiveness of Cultured Germ Cells*

We maintained the cells in serum-free medium for 10 days and compared with fresh purified germ cells to investigate sturgeon germ cell fate in our culture system. qPCR analysis confirmed that

cultured cells transcribed the germ cell marker genes *dead end*, *grip2*, *plk3*, *gfra1a*, and *ednrba*. Most marker gene expression showed no significant difference before and after 10 d culture with growth factor ($p > 0.05$, Figure 7), confirming that cultured cells remained similar to fresh germ cells.

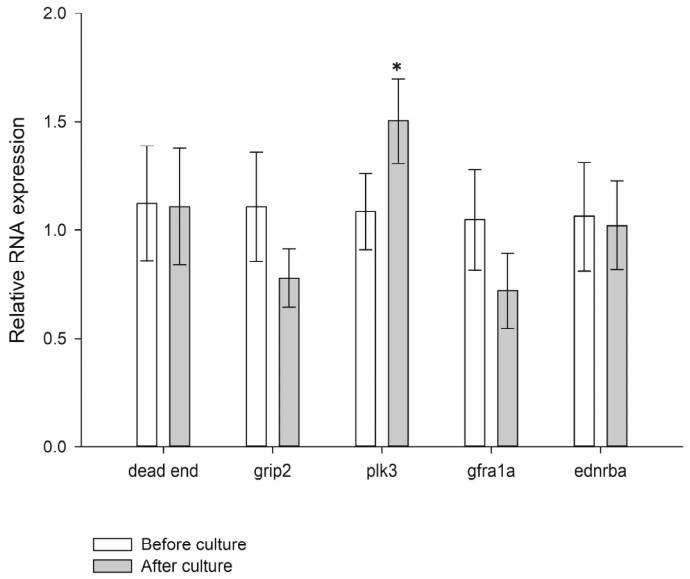


**Figure 7.** Expression of *dead end*, *grip2 plk3*, *gfra1a*, and *ednrba* in germ cells before and after 10-d culture. Data are shown as mean ± SEM ($n = 3$). Differences were considered significant at $p < 0.05$. The asterisk indicates significant difference of gene expression compared to before culture according to paired *t*-test.

To examine whether the transplanted germ cells could develop in recipient gonads, cells cultured for more than 40 d under serum-free conditions were injected into the body cavity of Russian sturgeon larvae. Before transplant, immunolabeling indicated that more than 91% of injected cells were germ cells (Figure 8A–L). Recipient fish were dissected one-month post-transplant. In 50%, attachment and development of the cultured cells in the recipient genital ridge could be detected (Figure 8M,N). After four months, PCR showed 33% of recipient fish to display sterlet-specific bands. In all gonad samples, fragments from both donor and recipient could be detected, which matched the fin tissues of the two species (Figure 8O).

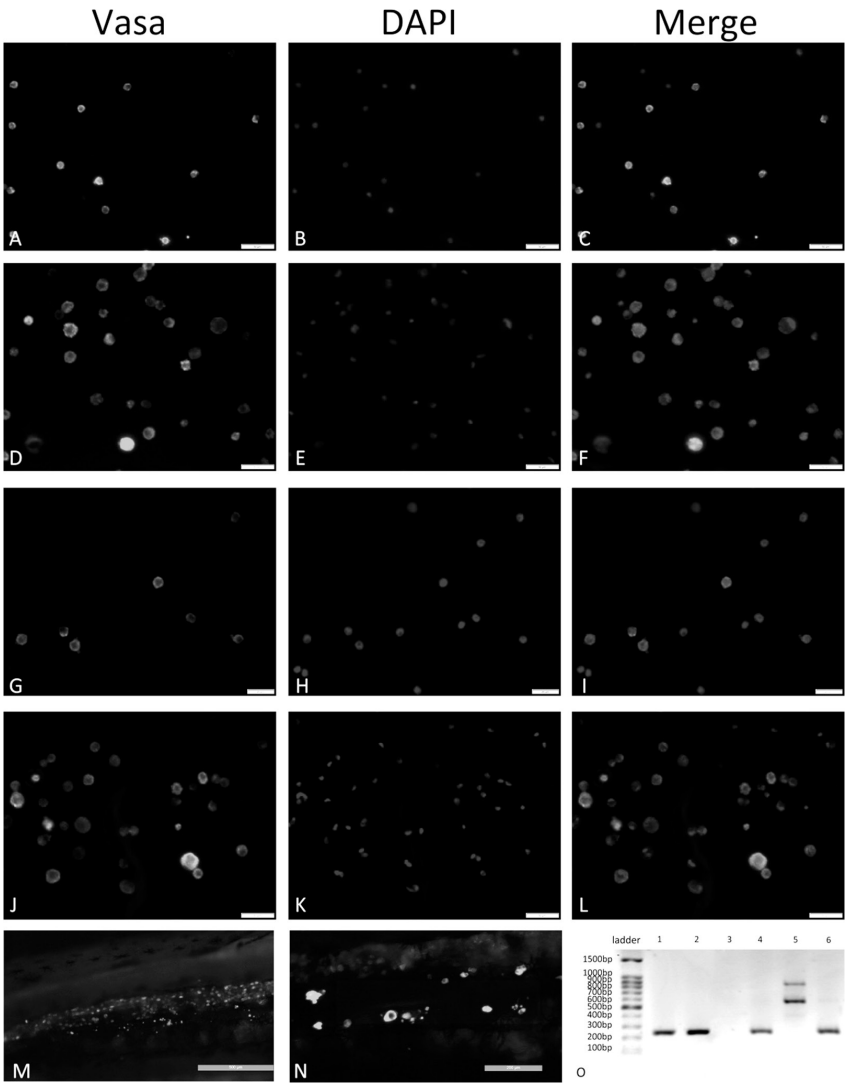


**Figure 8.** Immunofluorescence of sterlet germ cells in serum-free medium. Purified spermatogonia before culture (**A**–**C**) and before transplant after 40 d culture (**D**–**F**). Purified oogonia before culture (**G**–**I**) and before transplantation after 40d culture (**J**–**L**). Russian sturgeon larvae transplanted with cultured sterlet germ cells 40 dpt (**M**,**N**). Scale bars: 50 μm (**A**–**F**,**J**–**L**), 20 μm (**G**–**I**), 500 μm (**M**), 200 μm (**N**). Identification of sterlet from recipients by presence/absence of band (**O**). 1–3: Amplification of species-specific primer 247_ARp (sterlet positive). 1 = recipient gonads; 2 = normal sterlet fin; 3 = normal fin. 4–6: Amplification of species-specific primer 247_ARn (sterlet negative Russian sturgeon). 4: recipient's gonads. 5: normal fin from sterlet. 6: normal fin from Russian sturgeon.

## 4. Discussion

Brinster and Zimmermann [18] first described germ cell transplant in a mouse model. They dissociated germ cells using 0.1% collagenase and 0.25% trypsin in two steps. Okutsu et al. [3] performed xenogeneic spermatogonia transplant from rainbow trout to salmon (*Oncorhynchus masou*),

obtaining germ cells by dissociating rainbow trout testes with 0.5% trypsin in PBS. The donor-derived germ cells colonized embryonic gonads and produced functional gametes after migrating into the peritoneal cavity. Psenicka et al. [16] used 0.3% trypsin in PBS at 23 °C for dissociation of Siberian sturgeon testicular and ovarian cells. Shikina et al. [19] demonstrated disruption of some membrane proteins by trypsin, causing a reversible decrease in mitotic activity and reduced adhesiveness. Gonad cells digested with trypsin presented higher growth rate than collagenase H, dispase II and trypsin-EDTA, but the yield of cells dissociated by other enzymes showed slight variation depending on species and gonad stage. Gonad protein composition likely undergoes changes during gonad development. Further studies are required to confirm the effect of trypsin on sturgeon germ cell migration ability after short term culture. Germ cells proliferated at 17 °C, 21 °C, and 25 °C, but 21 °C was more effective than 17 °C and 25 °C, in agreement with the optimal temperature for sturgeon cells reported by Grunow et al. [20].

Increasing concentration of FBS was associated with increased mitotic activity of somatic cells. Proliferation of germ cells decreased with increased culture duration, suggesting that gonad somatic cells depleted the available nutrients and growth factor.

Gonad somatic cells may be involved in the regulation of germ cell survival, proliferation, and differentiation under physiological and pathological conditions [21]. We attempted to assess the effects on germ cells of sturgeon gonad somatic cells as feeder cells. In mammals, feeder cells have been commonly used to culture cells at low densities [22]. Some studies have shown that, when co-cultured with Sertoli cells, germ cell maintenance is lower than with other feeder cell types in mice [23]. Rat spermatogonia show extended propagation with Sertoli cells compared to with Sandos inbred mice embryonic fibroblasts feeders [24]. Feeder cells have been used for induction of cell differentiation in fish [25]. In our study, sturgeon germ cells attached and proliferated during 14 d culture, but showed reduced mitotic activity after 14 d, shedding the feeder layer gradually, suggesting that gonad somatic cells may be not optimal for germ cell proliferation and amplification in sturgeon. Whether these feeder cells can induce differentiation needs further investigation. We also investigated the propagation of germ cells under feeder-free culture conditions and found higher proliferation maintained longer than for cells cultured with feeders. This possibility that purified germ cells tend to retain initial characteristics during in vitro culture suggests that there may be cross-talk between germ cell clones.

With or without feeder cells, germ cell propagation continued no longer than 21 d. In an in vitro culture system, supplemental growth factor is essential for long-term culture and self-renewal of germ cells [26–28]. Recent research in zebrafish has reported that recombinant mammalian growth factors containing bFGF and insulin-like growth factor 1 (IGF-1) promoted spermatogonia proliferation in an in vitro culture system. In Nile tilapia, Tokalov and Gutzeit [29] observed that IGF and hCG induced spermatogonia proliferation in vitro. In rainbow trout, studies showed that spermatogonia proliferation could be enhanced by IGF, follicle-stimulating hormone (Fsh), growth hormone, and FGF-2. To maintain germ cell propagation of for a longer period, we added a cocktail of soluble growth factors. The addition of LIF and GDNF induced the highest mitotic activity. Sturgeon serum also significantly increased germ cell proliferation, and was associated with proliferation extension for 21 d. Leukemia inhibitory factor is reported to be an essential growth factor that supports SSC self-renewal in mice [30]. In mammalian testis, Fsh treatment is reported to stimulate the expression of GDNF from Sertoli cells, which binds GDNF family receptor alpha-1 (Gfra1) in undifferentiated spermatogonia A, enhancing their self-renewal and maintenance [31–33]. In fish, expression of Gfra1 has been observed in Nile tilapia [34], dogfish [35], and rainbow trout [36] type A spermatogonia. However, GDNF pathways in teleosts differ from that in mammals. Shikina and Yoshizaki [7] demonstrated that neither rat GDNF nor rat GFRA1-Fc fusion protein enhanced the proliferation of rainbow trout spermatogonia in in vitro culture systems. Expression of *gdnf* has been observed in germ cells but not in Sertoli cells; hence, in rainbow trout, GDNF most likely acts as an autocrine factor [37]; whereas, in zebrafish spermatogonia in vitro culture, recombinant human GDNF was observed to promote spermatogonia proliferation. In dogfish, GDNF could promote self-renewal of potential spermatogonia stem cells in

culture [38]. It would be interesting to determine how these factors influence the in vitro proliferation of sturgeon germ cells. In previous studies, serum of fish including carp and rainbow trout was reported to promote self-renewal of zebrafish or medaka stem cells [5,39]. However, an homologous fish serum for germ stem cell culture has not been reported. Use of serum from the same species might prevent rejection. Sturgeon serum may be a potential alternative to LIF and GDNF.

Barnes and Enat [40,41] reported that FBS contains complex unidentified materials such as plasma proteins, polypeptides, growth factors, hormones, and binding proteins that preserve cell survival when they attach to flasks [40,41]. It may also contain inhibitors of certain tissue-specific cells. An in vitro study of goat germ cell culture showed that high serum concentration may have detrimental effects on germ cell expansion and simultaneously stimulate extensive germ cell growth. Spermatogonia stem cells proliferated for seven days when supplemented with 1% FBS, but higher FBS levels impaired spermatogonia propagation [42]. Spermatogonia stem cell differentiation can be prompted by unidentified components in serum [40]. In our experiment, in the serum-free medium, mitotic activity of germ cells remained elevated for at least 34 d in vitro, and the growth of somatic cells was significantly inhibited.

qPCR analysis revealed no significant differences in marker gene expression before and after 10 d culture, indicating that the sturgeon germ cell population was able to expand in vitro while maintaining original characteristics. We also assessed whether cultured germ cells developed in recipients. Since no effective RNA primers distinguishing sterlet from Russian sturgeon are available, we injected pure cultured germ cells (91%) from sterlet into Russian sturgeon larvae and assessed with a sterlet-specific DNA primer. Results showed that cultured cells maintained their activity to incorporate into recipient gonads. Although it is uncertain whether the cells were germ cells or somatic cells, 91% of the transplanted cells were germ cells, and we suggest it is more likely that the cells developed in recipients were germ cells. Whether normal offspring can be obtained from cultured germ cells in our culture system requires further studies.

In conclusion, we established effective culture conditions that improved the mitotic activity of germ cells and maintained their survival for at least 40 d in culture with stable proliferation in vitro. The results offer useful details for culturing germ cells in sturgeon, representing a first step towards the establishment of germ cell lines that will be useful in germ cell xenotransplantation.

## 5. Conclusions

Enriched germ cells cultured in a serum-free medium proliferated 40 days in vitro. Cultured cells showed development in recipient genital ridges. The culture conditions that we describe could supply germ cells for transplantation and surrogate production of sturgeon and for other in vitro studies.

**Author Contributions:** Conceptualization and methodology, X.X. and P.L.; validation, X.X., M.P. and C.S.; formal analysis, X.X. and P.L.; investigation, M.P. and Q.W.; resources, H.Y., M.P. and Q.W.; data curation, C.L.; writing—original draft preparation, X.X. and P.L.; writing—review and editing, M.P. and Q.W.; project administration and supervision, M.P. and Q.W.

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## CHAPTER 4

# FLUORESCENCE-ACTIVATED CELL SORTING OF STERLET GERM CELLS BASED ON LIGHT SCATTER PROPERTIES

Xie, X., Kislik, G., Franěk, R., Fučíková, M., Steinbach, C., Tichopád, T., Pšenička, M., Fluorescence-activated cell sorting of sterlet germ cells based on light scatter properties. Manuscript.

My share on this work was about 70%.

# Fluorescence-activated cell sorting of sterlet germ cells based on light scatter properties

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**Abstract**

Sturgeons belong to the order Acipenseriformes, which are among the most ancient of actinopterygian fishes. Nowadays, many sturgeon species are critically endangered. Moreover, most sturgeon species are large and late maturing, which makes their culturing and conservation very costly and time-consuming. Germ cell culture and xenotransplantation could be used as an affordable and time-efficient method for surrogate production of endangered sturgeons. Thus, the development of new methods for enrichment of germ cell populations *in vitro* can be expected to increase the efficiency of culture and transplantation.

In our study, we established a method for identification and isolation of undifferentiated type A spermatogonia ($A_{und}$) from different developmental stages of testes using fluorescence-activated cell sorting (FACS). Flow cytometry analysis of freshly prepared whole testicular cell suspension showed a few distinguished cell populations formed according to different values of light scatter parameters. FACS of these different cell populations was performed directly on glass slides for further immunocytochemistry to identify germ cells. Results immunocytochemistry of post sorted cells, as well as immunohistochemistry and histology observation of whole testis sections, showed that the cell population in gate P1 on a flow cytometry plot (with high forward scatter (FSC-A) and high side scatter (SSC-A) parameter values) contains the highest amount of $A_{und}$ cells– over 90% among three different stages. We expect that the use of this FACS strategy can improve the production of sturgeons with surrogate broodstock and further study of cellular and molecular mechanisms of sturgeon germ cell development.

***Keywords:*** *Fluorescence-activated cell sorting, flow cytometry, germ cells, sturgeon, spermatogonia*

## Introduction

Sturgeons belong to the order Acipenseriformes, which are among the most ancient of actinopterygian fishes (Bemis et al. 1997). Sturgeon can be dated back to Upper Cretaceous and diverge from an ancient, pre-Jurassic teleost lineage approximately 300 million years ago (Inoue et al. 2005). Nowadays 27 species of sturgeon were listed as critically endangered in the International Union for Conservation of Nature and Natural Resources Red list, due to habitat alteration caused by damming of rivers, pollution and overharvesting (Birstein et al. 1997; Wei et al. 1997; Zhang et al. 2011). Although controlled propagation has been conducted successfully and proven to be essential for conservation, most species of sturgeon are late maturing, making culture and conservation costly and time-consuming (Bemis and Kynard 1997; Zhuang et al. 1997). Thus, new methods are needed to promote artificial propagation of sturgeon from both aquaculture and conservation viewpoints.

In past decades, germ cell xenotransplantation has been successfully established in rainbow trout (*Oncorhynchus mykiss*) and masu salmon (*Oncorhynchus masou*) (Takeuchi et al. 2004; Okutsu et al. 2007) and further employed in various commercially important fish species, as well as endangered species (Higuchi et al. 2011; Yoshikawa et al. 2017; Morita et al. 2015; Lujić et al. 2018). It proved only germ stem cells can incorporate into xenogeneic recipient's genital ridge, undergo gametogenesis, and generate functional donor-derived offspring in the gonads (Yoshizaki et al. 2012). In fish, undifferentiated type A spermatogonia ($A_{und}$), probably a small portion of the type A spermatogonia population, are considered as spermatogonial stem cells who remains the ability of self-renewal, differentiation, apoptosis and transforming into different cell types (pluripotency) (de Rooij and Russell 2000; Schulz et al. 2010) $A_{und}$ are isolated cells and also the largest germ cells in the fish testis, with large nucleus and high density of mitochondria (Xie et al. 2020). Additionally, the combination of germ stem cell *in vitro* culture, cryopreservation and transplantation is a powerful strategy for preserving precious fish genetic resources of endangered species (Yoshizaki and Yazawa 2019). In sturgeon, Pšenička et al. (2015) established a germ cell transplantation technique for Siberian sturgeon (*Acipenser baerii*) using Sterlet (*Acipenser ruthenus*) as recipients and showed colonization of donor cells in the gonads of recipients. Ye et al. (2017) demonstrated that isolated germ cells from Chinese sturgeon (*Acipenser sinensis*) could be colonized in Dabry's sturgeon (*Acipenser dabryanus*) larvae. With the establishment of germ cell *in vitro* culture (Xie et al. 2019), cryopreservation (Pšenička et al. 2016) and gene-editing techniques such as CRISPR/Cas (Baloch et al. 2019a,b), surrogate reproduction technology is expected to be a powerful method to preserve and recover endangered sturgeon species. Thus, a highly purified population of $A_{und}$ cells is essential to accomplish efficient germ cell *in vitro* culture, cryopreservation, and transplantation. However, the proportion of $A_{und}$ spermatogonia is low and decreasing, with the increasing proportion of differentiated germ cells throughout spermatogenesis. In past decades, Percoll gradient centrifugation was commonly employed on enrichment of germ cells in plenty of fish species (Pšenička et al. 2016; Rolland et al. 2009; Linhartová et al. 2014). Nevertheless, due to its low resolution capacity, Percoll is not accurate enough to enrich high pure homogenous cell populations with similar physiochemical properties, such as type A and type B spermatogonia. Fluorescence-activated cell sorting (FACS) is a precise and efficient method to collect target cells according to self-fluorescence property and specific antibodies conjugated with fluorescence microbeads. Mammalian SSCs have been sorted by FACS based on SSC surface markers, such as α6-integrin, CD9, SSEA-4, GFRA1, THY1, and MCAM (Kanatsu-Shinohara 2012; Kokkinaki et al. 2011; Valli et al. 2014). However, to date, fish SSC markers such as *sgsa-1*, *plzf*, *gfrα1*, *nanos2*, have been identified in only a few species (Xie et al. 2020). Some commercially important or endangered species

lack transgenic strains or specific molecular markers, presenting limitations to detecting and isolating SSCs (Xie et al. 2020). In past decades, type A and type B spermatogonia were enriched respectively from pvasa-GFP transgenic rainbow trout according to cell size and green fluorescent protein (GFP) intensity. Further studies have shown that $A_{und}$ were efficiently enriched only based on light scatters properties in rainbow trout, Japanese char (*Salvelinus leucomaenis*), Nibe croaker (*Nibea mitsukurii*) (Kise et al. 2012; Hayashi et al. 2014) and Pacific bluefin tuna (*Thunnus orientalis*) (Ichida et al. 2017). Therefore, light scattering properties are applicable to enriching type A spermatogonia without cell-labeling systems such as transgenes and cell surface antibodies. Remarkably, using this method, enriched live $A_{und}$ can be employed to produce monoclonal antibodies by inoculating into mice, following with screening with a combination of cell enzyme-linked immunosorbent assay, immunocytochemistry, flow cytometry (FCM), and immunohistochemistry.

In the present study, we used sterlet, the earliest maturity and smallest species, as an appropriate model species in Acipenseriformes. Cultured sterlet can be sexually mature in 3 years (Billard and Lecointre 2000). We investigate the morphology from sterlet testes in different maturing stages. Then we isolated an undifferentiated spermatogonial population from different maturing stages using FACS based on light scatter properties.

## Materials and methods

### Fish

Undifferentiated sterlet testes samples were collected from 4~6-month-old, (~10 cm in length and ~5g in weight), differentiated gonad samples were collected from 16~18-month-old, (~52 cm in length and ~200g in weight) and maturing gonad samples were collected from 2-year-old (~79 cm in length and ~432g in weight). Sterlet used in this study were cultivated in Faculty of Fisheries and Protection of Waters, University of South Bohemia. Deep anesthesia was induced by 0.05% 3-aminobenzoic acid ethyl ester methanesulfonate-222 (MS-222) (Sigma, St. Louis, MO, USA). Animal handling and experimentation were approved by the Ethics Committee on the Ministry of Agriculture of the Czech Republic (reference number: 53100/2013-MZE-17214).

### Preparation of testicular cells

Dissociation of testicular cells was performed, according to Xie et al. (2019). Gonads of sterlet were washed in phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA) containing antibiotics (50 μg/ml ampicillin, 200 U/ml penicillin, and 20 μg/ml streptomycin; all from Sigma, pH 8.0) and minced into 1-$mm^3$ pieces. Then pieces of tissue were dissociated by 0.25% trypsin (Gibco) for 2 h. The digestion was stopped by L-15 medium with 20% fetal bovine serum (FBS), filtered through a 40-μm pore-size nylon screen and centrifuged at 300g. The cell pellet was resuspended in PBS.

### Fluorescence-activated cell sorting of testicular cells

FACS of testicular single-cell suspensions was performed using a cell sorter – BD FACSAriaTM Fusion (BD Biosciences, USA). The cells were sorted using standard sterile setup of the cell sorter – 100 μm nozzle, 20 psi, PBS as a sheath fluid and precooled down to +5C sample holder. Single-cell suspensions of testicular cells prepared as described in (2.2) were sorted onto microscope poly-L-lysine coated slides for further morphology observation

and immunocytochemistry study. The isolation of different cell populations was performed according to their Forward and Side Scatter (FSC-A and SSC-A) values as demonstrated on flow cytometry plots in Fig.1. Preliminary in gating strategy debris, doublet discrimination, and live/dead cell separation was performed. Dead cells were possible to gate out because of their positive signal from Hoechst 33342 staining. For live/dead cells separation, the viability dye – Hoechst 33342 was added to all of the samples in the final concentration of 1ul/ml.

### Immunolabeling

For immunocytochemistry, the sorted cells from defined gates were mounted on microscope glass slides and fixed with 4% paraformaldehyde in PBS for 1 hour. The fixed cells were washed in PBS and permeabilized with 0.3% TritonX-100. The slides were blocked in PBS with 1% BSA and 0.05% Tween 20 (blocking solution) for 40 minutes. Then the slides were incubated overnight at 4 °C with DDX4 antibody (dilution 1:300), which is specific for sturgeon germline cells (Pšenička et al. 2015), and exposed for 1 h at room temperature with secondary antibody antirabbit immunoglobulin G–fluorescein isothiocyanate (FITC; F0382, Sigma, dilution 1:50) following stained with 4,6-diamidino-2- phenylindole solution (3 ng/mL). The slides were observed under a fluorescence microscope IX83 (Olympus, Japan) equipped with an ORCA R2 camera (Hamamatsu Photonics, Japan) and processed in the CellSens Olympus software. For each sample, minimum 500 cells were counted to calculate the proportion of vasa-positive cells, and the proportions were expressed as a percentage of total living testicular cells.

Immunohistochemistry of gonads was modified according to Pšenička et al. (2015). Testes serial sections were sliced by CM1850 Cryostat according to the standard cryo-section method. Subsequent blocking and incubation were processed as described in immunocytochemistry.

### Histology

Sterlet gonad samples, as well as sorted cell samples, were fixed with Bouin's solution overnight, then dehydrated and embedded following the standard paraffin method. The paraffin block was sliced into 4.5 mm serial sections. The paraffin sections were stained with hematoxylin and eosin. Different germ cell populations were identified and count according to the morphology description from Lacerda et al. (2014).

### Statistical analysis

The results were expressed as a ratio against each control as mean ± SEM. Statistical significance was analyzed by R with one-way analysis of variance (ANOVA) for the significant differences between group means. The Tukey-Kramer's multiple comparisons test was used for post-hoc tests. Probability values of $P<0.05$ were considered as significant.

## Results

### Histological observation and flow-cytometric analysis of undifferentiated testes

According to the histological observation, in the 4~6 -month-old testes of sterlet, spermatogonia were mostly large, single cells, surrounded by cytoplasmic extensions of one or two Sertoli cells (Fig 1. A). Immunohistochemistry of vasa antibody indicated that the large, single cells were vasa- positive cells (Fig1. B). Therefore, the vasa- positive cells were expected to be $A_{und}$ spermatogonia. The testes of sterlet are in maturity stage I: containing undifferentiated spermatogonia and somatic cells. 23.99% of cells were $A_{und}$ spermatogonia (Fig.1 C).

FCM sorting plot figure was demonstrated in Fig.1 D. We set three gates to isolate $A_{und}$ cells (Fig.1 E). Cells sorted from P1 gate demonstrated high FSC-A and high SSC-A. According to analysis of immunocytochemistry, 94.51% ±1.26% cells in P1 gate were vasa positive, which is significantly higher than other gates and unsorted cells ($P<0.01$, Fig 1. F, I). In P2 gate, FSC-A and SSC-A were lower than P1. Cells in P3 performed the lowest FSC-A and SSC-A. The vasa positive rate in P2, P3 gate and unsorted living cells were 36.41% ± 4.98%, 4.22% ±1.97% and 22.23% ± 5.63%. The cells sorted from P1, P2 and P3 showed uniform characteristics, respectively. Cells in P1 were >10um of diameter, while cells from P3 gate were smaller. Cells in P1 also showed round shape and big nucleus (Fig 1G, H).

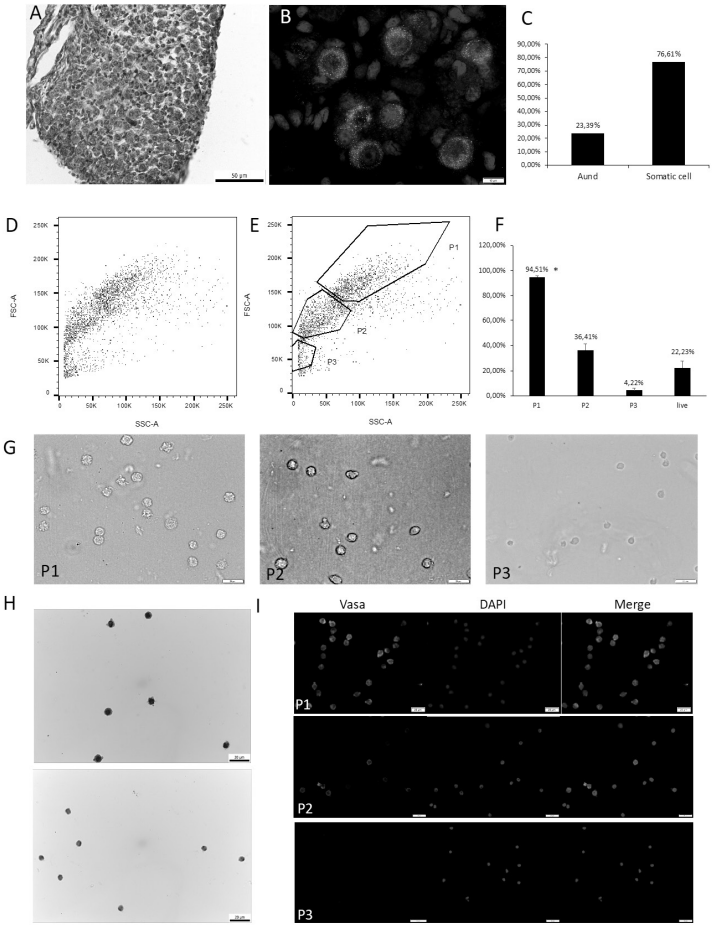


***Figure 1.*** ***Histological observation and light scattering property-dependent fractionation of 6 month-old sterlet testes.*** *A: Testes section stained with hematoxylin and eosin (HE). B: Testes section stained with anti-vasa antibody. C: The proportion of germ cells and somatic cells in testes. D, E: Bivariate histograms of flow cytometry data from dissociated testicular cells prepared from 6 month-old sterlets. Gates P1-P3 were set on the histogram and used for cell sorting. F: Vasa-positive rates of sorted cells in gates P1-P3 and unsorted live cells [mean ± standard error of mean (SEM)]. Means with asterisk indicate significant differences ($P < 0.05$, F). G: Microscopic observation of sorted cells using gates P1-P3. H: HE stained cells from gates P1-P3. I: Immunofluorescent staining for detection/calculation of vasa positive cells in different gates. Blue signal – DAPI staining, Green signal – vasa antibody staining.*

Germ cell cysts can be observed in 18-month-old testes, containing 4–16 spermatogonial cells (Fig.2 A). Compared with $A_{und}$ in 6 month-old testes, most spermatogonial cells showed smaller size, had one or more nucleoli and a relatively large cytoplasmic volume. They are also connected cells with round to oval shape, indicating that $A_{und}$ have differentiated into $A_{diff}$ and type B spermatogonia in cysts. $A_{und}$, $A_{diff}$ and Type B spermatogonia were tested as vasa positive by immunohistochemistry (Fig2. B). The proportion of various germ cells was shown in Fig2. C. Thus, the testes of sterlet are in maturity stage II: containing $A_{und}$, $A_{diff}$ and Type B spermatogonia and somatic cells, but spermatogenesis did not occur.

The same gates set in 3.1 were applied to analyze FCM sorting plot of testicular cells from 18-month-old gonads(Fig 2.D, E). As a result, cells collected- in P1 gate displayed 94.90% ±1.3% vasa positive which is significantly higher than other gates and unsorted cells ($P<0.01$, Fig.2 F). Cells also showed similar diameters and large nucleus (Fig2. G, H). The number of cells located in P2 gate increased, that more than 66.99% ±11.04% were tested as vasa positive cells. Cell size was approximately 8–10μm, relatively smaller than those cells sorted in P1 gate (Fig2. G, H). 5.31% ±3.06% cells showed vasa positive in P3($P<0.01$ Fig. 2 E).

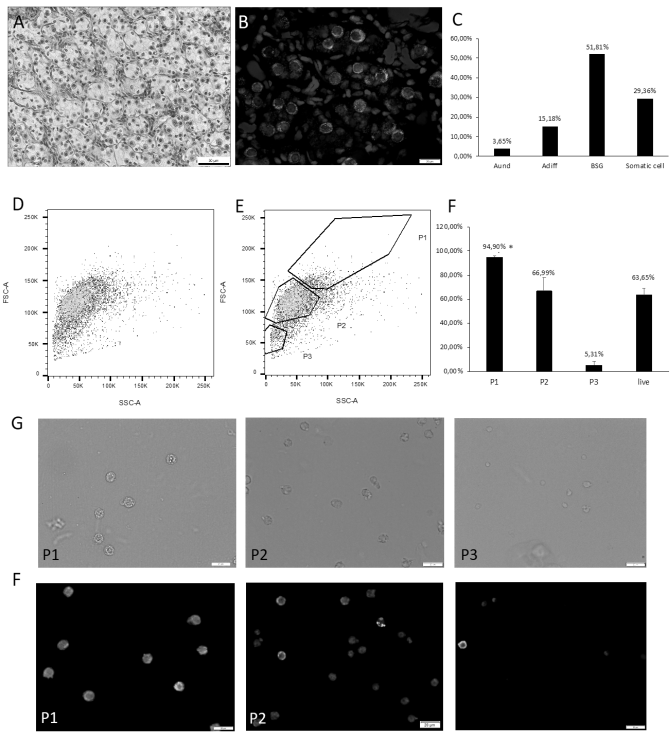


***Figure 2.*** ***Histological observation and light scattering property-dependent fractionation of 18 month-old sterlet testes.*** *A: Testes sections stained with hematoxylin and eosin (HE). B: Testes sections stained with anti-vasa antibody. C: The proportion of various germ cell populations and somatic cells in testes. D, E: Bivariate histograms of flow cytometry data from dissociated testicular cells prepared from 18 month-old sterlets. Gates P1-P3 were set on the histogram and used for cell sorting. F: Vasa-positive rates of sorted cells in gates P1-P3 and unsorted live cells [mean ± standard error of mean (SEM)]. Means with asterisk indicate significant differences ($P < 0.05$). G: Microscopic observation of sorted cells using gates P1-P3. H: Cells from gates P1-P3 stained with anti- vasa antibody and DAPI.*

**Histological observation and fractionation of spermiogenic testes using FCM**

In 2-year-old testes, various germ cell populations can be found in testes, including $A_{und}$, $A_{diff}$, Type B and spermatocytes (Fig 3. A), indicating the testes developed into maturity stage III. The proportion of various germ cells was shown in Fig 3. B. The whole testicular cell suspension was sorted in the same setting in 3.1(Fig 3. C, D). As a result, the rate of vasa-positive cells in P1 was 90.76% ± 3.78%, which was significantly higher than other gates ($P<0.01$, Fig 3. E). Cells located in P1 area were the largest cells compared with cells in P2 and P3 (Fig 3. F, G). The rate of P2, P3 and unsorted living cells were 69.76% ± 6.72%, 37.35% ± 7.10%, and 85.47% ± 0.37%, respectively (Fig 3. E).

Among testes in stage I, II and III, cells sorted from P1 demonstrated similar characteristics, such as large cell size, high karyoplasmicratio. They showed a stable rate of vasa-positive cells ($P>0.05$). Thus, it is expected that the population sorted from P1 gate was undifferentiated spermatogonia.

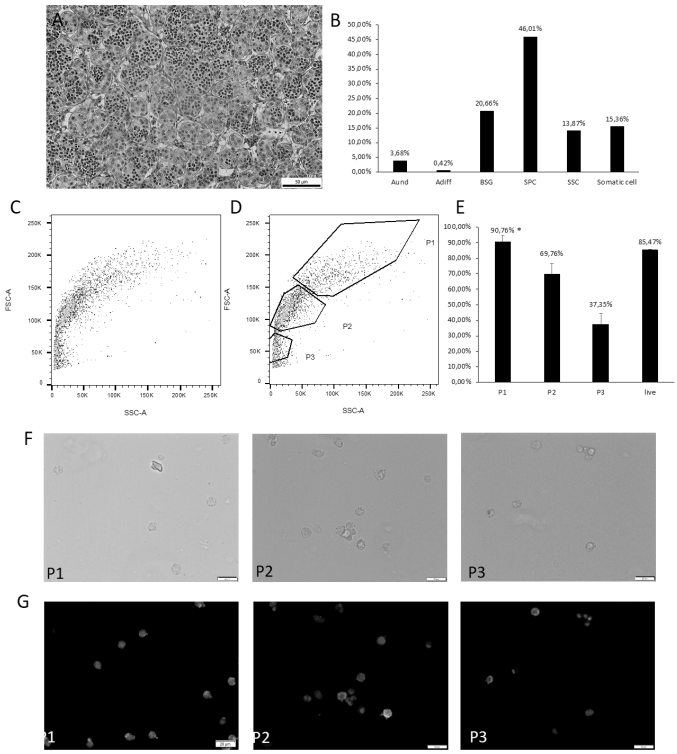


***Figure 3.*** ***Histological observation and light scattering property-dependent fractionation of 2-year-old sterlet testes.*** *A: Testes sections stained with hematoxylin and eosin (HE). B: The proportion of various germ cell populations and somatic cells in testes. C, D: Bivariate histograms of flow cytometry data from dissociated testicular cells prepared from 2-year-old sterlets. Gates P1-P3 were set on the histogram and used for cell sorting. E: Vasa-positive rates of sorted cells in gates P1-P3 and unsorted live cells [mean ± standard error of mean (SEM)]. Means with asterisk indicate significant differences ($P < 0.05$). F: Microscopic observation of sorted cells using gates P1-P3. G: Cells from gates P1-P3 stained with anti-vasa antibody and DAPI.*

## Discussion

In the present study, we established a method to enrich undifferentiated type A spermatogonia of sterlet from testes in maturity stage I, II and III. According to the histological observation of fish testes, undifferentiated type A spermatogonia are the largest cells among all the germ cells throughout the developmental stage and possess the ability of "stemness" (Xie et al. 2020). In maturity stage I, most germ cells are $A_{und}$ cells, surrounded by Sertori cells. Our results demonstrated that cells sorted from P1 gate (both high FSC-A and SSC-A value) showed a very high vasa positive rate. The diameter of the cells was identical to the average size of $A_{und}$ in histological observation. Besides, low FSC-A and SSC-A value area were defined as P3 gate. Most cells from P3 were vasa negative cells. Therefore, we can conclude that vasa-positive cells sorted from P1 gate are undifferentiated type A spermatogonia and vasa-negative cells from P3 gate are somatic cells.

Testes in maturity stage II contain a few amount of $A_{und}$ and $A_{diff}$, and a large number of type B spermatogonia but no spermatocyte. The size of germ cells gradually decreased along with their developmental stage (Schulz et al. 2010). Vasa-positive cells collected from P1 showed similar characteristics as those in undifferentiated testes, indicating that vasa positive cells collected from this gate are $A_{und}$. Interestingly, in this stage, sorting plot based on light scattering property has been changed obviously. More cells located in P2 gate, showing relatively low FSC and SSC, suggesting these cells are smaller than cells in P1 and have simple intracellular structure. The vasa-positive rate of cells in P2 was higher, compared with cells in P1 from stage I testes. On the other hand, histology of testes revealed that $A_{diff}$ and type B spermatogonia have increased in this stage. Thus, the difference of sorting plot may cause by change of the proportion of germ cell populations and vasa-positive cells in P2 gate are expected to contain $A_{diff}$ and type B spermatogonia. In stage III, various germ cell populations were found in testes, including $A_{und}$, $A_{diff}$, type B and spermatocytes. The proportion of cells in P2 gate showed a reduction in sorting plot compared with stage II. Vasa positive cells sorted from P1 gate still demonstrated the same characteristic as $A_{und}$ populations. The vasa-positive cells were also detected in P3 gate, probably because the spermatocytes showed close FSC-A and SSC-A as somatic cells.

The other interesting phenomenon we observed in this study was that side scatter value of the whole testicular cells increased along with forward scatter growing. The factors affecting total light scattering, including the membrane, nucleus, internal complexity of the cell, cell shape and surface topography. ASGs of Pacific bluefin tuna were enriched in the fraction with high FSC and relatively low SSC value (Ichida et al. 2017), as well as in Nibe croaker, rainbow trout and masu salmon (Kise et al. 2012), indicating ASGs possess the largest size and relative simple internal cell structure among above species. It assumed that the light scattering properties of ASGs might be widely conserved throughout the evolution of Perciformes. However, in the present study, ASGs in high FSC fraction always showed high SSC, especially in undifferentiated testes, that most germ cells are undifferentiated spermatogonia. Studies intracellular ultrastructure of sturgeon germ cells is needed in the future. On the other hand, a similar sorting plot was reported in medaka (*Oryzias latipes*). Spermatogonia at early stage were Cells enriched in a fraction in which both FSC and SSC signals were expected as spermatogonia at early stage (Satoh et al. 2019). Monitoring light scatter properties of human pluripotent stem cells, Ramirez et al. (2013) revealed that high SSC cells were characterized by more frequent simultaneous expression of the cell surface pluripotency factors and displayed a higher mitochondrial content. High SSC cells were more likely to generate colonies upon single-cell passage than low SSC cells. Therefore, it will be of interest to reveal whether the side scatters intensity also reflects potential of differentiation in fish germ stem cells.

Enrichment of $A_{und}$ spermatogonia by FACS could help to increase transplantation efficiency (Kise et al. 2012). Flow cytometry is also a powerful tool for transcriptomic or proteomic analyses of various germ cell populations, which can contribute to generating specific cell surface antibodies to identify different germ cell populations (Hayashi et al. 2019; Ichida et al. 2019). In the present study, we established an efficient method using FACS to enrich undifferentiated spermatogonia from sterlet. The purified undifferentiated spermatogonial population could be an ideal material to characterize sturgeon germ cells in transcriptomic or proteomic levels and accelerate various studies regarding the basic and applied biology of fish spermatogonia.

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## CHAPTER 5

# A NOVEL MONOCLONAL ANTIBODY FOR IDENTIFICATION OF TYPE A SPERMATOGONIA IN STURGEON

Xie, X., Ichida, K., Iwasaki-Takahashi, Y., Yoshizaki, G., Steinbach, C., Franěk, R., Pšenička, M., A novel monoclonal antibody for identification of type A spermatogonia in sturgeon. Manuscript.

My share on this work was about 60%.

## A novel monoclonal antibody for identification of type A spermatogonia in sturgeon

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**Abstract**

Sturgeons are ancient species and most of them are under threaten of endangered. So far, identification of various germ cell populations in sturgeon is still lack of knowledge. Although several antibodies were produced, such as Vasa, Dead end and Nanos1, specific antibody that identify of type A spermatogonia (ASGs) has not been found yet. In previous study, monoclonal antibodies were generated by inoculating ASGs of Pacific bluefin tuna (*Thunnus orientalis*) and rainbow trout (*Oncorhynchus mykiss*) into mice. In this study, we detected above monoclonal antibodies to find suitable antibodies that can specifically recognize sterlet ASGs. Two antibodies, no. 20 from Pacific bluefin tuna and no. 180 from rainbow trout, showed strong and specific signal of ASGs. Then, we performed flow-cytometry to isolate different developmental stages of germ cells and somatic cells. According to immunocytochemistry analysis, we found that no. 20- positive cells are not significantly different from vasa-positive cells in P1 fraction, which enrich undifferentiated germ cells, but the rate of no. 20-positive cells were lower than vasa-positive cells in P2 gate, which was assumed containing differentiated spermatogonia. However, no signals of nos. 80 antibody was detected in cell fractions. Therefore, no. 20 antibody derived from ASGs of Pacific bluefin tuna is expected to be a powerful tool to identify sterlet ASGs accurately.

***Keywords:*** *monoclonal antibody; immunohistochemistry; flow-cytometry; sturgeon; type A spermatogonia.*

## Introduction

Sturgeons (Family Acipenseridae, Order Acipenseriformes) are among the most ancient Actinopterygian fishes which are referred to as "living fossils" (Bemis et al. 1997). In past decades, due to human activities, such as damming of rivers, poaching, pollution, habitat degradation and overexploitation for the harvesting of caviar (Birstein et al. 1997; Wei et al. 1997; Zhang et al. 2011), Twenty- seven species of sturgeon were listed as critically endangered in the International Union for Conservation of Nature and Natural Resources Red list and 63% of them were under threatened as critically endangered. Sterlet (*Acipenser ruthenus*), as the earliest maturity and smallest species of Acipenseridae family, is an important aquaculture species. Cultured sterlet, which can be sexually mature in 3 years (Billard and Lecointre 2000), was expected to be an appropriate model species in Acipenseriformes. To date, the surrogate production technique has been firstly established between Siberian sturgeon (*Acipenser baerii*) and Sterlet (*Acipenser ruthenus*) and further applied on Chinese sturgeon (*Acipenser sinensis*) and Dabry's sturgeon (*Acipenser dabryanus*) (Pšenička et al. 2015; Ye et al. 2017). Moreover, *in vitro* cell culture (Xie et al. 2019), cryopreservation (Pšenička et al. 2016) and gene-editing techniques such as CRISPR/Cas and nanoparticles (Baloch et al. 2019a,b) have been employed on sturgeon germ cells. However, since only germ stem cells can colonize in genital ridge of recipients and generate functional donor-derived offspring in the gonads (Yoshizaki et al. 2012), identification and characterization of germ stem cells are essential for efficient transplantation.

Germ cells form complex populations that are involved in gonadal development and gametogenesis. In fish testes, a small portion of spermatogonia, type A undifferentiated spermatogonia, possess "stemness" that are capable of self-renewal or differentiation into daughter cells. To date, Although available information on fish spermatogonial stem cells (SSCs) has expanded in recent decades, markers for SSCs have been identified in only a few species (Lacerda et al. 2014), presenting limitations to detecting and isolating SSCs. The knowledge of fish spermatogonial stem cells has increased in recent decades, but SSC markers, such as *plzf*, *gfrα1,* and *nanos2*, have been found in only a few species, which limits the detection and isolation of SSCs. In sturgeon, fertility genes, such as *boule*, *dazl* (Ye et al. 2015) *dend end* (Linhartová et al. 2015; Yang et al. 2015), *vasa* (Ye et al. 2016) and *piwi* (Yang et al. 2020) have been identified in sturgeon gonads. However, cellular and subcellular localization indicated that the above genes showed differential expression in various developmental stages of germ cells. Still, no gene was found that only specifically expressed in type A spermatogonia (ASGs) yet. What's more, the characteristics of various germ cell populations are also not well understood. Therefore, using antibodies against cell epitopes could be one of the most powerful approaches to recognize ASGs in perspective of protein level. Plenty of antibodies against SSC-specific surface markers have been identified in mammals and further employed for enriching SSCs, including GFR$\alpha$1 (Buageaw et al. 2005), $\alpha$6-integrin (Alipoor et al. 2009; Nickkholgh et al. 2014), CD9 (Kanatsu-Shinohara et al. 2013; Zohni et al. 2012), and Thy-1 (Kubota et al. 2004; Abbasi et al. 2013; Reding et al. 2010). Although cell surface markers would exhibit lower species-specificity if they recognized sugar chains in cell membrane as epitopes, mammalian antibodies are not always suitable for work with fish spermatogonia (Nagasawa et al. 2012; Nakajima et al. 2014). Thus, Hayashi et al. generated novel monoclonal antibodies (mAbs), which specifically identify the cell surface of ASG in rainbow trout (Hayashi et al. 2019). Fluorescent dye-conjugated monoclonal antibodies were produced, which achieve to recognize, visualize, and trace live ASGs in Pacific bluefin tuna (Ichida et al. 2019). In present study, we applied these mAbs in sterlet testes to screen the special antibodies that could recognize the antigens of ASG in sterlet.

## Materials and method

### Fish

Sterlet cultivated in Faculty of Fisheries and Protection of Waters, University of South Bohemia were used for present study. Gonad samples were collected from 8 month-old (~15 cm in length and ~26g in weight) and 24 month-old sterlet (~79 cm in length and ~436g in weight). Deep anesthesia was induced by 0.05% 3-aminobenzoic acid ethyl ester methanesulfonate-222 (MS-222, Sigma, St. Louis, MO, USA). Animal handling and experimentation were approved by the Ethics Committee on the Ministry of Agriculture of the Czech Republic (reference number: 53100/2013-MZE-17214).

### Histology

Sterlet testes were fixed with Bouin's solution at 4 °C overnight, then dehydrated and embedded as the standard paraffin method. The paraffin block was cut into 4.5 mm serial sections. The sections were stained with hematoxylin and eosin (HE).

### Screening of mAbs for A-SG recognition in immature testes

The monoclonal antibodies applied in present experiment were generated from Ichida et al. (2019) and Hayashi et al. (2019). Immunohistochemistry (IHC) was modified from Ichida et al. (2019). The testes of sterlet with all the developmental stages of germ cells were fixed in 4% paraformaldehyde (PFA) 16h at 4 °C. Then tissue was dehydrated and embedded as standard methods. The wax blocks were cut into 4.5 μm serial sections following detaxation and rehydration through a xylene-ethanol series. Antigen retrieval treatment was conducted using Histo VT One solution (Nacalai Tesque, Kyoto, Japan) at 90 °C for 20 min. Subsequently, the sections were washed in PBS containing 0.1% Tween 20 (Merck KGaA, PBST) for 5 min three times. To block the nonspecific antibody, the sections were incubated with Block-Ace (DS Farmer Biomedical, Osaka, Japan) at room temperature for 30-40 min. Then the sections were incubated with mAbs, which were diluted 100-fold in Can Get Signal® immunostain solution B (Toyobo Co., Osaka, Japan) for 16 h at 4 °C following washed three times for 5 min each with PBST. The sections were incubated for 1 h at room temperature in Alexa Fluor 488 Goat anti-Mouse IgG (H + L) (Thermo Fisher Scientific) diluted with Can Get Signal® immunostain solution A (1:200, Toyobo Co.). Finally, the sections were washed three times with PBST for 5 min and observed under a fluorescence microscope (model BX53; Olympus). The slides were then counterstained with hematoxylin and eosin.

### Preparation of live testicular cells

Preparation of sterlet testicular cells was performed according to Xie et al. (2019). Testes were washed in phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA) supplemented with antibiotics (50 μg/ml ampicillin, 200 U/ml penicillin, and 20 μg/ml streptomycin; all from Sigma, pH 8.0). Then the testes were cut into 1-$mm^3$ pieces and dissociated by 0.25% trypsin (Gibco) for 2 h. L-15 medium with 20% fetal bovine serum (FBS) was added to stop the digestion. After filtered through a 40-μm pore-size nylon screen, the cell suspension was centrifuged at 300g and resuspended in PBS.

### Cell sorting and immunocytochemistry

The experiment of flow cytometry (FCM) of sterlet testicular cells was performed according to Chapter 4. Cells were sorted from defined gate P1 and total living cells. The method of immunocytochemistry (ICC) was modified according to immunohistochemistry in 2.3. Sorted cells were mounted on microscope glass slides, fixed in PFA and permeabilized with 0.3% TritonX-100. Then the cells were blocked with Block-Ace at room temperature for 30–40 min. Then the slides were incubated 16 at 4 °C with DDX4 antibody (dilution 1:300) and selected monoclonal antibodies, which is specific for sturgeon germline cells. After washed three times for 5 min with PBST, the slides were incubated with anti-rabbit IgG–fluorescein isothiocyanate (FITC; F0382, Sigma, dilution 1:50) and Alexa Fluor 647 Goat anti-Mouse IgG (H + L) (ab150115, Abcam, dilution 1:200), both diluted in Can Get Signal® immunostain solution A. After stained with 4,6-diamidino-2- phenylindole solution (DAPI, 3 ng/mL). For each sample, minimum of 500 cells were counted to calculate the proportion of cells with positive signals, and the proportions were expressed as a percentage of total living testicular cells.

### Statistical analysis

The results were expressed as a ratio against each control as mean ± SEM. A two-tailed Student t-test was used to evaluate whether there was a significant difference in the mean of vasa and No.20 antibodies (using an F-test to check that the variances of the populations were equal). Probability values of $P<0.05$ were considered as significant.

## Results

### Screening of antibodies capable of recognizing ASGs

In present study, to identify available antibodies for sterlet ASGs, we screened 383 antibodies, which were generated from direct inoculation of female BALB/c mice with enriched live Pacific bluefin tuna ASGs (Ichida et al. 2019). IHC was performed on histological sections of 20 month-old sterlet testes. In this age, all developmental stages of germ cells, from ASGs to spermatozoa, were observed in testes (Figure 1, HE).

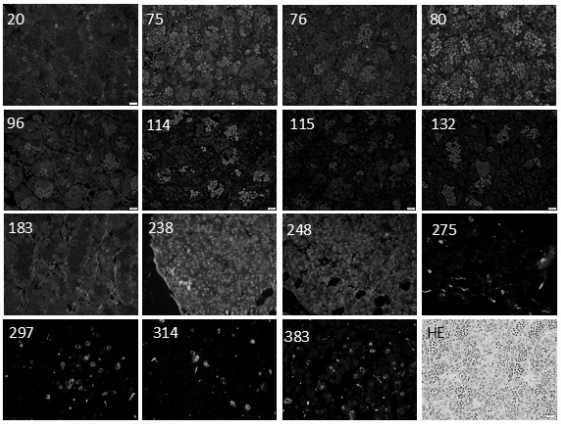


***Figure 1.*** ***Immunohistochemistry analysis of testes from 24-month-old sterlet.*** *Green fluorescence micrographs are shown. The antibody number is shown at the upper left corner of each panel. Histology of 24-month-old sterlet testes is shown in the last photo (HE). Scale bar = 20 μm.*

Fluorescence signals were observed in 15 of 384 antibodies, indicating that these antibodies could identify germ cells (Figure 1). However, 14 of them showed positive signals in various types of germ cells, including type B spermatogonia and spermatocytes. When using antibody No. 20 antibody, a particularly clear and specific signal was observed in ASGs, and minimal signals were detected in other types of germ cells and somatic cells (Figure 2, A). Noticeably, No. 20 antibody labeled speckles in the cytoplasm of ASGs instead of cell membrane. Except for the above antibodies, the remaining antibodies were not detected positive signals in a portion of germ cells.

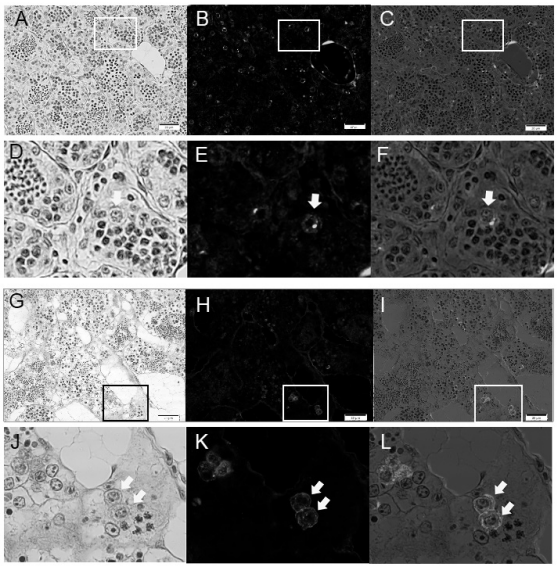


***Figure 2.*** ***Immunohistochemistry with monoclonal antibody No. 20 from Pacific bluefin tuna (A - F) and No. 80 from rainbow trout (G - L) in the testes of mature sterlet.*** *HE stained (A, D, G, and J), fluorescence stain (B, E, H, and K) and merged (C, F, I, and L) views of paraffin sections of mature sterlet testes are shown. High magnifications of the insets in A - C and G - I are shown in D - F and J - L, respectively. Antibody signals were detected on the type A spermatogonia (ASGs; arrowheads).*

On the other hand, IHC was also performed in the antibodies produced by immunizing ASGs from rainbow trout (Hayashi et al. 2019). In total, 59 of antibodies were detected to filter the suitable antibodies that can recognize sterlet ASGs. A strong and specific signal was detected in ASGs using No. 80 antibody, but not in other developed germ cells and somatic cells (Figure 2. B, C). The other antibodies had the capability to distinguish germ cells, while ASGs were not the unique population showing positive signals.

### Flow-cytometry and immunocytochemistry of testicular cells

To evaluate whether No. 20 and No. 80 antibodies respectively against ASGs of Pacific bluefin tuna and rainbow trout, we dissociated 8-month-old testicular cells and performed FCM to enrich undifferentiated type A spermatogonia ($A_{und}$). In this age, cysts could be observed in sterlet testes, which contained $A_{und}$, differentiated A spermatogonia ($A_{diff}$), and few type B spermatogonia (Figure. 3, A). IHC using No. 20 antibody showed positive signals in ASGs of immature testes similar to vasa antibody (Figure. 3, B - D).

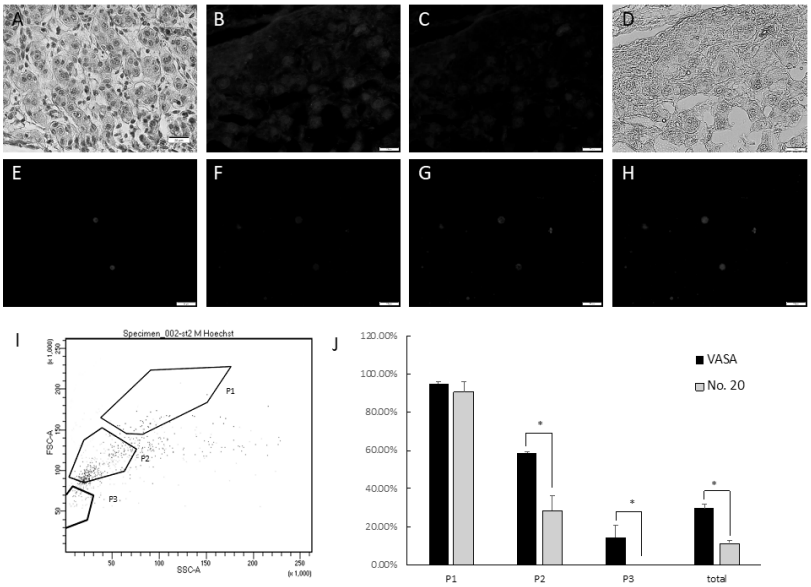


***Figure 3.*** ***Immunohistochemistry with monoclonal antibody No. 20 from Pacific bluefin tuna in the testes of 8-month-old sterlet.*** *Paraffin sections were stained by HE (A), vasa antibody (B), No.20 antibody (C), and bright field (D). Cells sored from P1 were labeled by DAPI (E), No. 20 antibody (F), vasa antibody (G), and merge (H). Flow cytometry sorting plot from dissociated testicular cells prepared from 8-month-old sterlets (I). Gates P1-P3 were set on the histogram and used for cell sorting. Vasa-positive and No.20-positive rates of sorted cells in gates P1-P3 and unsorted live cells [mean ± standard error of mean (SEM), ]]. Means with asterisk indicate significant differences (P < 0.05).*

Cells sorted by FCM were performed clear and specific signals using No. 20 antibody (Figure 3. E - H). However, we did not observe positive signal in dissociated cells using No. 80 antibody. Cells sorted from three defined gates were detected with vasa, No. 20 and No. 80 antibodies. FCM analysis revealed that 90.42% ± 5.42% of P1 cells were identified as positive using No. 20 antibody. 94.76% ± 1.2% of vasa-positive cells were observed in P1, which was not significantly different between both antibodies (P>0.05). 58.78% ± 0.64% of P2 cells were vasa-positive, while 28.7% ± 7.77% of them showed positive signal to No. 20 antibody, which

is significantly lower than vasa ($P< 0.05$, Figure 3. I - J). The positive rate of vasa and No. 20 in P3 fractions were 14.32% ± 6.51% and 0%, respectively. 29.68% ± 2.26% of cells were vasa-positive and 11.59% ± 1.31% of cells were No.20-positive among live testicular cells.

## Discussion

In some endangered or aquaculture species, transgenic method is not suitable for their conservation and applications because the offspring produced by transgenic fish germ cells also carry those transgenes. Recently, Ichida and colleagues (Ichida et al. 2019) inoculated enriched live ASGs into mice and produced mAbs that could recognize the cell surface antigens of ASGs in Pacific bluefin tuna (*Thunnus orientalis*). As a result, live ASGs could be visualized and traced by available antibodies in one-step immunocytochemistry which was conjugated with fluorescent dye. Monoclonal antibodies were also generated from rainbow trout to identify its ASGs (Hayashi et al. 2019). Generally, some surface antibodies show lower species specificity that some markers from mouse can be employed on human germ cells (Oatley and Brinster 2008; Kanatsu-Shinohara et al. 2012; Kokkinaki et al. 2011; Altman et al. 2014; Valli et al. 2014), even mouse anti-Thy1.2 (CD90.2) were capable of identifying carp ASGs through magnetic-activated cell sorting (MACS, Panda et al. 2011). Therefore, in present experiment, we screened these antibodies produced from Pacific bluefin tuna, expecting to find suitable antibodies that can label sterlet ASGs specifically.

Results of IHC indicated that No. 20 antibodies from Pacific bluefin tuna and No. 80 from rainbow trout had the capability to recognize sterlet ASGs. Subsequently, we detected whether these two antibodies work on ICC as well as on IHC. According to the sorting experiments in chapter 3, testicular cells containing $A_{und}$, $A_{diff}$ and few type B spermatogonia were sorted as three gates to figure out the difference of staining between vasa antibody and mAbs. As a result, P1 cells, which were expected to be $A_{und}$, showed no different staining efficiency of vasa and No. 20, while vasa-positive rate in P2 was higher than No. 20-positive. Since cells in P2 were considered to contain type B spermatogonia, we assumed this difference between vasa and No. 20 was caused by type B spermatogonia. However, we did not detect signals from No. 80. Thus, No. 20 antibody from Pacific bluefin tuna could be a suitable antibody to identify ASGs of sterlet.

Interestingly, the localization of No. 20 antibody staining was in the cytoplasm of ASGs, although those antibodies were produced against epitopes of the cell surface membrane. In this study, the epitopes of antibody No. 20 has not been identified yet. However, a preliminary study on N-glycosidase digestion of these antibodies from bluefin tuna revealed that several antibodies recognize sugar chains as their epitopes (Ichida et al. 2019). Generally, antibodies that recognize epitopes of sugars show lower species specificity compared with antibodies that recognize epitopes of proteins. The epitopes of No. 20 antibody is of interest to identify in our future studies.

In present study, we identified a novel antibody that can specifically recognize sterlet ASGs. Some studies reported sturgeon germ cell antibodies such as vasa (Ye et al. 2016), dead end (Yang et al. 2015) and nanos (Ye et al. 2012), but these antibodies expressed in all developmental stages of germ cells. Thus, No. 20 antibody from Pacific bluefin tuna could be a convenient tool for sterlet to evaluate the efficiency of studies regarding ASGs. For example, No. 20 antibody can achieve the accuracy of the ASGs identification *in vitro* cell culture, which is vital to investigate the culture conditions for germ cell expansion and long period maintain with original cellular characteristics. The generation of monoclonal antibody provided a notable method which is efficient to enrich ASGs and practical for aquaculture and it can also readily be applied to various species since transgenic fish offspring possess transgenes, which are not suitable for aquaculture applications and endangered species conservation.

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## CHAPTER 6

## General discussion

Spermatogenesis is a continuous and dynamic developmental process that spermatogonial stem cells (SSC) undergo proliferation and differentiation to form a mature spermatozoon. Isolation, enrichment, and *in vitro* culture of germ cells are expected to provide a large amount of pure SSCs for further manipulation, such as cryopreservation and transplantation (Chapter 2). An optimal *in vitro* culture condition of germ cell was established in sturgeon. Proliferation and survival of germ cells were increased by elimination of gonadal somatic cells, the addition of growth factors, and the replacement of fetal bovine serum (FBS). Cultured germ cells were capable of incorporating into the recipient's genital ridge after transplantation (Chapter 3). A protocol of enrichment of undifferentiated type A spermatogonia ($A_{und}$) was established in sterlet (*Acipenser ruthenus*). A high purity population of $A_{und}$ could be isolated from sterlet testes in different stages using fluorescence-activated cell sorting (FACS) based on light scatter properties (Chapter 4). A novel monoclonal antibody derived from Pacific bluefin tuna type A spermatogonia (ASG) can recognize ASGs in sterlet (Chapter 5).

### Fish germ cell culture

Spermatogenesis is a continuous and orderly developmental process in which a single diploid spermatogonial stem cell (SSC) proliferates and differentiates to form mature spermatozoa. In teleosts, $A_{und}$ are regarded as SSCs, which are singly isolated and encapsulated by Sertoli cells (Lacerda et al. 2014). The balance between SSC self-renewal and differentiation is maintained in a specialized microenvironment in the testes known as the testicular niche (de Rooij 2001, 2006, 2018; Nóbrega et al. 2010; de Cuevas and Matunis 2011; Lacerda et al. 2012; de Siqueira-Silva et al. 2019), which provides growth factors and cell-to-cell interactions (Chapter 2). Furthermore, self-renewal and differentiation of ASGs are regulated by endocrine and paracrine in complex pathways, such as Wnt5a and insulin-like peptide 3 (Insl3) (Safian et al. 2018, Assis et al. 2016), expressed in Leydig cells, and Tgf-β and Amh, expressed in Sertoli cells (Skaar et al. 2011; Morais et al. 2017; Adolfi et al. 2019). On the other hand, surrogate reproduction technology has developed in various fish species (Yoshizaki and Yazawa, 2019), which accelerates the development of transplanting germ cells from different species and produces donor-derived gametes in recipients. Germ cell manipulation, including isolation, enrichment, and cell culture to support their proliferation without differentiation, will help to overcome the limitations of low germ cell numbers for cryopreservation and transplantation (Chapter 2). Surrogate reproduction technology is regarded as a delightful strategy to recover endangered sturgeon resources. To date, germ cell transplantation has been established on several sturgeon species (Pšenička et al. 2015; Ye et al. 2017).

To obtain specific germ cell populations, efficient purification and long-term amplification are required to accomplish a stable supply. We established an *in vitro* culture condition of germ cells in sturgeon and optimized it for longer survival and higher proliferation rate (Chapter 3). It commonly believed that collagenases and dispase are relatively mild enzymes to minimize damaging effects on cells and widely applied to various species (Ohgawara et al. 1987). The disruption of epitopes from cell membrane surface has been observed during trypsin digestion, which may impair germ cell function (Zou et al. 2011; Garcia and Hofmann 2012; Shikina et al. 2013). In the testicular culture system of Japanese eel (*Anguila japonica*), trypsin was found to be an essential factor in the control of germ cell development (Miura et al. 2009). However, as it showed by Pšenička et al. (2015) and Ye et al. (2017), trypsin was the preference to digest gonads in sturgeon. We observed that the type of enzyme did not significantly affect

proliferation of germ cells (Chapter 3). In rainbow trout (*Oncorhynchus mykiss*), germ cells digested by trypsin could be recovered in short term culture (Shikina et al. 2013). it would be of interest whether trypsin damage epitopes of sturgeon germ cells in the future.

On the other hand, as the summary in Chapter 2, different types of somatic cells will influence spermatogonia on their self-renewal vs. differentiation. The mixture of somatic cells contained multiple cell types that were used as feeder cells in Chapter 3. However, feeder cells did not increase the proliferation rate of germ cells. Probably factors secreted by feeder cells were antagonistic with the component in the culture medium.

**Interaction of somatic cells and germ cells**

In past decades, the function of testicular somatic cells as feeder cells has been revealed along with the investigation of cell signalling pathways of various types of somatic cells. Meanwhile, proper soluble growth factors were figured out to support self-renewal or differentiation of spermatogonia/ oogonia. Here we summarized the timeline of developments of feeder cells and factors in fish germ cell culture, mainly on zebrafish, medaka, and rainbow trout, in past 40 years (Figure 1). Germ cell *in vitro* activity may also occur partly through the intercellular junction and not only through paracrine signals from Sertoli cells. Cell-cell interactions are involved in the maintenance and regulation of spermatogenesis in mammals gonads (Russel 1993).

In fish, it was revealed that cellular interactions play an essential role in structure and function. Studies showed that that Sertoli cells and germ cells communicate mainly through cell-cell junctions, such as adhesive junctions, gap junctions, and tight junctions (Pointis et al. 2005). As shown in Chapter 2, incomplete cytokinesis occurs at the end of mitosis during spermatogenesis. The two newly-generated spermatogonia remain connected by a cytoplasmic bridge, instead of forming individual cells. But the cytoplasmic bridge is not present in the descendants of an SSC that enters a self-renewal pathway in which two single, isolated daughter cells are generated. What's more, it is also reported in literatures that germ cell membranes are apparently in contact with each other, as germ cells within a cyst are not normally separated by Sertoli cell processes (Loir et al. 1995). Another interesting characteristic is that germ cells were interconnected through strong adhesive junctions in trout testes (Loir et al. 1995). This evidence indicates that Sertoli cell and germ cell junctions might be involved in the regulation of germ cell development via coordinating mechanisms (Batlouni et al. 2005). There is evidence of the interaction of somatic cells and germ cells in *in vitro* culture.

In Chapter 3, we found that germ cells maintained better proliferation in feeder-free culture than cultured with somatic cells. The addition of proper soluble growth factors is essential for sturgeon germ cell survival and proliferation in long-term culture (Chapter 3). In coculture with somatic cells, there is still the fact that germ cells are more likely to attach on somatic cells, form clumps, expand their number, and then initiate spermatogenesis (Sakai 2002). It seems uncertain that formation of clumps is the signal of differentiation (Sakai 2002; Hong et al. 2004; Shikina and Yoshizaki 2010). In other words, how germ cell interaction plays a role in spermatogenesis? In feeder-free culture, spermatogenesis was automatically induced when cells were kept at high confluence without subculture in medaka (*Oryzias latipes*) testis (Hong et al. 2004). However, enriched carp (*Labeo rohita*) SSCs formed colonies, remained undifferentiated, and proliferated for more than 60 days in *in vitro* culture (Panda et al. 2011). Thus, our experiment would provide information about the role of intercellular junctions on germ cell development, and whether there is synergistic/antagonism between soluble growth factors and the cellular junctions. As feeder cells used in our experiment were derived from

testes, they might contain various somatic cell types. Further assessment needs to be done to establish a pure Sertoli cell line derived from sturgeons to investigate their function to germ cell proliferation.

FBS is the most commonly used in cell culture. It is believed that serum has been applied to supply essentials nutrients for *in vitro* cell growth (Creemers et al. 2002). Serum contains not only plasma proteins, polypeptides, growth factors, hormones, binding proteins, and contact and extension factors that protect cells from damage when they adhere to the culture plates, but also some unknown components that affect cell growth (Barnes and Sato 1980).

In fish germ cell culture, FBS was used at beginning to support germ cell proliferation (Creemers et al. 2002). However, the culture of trout ASGs demonstrated that ASGs proliferate under culture condition with high concentration of serum but eventually disappeared (Shikina et al. 2008). Somatic cells gradually occupied space and nutrition in a given culture system, especially in higher serum concentration since trout ASGs have an extremely slow cell cycle (Shikina et al. 2008). In low serum concentration, mouse SSCs attach to somatic cells and begin to proliferate after somatic cell attachment and proliferation on flasks first (Shikina et al. 2008). On the contrary, in a higher serum concentration, SSCs formed colonies and propagated but finally stop growth and detached due to the extensive growth of fibroblasts (Kanatsu-Shinohara et al. 2005).

FBS contains a wide-range of both inhibitory and stimulatory factors. Germ cells detached from feeder cells or the flask and their morphology changed in the later period of culture, which was considered to be apoptosis or differentiation. The pathways of cell proliferation and apoptosis are coupled (King and Cidlowski 1998; Durrieu et al. 2000; Kuranaga and Masayuki 2007; Charvet and Striedter 2004). Germ cells and/or their associated Sertoli cells were responsive to both inhibitory and stimulatory signals, which result in a conflict of signals and ultimately cell death (Bertolero et al. 1986; Zheng et al. 2006). Under *in vivo* conditions, these antagonistic factors probably act in synergy to maintain physiological balance with the regulation of pituitary, while this may not be the case in *in vitro* culture.

It reported BMP-4 could induce spermatogonial differentiation (Kodaira et al. 2006) both *in vivo* and in spermatogonial cultures in zebrafish (Wong and Collodi 2013) and mouse (Neumann et al. 2011; Nagano et al. 2003; Pellegrini et al. 2003). To overcome these disadvantages, serum-free culture of mouse SSCs was first established by Kubota (Kubota et al. 2004). In fish, to suppress the overgrowth of testicular somatic cells, Shikina and Yoshizaki developed a new culture condition by replacing FBS by adding soluble factors (Shikina and Yoshizaki 2010). This culture system could extend the period of ASG proliferation, maintaining their original morphology and GFP intensity similarly to vas::GFP trout of spermatogonia without promotion of somatic cell overgrowth (Shikina and Yoshizaki 2010). Subsequently, low serum or serum-free culture conditions were developed in more fish species. For example, in zebrafish, female germline stem cells could remain proliferation more than six weeks *in vitro* based on a serum-supplemented proprietary medium (Stempro-34, Wong et al. 2013a).

It is worthy of establishing a well-defined medium for mixed cell populations in a way that nontarget cells are inhibited to growth. At the same time, the target cells are stimulated to proliferate by specific growth factors present in the medium.

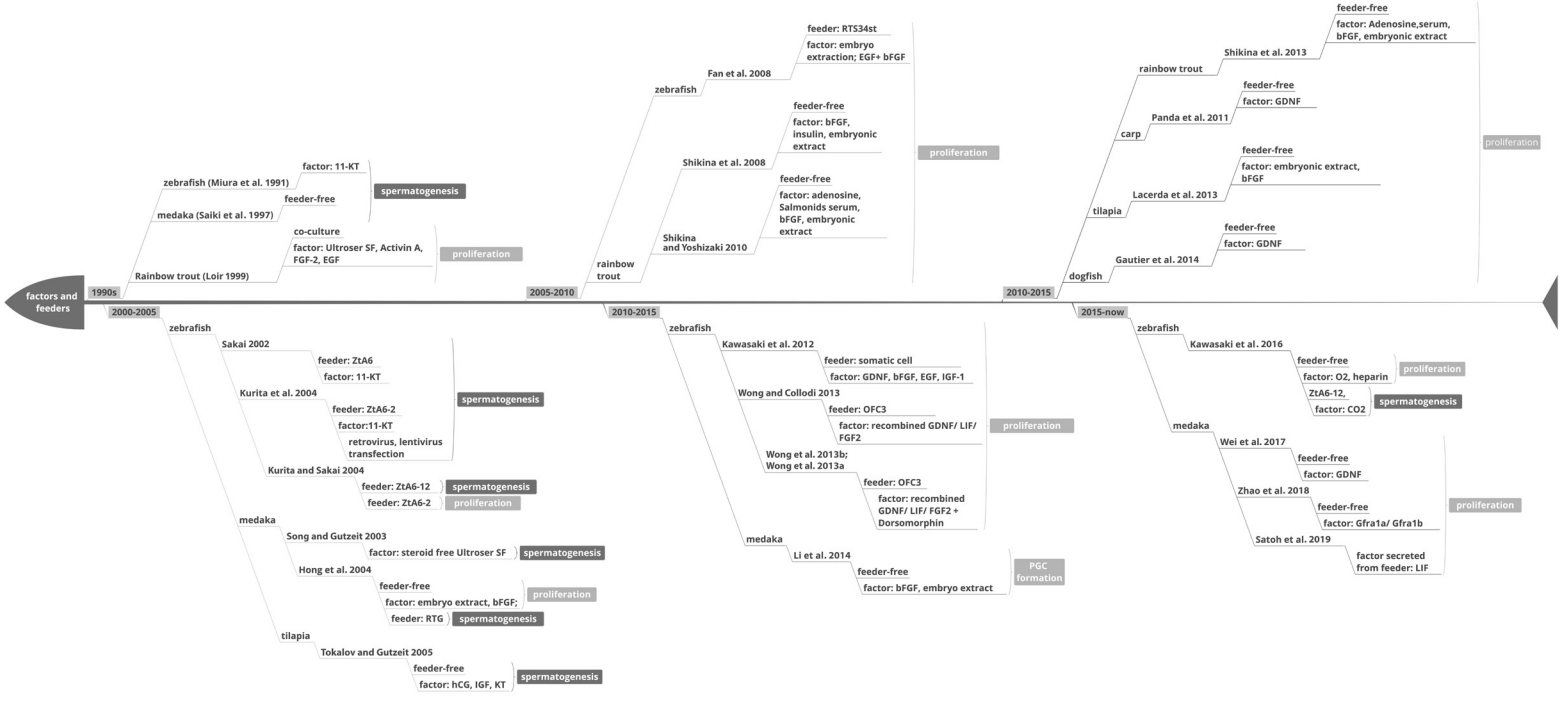


***Figure 1.*** *Major landmarks concerning fish germ cell culture and previous related studies regarding feeder cells and growth factors from the 1990s to now.*

**Application of fluorescence-activated cell sorting on fish**

In summary, we established effective *in vitro* culture conditions of sturgeon germ cells in Chapter 3. Under this condition, germ cells were capable of proliferating for at least 40 days. Morphology remained similar to that of fresh germ cells. Germ cells cultured more than 40 days showed development in recipient genital ridges after transplantation. So far, the immortality of fish germ cells only achieved in few species (Hong et al. 2004). The investigations of different factors can offer useful details to reveal regulation of sturgeon germ cell development, representing a first step towards the establishment of germ cell lines in sturgeon. What's more, combined with cryopreservation (Pšenička et al. 2015), the culture conditions that we described could maintain a long term supply of germ cells for surrogate production and other *in vitro* studies.

In Chapter 3, we isolate sturgeon germ cells by differential plating. The purity of rainbow trout spermatogonia can even reach to >95% using differential plating (Shikina et al. 2008; Shikina and Yoshizaki 2010). Compared with density gradient centrifugation discontinuous, such as Percoll or Ficoll, differential plating is an effective method to obtain high purity of germ cells without causing damage. However, enrichment usually takes five to seven days. It should be taken into account that cellular properties might change during the *in vitro* culture period. Besides, regardless of differential plating or density gradient centrifugation discontinuous, it is hard to isolate specific germ cell subpopulations, especially ASGs. A small portion of the ASGs are undifferentiated, which remain the ability of self-renewal, differentiation, apoptosis, and transforming into different cell types (de Rooij and Russell 2000; Schulz et al. 2010). Therefore, we established a method to enrich undifferentiated spermatogonial population from different ages using FACS based on light scatter properties (Chapter 4). The sorting plots were different from maturity I, II and III, but we figured out that cells sorted from P1 gate, which performed both high forward scatter (FSC-A) and side scatter (SSC-A) value, showed very high vasa positive rate. Since there were no suitable antibodies to test ASGs specifically, morphological characteristics were also observed to distinguish cell type. No matter which stage the gonad was, cells in P1 gate possessed the biggest cell size and nucleus. Especially testes in undifferentiated stage mainly contained two populations, undifferentiated spermatogonia, and somatic cells. Thus, we hypothesized that cells performed high FSC-A and SSC-A properties were undifferentiated type A spermatogonia.

To date, enrichment of type A spermatogonia was reported in few fish species. Nagasawa and colleagues used pvasa-GFP transgenic rainbow trout and sorted cells based on diameter and Gfp intensity (Nagasawa et al. 2010; 2013). Further studies have shown that physiochemical characteristics of FACS, such as light scatters, could be used for sorting $A_{und}$ from Gfp-transgenic rainbow trout (Kise et al. 2012). Transplantation efficiency could be increased by enrichment of $A_{und}$. For transgenic fish, target cells can be sorted by FACS according to their self-fluorescence property. But for some endangered or aquaculture species, transgenic method is not suitable or available. Ichida et al. have established a technique to purify ASGs of immature, maturing and spermiogenic testes of Pacific bluefin tuna (*Thunnus orientalis*) based on light-scattering properties, indicating light scattering properties are applicable to enriching type A spermatogonia without cell-labeling systems such as transgenes and cell surface antibodies (Ichida et al. 2017).

In Chapter 4, we established an efficient method using FACS to enrich undifferentiated spermatogonia from sterlet. Type A spermatogonia cell sorting cells are expected to be an ideal material to characterize sturgeon germ cells in transcriptomic or proteomic level. This is also an important advantage when considering its application to commercially valuable or endangered species that lack the transgenic strains or specific molecular markers necessary to identify specific cell lineages.

In fact, the development of FACS revolutionized single-cell analysis. In the present thesis, sorted cells can be collected into multi-well plates as single cell for downstream experiments (data not performed). On the one hand, target cell characteristics such as morphology, viability, and surface markers indicated by light scattering and fluorescence are captured and analyzed, and cells with defined signals can be collected. On the other hand, FACS enables data exploration, quality control, and testing in single-cell qPCR-based gene expression experiments. Therefore, some cells sorted by FACS using surface markers were supposed to be monolithic. Still, mRNA expression of specific genes within these cells can be heterogeneous (Dalerba et al. 2011), indicating that cells consist of subpopulations. More and more studies based on the flow cytometry platform have made essential contributions to the classification and quantification of dozens of single-cell parameters for specific cells (Bendall et al. 2011; Bandura et al. 2009; Müller and Nebe-Von-Caron 2010). A rare circulating tumor cell (CTC) was recently isolated from a pancreatic ductal adenocarcinoma based on a novel flow cytometer.

In addition, imaging flow cytometry (IFC) has been widely utilized to characterize dielectric properties of the cytoplasm and morphological and molecular localization information by capturing single cells and tracking single-molecule in individual cells within a living organism (Spitzer and Nolan 2016; Muñoz et al. 2018; Hyka et al. 2013). Moreover, Since IFC is capable of separation of recombinant cell lines at the single-cell level, it helps to quantify organelles, peptides, and messenger RNA (Pekle et al. 2019) which possess large potential for clinical application.

In general, flow cytometry becomes increasingly crucial in the study of complex single-cell systems. Although there have not been many reports on germ stem cells, the application of flow cytometry in screening tumor cells and other types of stem cells has provided new insights for characterization of germline stem cells.

With the demanding of identification of ASGs, we tested the monoclonal antibodies derived from Pacific bluefin tuna on sterlet testes to screen the special antibodies that could recognize the antigens of ASG in sterlet (Chapter 5). In the present thesis, we summarized the spermatogonial stem cell markers in fish (Chapter 2). The knowledge of SSCs was increasing, but SSCs markers have been found in only a few species. Several surface markers were found to sort spermatogonia in mammals differentially. For example, GFRα1 (Buageaw et al. 2005), α6-integrin (Alipoor et al. 2009; Nickkholgh et al. 2014), CD9 (Kanatsu-Shinohara et al. 2013; Zohni et al. 2012), and Thy-1 (Kubota et al. 2004; Abbasi et al. 2013; Reding et al. 2010) have been used to isolate SSCs in human, mouse, and bull. Thy1.2 (CD90.2) antibody from mouse was proved to recognize ASGs in carp (Panda et al. 2011) and catfish (Nayak et al. 2016). In sturgeon, differential expression of fertility genes, such as *boule*, *dazl* (Ye et al. 2015) *dend end* (Linhartová et al. 2015; Yang et al. 2015), *vasa* (Ye et al. 2016) and *piwi* (Yang et al. 2020) have been identified in sturgeon gonads. Still, so far, the gene specially expressed in ASGs has not been found yet. Monoclonal antibodies (mAbs), which specifically recognize the cell surface antigens of ASGs were generated from rainbow trout and Pacific bluefin tuna, respectively (Ichida et al. 2019; Hayashi et al. 2019). In Chapter 5, we applied these mAbs in sterlet testes to screen the special antibodies that could recognize the antigens of ASG in sterlet. Immunolabeling demonstrated that No. 20 antibody from Pacific bluefin tuna could acknowledge sterlet ASGs specifically. The identification of ASGs by mAbs No. 20 provides a convenient tool to evaluate the efficiency of germ cell manipulation. It will be of interest to identify the epitopes of No. 20 antibody in the future study.

## Germ stem cell applications

### Germ cell transplantation

Combined with techniques, such as cryopreservation and transplantation, germ stem cells can be a robust material for studying gonadal development of model, aquaculture, and endangered species. Germ stem cell isolation, enrichment, and culture conditions that support their expansion without differentiation will help to overcome the limitation of the low germ cell number before cryopreservation and transplantation purposes.

Surrogate reproduction is one of the most useful technologies of germ stem cells that produce donor-derived gametes in a recipient by transplanting germ cells of a donor into a recipient of a different strain or species. This technique is a complex strategy, involving amplification and cryopreservation of germ cells, preparation of sterile recipients, transplantation, and production of donor-derived offspring in recipients. In past decades, surrogate reproduction has been successfully established in rainbow trout (*Oncorhynchus mykiss*) and masu salmon (*Oncorhynchus masou*) (Takeuchi et al. 2004; Okutsu et al. 2007) and further employed in various commercially important fish species, as well as endangered species (Higuchi et al. 2011; Yoshikawa et al. 2017; Morita et al. 2015; Lujić et al. 2018). It proved only germ stem cells can incorporate into xenogeneic recipient's genital ridge, undergo gametogenesis, and generate functional donor-derived offspring in the gonads (Yoshizaki et al. 2012). In sturgeon, Pšenička et al. (2015) established a germ cell transplantation technique for Siberian sturgeon (*Acipenser baerii*) using Sterlet (*Acipenser ruthenus*) as recipients and showed colonization of donor cells in the gonads of recipients. Ye et al. (2017) demonstrated that isolated germ cells from Chinese sturgeon (*Acipenser sinensis*) could be colonized in Dabry's sturgeon (*Acipenser dabryanus*) larvae. With cryopreservation (Pšenička et al. 2016) and gene-editing techniques such as CRISPR/Cas (Baloch et al. 2019a,b), surrogate reproduction technology is expected to be a powerful method to preserve and recover endangered sturgeon species. Thus, a highly purified population of $A_{und}$ cells is essential to accomplish sturgeon surrogate reproduction strategy. Moreover, instead of using fresh germ stem cells from individual donor fish, germ stem cells expanding by *in vitro* culture condition can overcome the limitation of germ stem cells in individual fish. The establishment of germ cell *in vitro* culture (Chapter 2) and efficient purification of $A_{und}$ (Chapter 3) are expected to accomplish the potential improvement of transplantation efficiency. Further assessment is needed to confirm that whether the proliferation of sorted $A_{und}$ cells based on Chapter 3 can be supported by the optimized sturgeon germ cell culture condition and the success rate of transplantation can be enhanced.

### Future prospects of germ cell manipulation in fish

Germ cell culture is an ideal system for studying the mechanisms regarding self-renewal and differentiation, meiosis of germ cells and their regulations. Combine with germ cell culture, gene editing techniques such as CRISPR/Cas, transfection, and immortality are expected to utilize for germ-line transmission by cell transplantation, nuclear transfer, and/ or *in vitro* sperm production followed artificial insemination. For example, Kurita and Sakai isolated and established a zebrafish spermatogonial cell culture condition, which is capable of sperm production (Kurita and Sakai 2004). transgenic zebrafish strain could be obtained from cultured sperm that the sperm were differentiated from premeiotic germ cells infected with a pseudotyped retrovirus *in vitro* (Kurita et al. 2004). Compared with the traditional method used for generating transgenics in zebrafish, retrovirus transfection is efficient because the transgenic fish strains are not mosaic, which reduces the screening of transgenics and fish

rearing and skip a whole generation. In mammals, germline modification is also expected to be enormously valuable applications combined with germ cell culture (Brinster 2002). Subsequent development of long-term culture systems has allowed a variety of techniques to be used for genetic modification of SSCs, including knock-out (Kanatsu-Shinohara et al. 2006), TALEN (Sato et al. 2015), Besides, in long-term culture systems of rat, transgenic rats were first generated using a lentiviral vector (Hamra et al. 2002; Ryu et al. 2007) and the CRISPR/Cas9 system has also been utilized on rat SSCs subsequently (Chapman et al. 2015). Off-target effects and mosaic are two major problems that occur in CRISPR/Cas9 editing, but culturing SSC can avoid these problems because off-target SSC clones can be identified. A suitable alternative SSC can be selected to produce sperm (Kubota and Brinster 2018). Although there is still much progress to be done, knowledge of germ stem cells has evolved rapidly and has revealed novel and promising approaches to germ stem cell manipulation.

## Conclusion

Surrogate reproduction technique is a complex strategy involving amplification and cryopreservation of germ cells, preparation of sterile recipients, transplantation, and production of donor-derived offspring in recipients. Germ stem cells (GSC) from large body size fish with long generation can be transplanted into closely related small size recipients with shorter generation. The recipients are able to produce sperm or egg-derived from the donor germ cells, which simplify the donor fish cultivation and perform in small facilities at lower costs. This technique was first established on rainbow trout (Okutsu et al. 2007; Yoshizaki et al. 2010) and gradually wide applied on aquaculture fish (Franěk et al. 2019) and endangered species (Pšenička et al. 2015; Ye et al. 2017; Octavera and Yoshizaki 2018). On the other hand, cryopreservation of sperm is well developed in various fish species, while it is not available for eggs. In this case, germ cell cryopreservation following transplantation could be an efficient alternative to undergo gonadal development and generate functional offspring in recipients. To accomplish surrogate reproduction strategy, a continuous supply of large amounts of GSCs is required for the transplantation system. Instead of using fresh GSCs from individual donor fish, GSCs expanding by *in vitro* culture condition can overcome the limitation of GSCs in individual fish.

Results of this Ph. D thesis provide new insights into the study of sterlet and sturgeon germ cells. Combined with cryopreservation, the establishment of sturgeon germ cell culture condition is expected to recover endangered, even extinct sturgeons. Furthermore, as $A_{und}$ are required for transplantation, the protocol of isolation of purified $A_{und}$ population can improve the efficiency of culture and transplantation. Meanwhile, the novel monoclonal antibody provides a powerful tool to identify ASGs in sturgeon.

**The specific conclusions are as follows:**

- Germline stem cells (GSC) are kind of cells that are capable of self-renewal to produce more stem cells or differentiation into daughter cells dedicated to gametogenesis. The mechanism to balance self-renewal and differentiation are regulated by complex physical and paracrine interactions. Germ stem cell isolation, enrichment, and culture conditions to support their proliferation without differentiation will help to overcome the limitations of low germ cell numbers for cryopreservation and transplantation (Chapter 2).
- An *in vitro* germ cell culture condition was established and optimized. Through the elimination of gonadal somatic cells, addition of growth factors, and replacement of FBS, proliferation and survival of cells could prolong for more than 40 days. Cultured

cells were able to incorporate into the recipient's genital ridge after transplantation (Chapter 3).
- The fluorescence-activated cell sorting (FACS) plot can reflect the composition of cell populations in sturgeon testes. A high purity population of undifferentiated type A spermatogonia could be isolated from sterlet testes in different stages using FACS based on light-scatter properties (Chapter 4).
- A novel monoclonal antibody derived from Pacific bluefin tuna can recognize type A spermatogonia in sterlet (Chapter 5).

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## English summary

### *In vitro* culture of sturgeon germ stem cells

Xuan Xie

Germ stem cells such as spermatogonia type A (ASGs) are a unique stem cell population capable of both self-renewal or differentiation into gamete-fated daughter cells. Gametogenesis, the complex developmental process germ stem cell differentiation, is regulated by the activity of local signaling systems involving sex steroids, growth factors, small RNAs and epigenetic switches. To date, numerous research efforts have been conducted on a growing number of fish species to improve the in-vivo transfer of germ stem cells from host to donor, technology known as surrogate reproduction. Application of surrogate reproduction techniques enhances the reproductive capabilities in culture of relevant conservation and commercial fishes. For example, breeding programs of fish with large body size and slow sexual maturation such as bluefin and sturgeon may require significant space, labor, and capital. Thus, surrogate reproduction provides a rapid and low-cost alternative to enhance propagation of high value and endangered fish species.

Studies regarding sturgeon ASG *in vitro* culture, isolation, and identification were performed in the present thesis. The necessary *in vitro* culture condition of sturgeon germ cells has been established and optimized by elimination of gonadal somatic cells, addition of growth factors, and replacement of FBS. Germ cells cultured for 40 days were able to develop in genital ridge of recipients after transplantation. The *in vitro* culture system of sturgeon germ cells is expected to maintain a long-term supply of germ cells for surrogate production and provide a platform for studying the mechanisms underlying mitotic proliferation, differentiation of stem cells, meiosis, and stem cell regulation *in vitro*. It is also of interest to combine germ cell culture with gene editing techniques such as CRISPR/Cas9 for germ-line transmission by cell transplantation and nuclear transfer.

Isolation of a high purity population of ASGs was achieved through fluorescence-activated cell sorting from testicular tissue of sterlet of various ages. Cells with similar forward light scatter and side light scatter were grouped. These groups of cells were tested for vasa positivity and the cluster with relative high vasa positivity and appropriate relative shape and density was assumed to be ASGs. This purified ASG population could be an ideal material to generate a transcriptomic or proteomic profile of sturgeon germ cells. Furthermore, purified ASG populations were sorted based on light scatter properties, which do not saturate cell epitopes and interfere with downstream applications.

Recognition of ASGs is necessary to identify the developmental stage of testes and evaluate the results of germ cell manipulations. In the past, several fertility related genes in sturgeon gonads have been identified, but gene expression specific to germ cells has not been found yet. Recently, identification of ASGs was achieved with monoclonal antibodies (mAbs) specific to cell-surface antigens in Pacific bluefin tuna and rainbow trout. In the present thesis, the mAbs were applied to sterlet. Results revealed that No.20 antibody of Pacific blue fin tuna were monospecific to sterlet ASGs and can subsequently be used as an accurate identification method. The ability to detect this cell population in culture is relevant to evaluate the appropriate culture conditions for maintaining original cellular characteristics following germ cell expansion and long culturing periods.

In summary, the present thesis reviewed fish spermatogonial cell morphology and identification, and the endocrine and paracrine regulation of spermatogenesis. The aforementioned methods of identification, enrichment and *in vitro* culture of germ cells provides a novel and accurate platform to investigate the regulatory network that determines sturgeon germ cell fate, and is expected to have various potential biotechnological applications.

## Czech summary

### *In vitro* kultivace zárodečných kmenových buněk jesetera

Xuan Xie

Zárodečné kmenové buňky, jako jsou spermatogonie typu A (ASG), jsou unikátními populacemi kmenových buněk, které mají schopnost sebe obnovení nebo diferenciace do dceřiných buněk. Gametogeneze je komplexní a dynamický vývojový proces regulovaný aktivitou lokálních signálů zahrnujících pohlavní steroidy, růstové faktory, malé RNA a epigenetické faktory. Aktuálně je mnoho výzkumného úsilí investováno ke zlepšení *in vivo* transferu zárodečných kmenových buněk, technologie známé jako náhradní reprodukce. Aplikace techniky náhradní reprodukce zlepšují reprodukční vlastnosti v chovu ryb relevantních pro konzervaci i komerční chov. Například chovné programy ryb s velkými rozměry nebo pomalým pohlavním dospíváním, jako jsou tuňák nebo jeseter, mohou vyžadovat významné finanční náklady, prostor pro odchov a pracnost. Techniky náhradní reprodukce nabízejí rychlou a levnou alternativu efektivní reprodukce cenných a ohrožených druhů ryb.

V této dizertační práci byla studována *in vitro* kultivace, identifikace a izolace ASG jesetera.

Nezbytné podmínky *in vitro* kultivace ASG jesetera byly stanoveny a optimalizovány pomocí eliminace somatických buněk gonády, přídavkem růstových faktorů a nahrazením FBS. ASG kultivované po dobu 40 dnů se byly schopné vyvíjet v zárodečné rýze recipientů po transplantaci. *In vitro* systém kultivace ASG jesetera může zajistit dlouhodobé zásobení zárodečnými buňkami pro náhradní reprodukci a zároveň poskytnout platformu pro studium mechanizmů mitotické proliferace, diferenciace kmenových buněk, meiózy a *in vitro* regulace kmenových buněk. Dalším zájmem může být kombinace kultivace zárodečných buněk s technikami genové editace, jako jsou CRISPR/Cas9 pro transmisi zárodečné linie pomocí transplantace buněk nebo jaderného transferu.

Izolace vysoce purifikované populace ASG bylo dosaženo pomocí techniky fluorescenčně aktivovaného sortování buněk z testikulární tkáně jesetera malého v různých stadiích vývoje. Buňky byly sortovány na základě přímého a bočního světelného rozptylu (FSC, SSC). Tyto populace buněk byly testovány na Vasa protein. Klastr buněk s relativně silným signálem na Vasa a s příslušným tvarem a hustotou byl považován za ASG. Takto purifikované ASG populace by mohly být ideálním materiálem pro charakterizaci zárodečných buněk jeseterů na úrovní transkriptomu nebo proteomu. Navíc byly ASG sortovány na základě vlastností rozptylu světla, což nesaturovalo buněčné epitopy a nezabraňuje následujícím aplikacím.

Rozpoznání ASG je nezbytné k vyjádření stupně vývoje testes a hodnocení výsledku manipulace zárodečných buněk. V minulosti bylo několik pohlavních genů identifikováno v gonádách jesetera, ale geny, které by se exprimovaly specificky v zárodečných buňkách doposud nalezeny nebyly. V současné době bylo dosaženo identifikace ASG s monoklonálními protilátkami (mAbs) specifickými na povrchové antigeny u tuňáka a pstruha. V této práci byly mAbs aplikovány na jesetera malého. Výsledky odhalily, že protilátka na tuňáka číslo 20 je monospecifická na ASG a může být následně využita jako přesná identifikační metoda. Schopnost detekce této buněčné populace je relevantní k hodnocení vhodných podmínek kultivace se zachováním původních buněčných vlastností při proliferaci a dlouhodobé kultivaci.

Předkládaná dizertační práce popisuje morfologii, identifikaci a endokrinní a parakrinní regulaci spermatogoniálních buněk. Výše zmíněné metody identifikace, izolace a *in vitro* kultivace zárodečných buněk poskytují novou a přesnou platformu, pomocí které můžeme zkoumat regulační síť, která determinuje buněčný osud a lze je využít i v různých biotechnologických aplikacích.

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- National Agency for Agricultural Research, project QK1910248.
- Czech Science Foundation projects 17-19714Y, 20-23836S.

## List of publications

### Peer-reviewed journals with IF

**Xie, X.**,Nóbrega, R., Pšenička, M., 2020. Spermatogonial stem cells in fish: Characterization, isolation, enrichment, and recent advances of *in vitro* culture systems. Biomolecules 10, 644. (IF 2019 = 4.082)

Yang, X., Yue, H., Ye, H., Shan, X., **Xie, X**., Li, C., & Wei, Q., 2020. Identification and characterization of two piwi genes and their expression in response to E2 (17β-estradiol) in Dabry's sturgeon *Acipenser dabryanus*. Fisheries Science, 1–11. (IF 2019 = 1.173)

Franěk, R., Tichopád, T., Steinbach, C., **Xie, X**., Lujić, J., Marinović, Z., Horváth, Á, Kašpar, V., Pšenička, M., 2019. Preservation of female genetic resources of common carp through oogonial stem cell manipulation. Cryobiology 87, 78–85. (IF 2018 = 2.141)

**Xie, X.,** Li, P., Pšenička, M., Ye, H., Steinbach, Ch., Li, Ch., Wei, Q., 2019. Optimization of *in vitro* culture conditions of sturgeon germ cells for purpose of surrogate production. Animals 9, 106. (IF 2018 = 1.832)

Li, J., Zhang, H., Lin, D., Wu, J., Wang, C., **Xie, X.**, Wei, Q., 2016. Spatiotemporal distribution and assemblages of fishes below the lowermost dam in protected reach in the Yangtze River main stream: Implications for river management. BioMed Research International, 2016. (IF 2015 = 2.134)

Li, L., Wei Wei, Q., Wu, J.M., Zhang, H., Liu, Y., **Xie, X.**, 2015. Diet of *Leptobotia elongata* revealed by stomach content analysis and inferred from stable isotope signatures. Environmental Biology of Fishes 98(8): 1–14. (IF 2014 = 1.570)

### International conferences

**Xie**, **X.**, Kislik, G., Abaffy, P., Šindelka, R., Franěk, R., Pšenička, M., 2019. Fluorescence-activated cell sorting (FACS) of sterlet (*Acipenser ruthenus*) spermatogonia based on light scatter properties. 7th International Workshop on the Biology of Fish Gametes, Rennes, France, September 2–6, 2019.

**Xie, X.,** Li, P., Pšenička, M., Ye, H., Pocherniaieva, K., Zeng, L., Ma, J., Li, C., Wei, Q., 2018. Effect of growth factors and gonadal somatic cells on germ cell proliferation in sturgeon. 11th International Symposium on Reproductive Physiology of Fish. Manaus, Brazil, June 3–8. 2018.

**Xie, X.,** Li, P., Pšenička, M., Ye, H., Pocherniaieva, K., Zeng, L., Ma, J., Li, C., Wei, Q., 2018. Effect of feeder cells on germ cell proliferation in sturgeon. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation. Arendal, Norway, June 17–19, 2018.

**Xie, X.,** Li, P., Pšenička, M., Ye, H., Pocherniaieva, K., Zeng, L., Ma, J., Li, C., Wei, Q., 2017. Establishment of *in vitro* culture conditions of sturgeon germ cells. 6th International Workshop on Biology of Fish Gametes. Ceske Budejovice, Czech Republic, September 4–7, 2017.

## Training and supervision plan during study

| | |
|---|---|
| **Name** | Xuan Xie M.Sc. |
| **Research department** | 2016–2020: Laboratory of Germ Cells of FFPW |
| **Supervisor** | Assoc. Prof. Martin Pšenička |
| **Period** | 2016–2020 |

| **Ph.D. courses** | **Year** |
|---|---|
| Pond aquaculture | 2017 |
| Applied hydrobiology | 2017 |
| Ichthyology and fish taxonomy | 2017 |
| Basic of scientific communication | 2018 |
| English language | 2019 |

| **Scientific seminars** | **Year** |
|---|---|
| Seminar days of RIFCH and FFPW | 2017<br>2018<br>2019<br>2020 |

| **International conferences** | **Year** |
|---|---|
| **Xie**, **X.**, Kislik, G., Abaffy, P., Šindelka, R., Franěk, R., Pšenička, M., 2019. Fluorescence-activated cell sorting (FACS) of sterlet (*Acipenser ruthenus*) spermatogonia based on light scatter properties. 7th International Workshop on the Biology of Fish Gametes, Rennes, France. September 2–6, 2019. | 2019 |
| **Xie, X.,** Li, P., Pšenička, M., Ye, H., Pocherniaieva, K., Zeng, L., Ma, J., Li, C., Wei, Q. 2018. Effect of growth factors and gonadal somatic cells on germ cell proliferation in sturgeon. 11th International Symposium on Reproductive Physiology of Fish. Manaus, Brazil. June 3–8, 2018. | 2018 |
| **Xie, X.,** Li, P., Pšenička, M., Ye, H., Pocherniaieva, K., Zeng, L., Ma, J., Li, C., Wei, Q., 2018. Effect of feeder cells on germ cell proliferation in sturgeon. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation. Arendal, Norway. June 17–19, 2018. | 2018 |
| **Xie, X.,** Li, P., Pšenička, M., Ye, H., Pocherniaieva, K., Zeng, L., Ma, J., Li, C., Wei, Q. 2017. Establishment of *in vitro* culture conditions of sturgeon germ cells. 6th International Workshop on Biology of Fish Gametes. Ceske Budejovice, Czech Republic, September 4–7, 2017. | 2017 |

| **Foreign stays during Ph.D. study at RIFCH and FFPW** | **Year** |
|---|---|
| Dr. Jean-Jacques Lareyre, Laboratory of Fish Physiology and Genomics, INRA, Rennes, France, germ cell manipulation in rainbow trout, two month. | 2018 |
| Prof. Goro Yoshizaki, Tokyo University of Marine Science and Technology, Tokyo, Japan. Germ cell culture in rainbow trout, two months. | 2017 |

| **Pedagogical activities** | **Year** |
|---|---|
| Leading of Summer school project entitled "Sorting and purification of sturgeon germ cell populations based on flow-cytometric light scatters". | 2019 |
| Lecturing of students in fish germ cell culture in range of 90 hours. | 2019–2020 |

## *Curriculum vitae*

**PERSONAL INFORMATION**

**Name:** Xuan
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**RESEARCH INTEREST**

Reproductive biology in fish, germ cell development
Germ cell biotechnologies – germ cell culture, transplantation.

**EDUCATION**

**2016–present** Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic
**2013–2016** M.Sc. in hydrobiology. Co-educated student of Southwest University, Chongqing and Yangtze River Fisheries Research Institute, Wuhan, China
**2010–2013** B.Sc. in Biology Science. College of Life Science, Yuncheng University, China

**PROFESSIONAL EXPERIENCE**

**6 –10/2016** Worker in Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China

**COMPLETED COURSES**

Basic of scientific communication, Ichthyology and fish taxonomy, Pond aquaculture, Applied hydrobiology, English language

**RESEARCH STAY**

**5.10 – 1.12/2018** Dr. Jean-Jacques Lareyre, INRA Laboratoire de Physiologie et Génomique des Poissons, Rennes, France
**14.2–14.4/2019** Prof. Goro Yoshizaki, Tokyo University of Marine Science and Technology, Tokyo, Japan