Aquaculture 472 (2017) 4–20

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

# Biology of teleost primordial germ cells (PGCs) and spermatogonia: Biotechnological applications

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ARTICLE INFO

*Article history:*
Received 3 June 2015
Received in revised form 16 February 2016
Accepted 3 March 2016
Available online 7 March 2016

*Keywords:*
Germline
Cryopreservation
Cell culture
Surrogate production
Xenotransplantation

ABSTRACT

This review provides a general view on teleost germline development using primordial germ cells (PGCs) or spermatogonia (SG), highlighting recent progress in research on these two cell types in teleost fishes. Due to the interest of these cells for gene banking purposes, this chapter also reviews the available protocols for cryopreservation and the techniques that have been employed to assess cellular and molecular status after the process. Protocols for *in vitro* culture and the possibility of generating PGCs *in vitro* from non-committed embryonic cells are also discussed. Furthermore, the potential of these cells in surrogate production is presented, analyzing the transplantation and xenotransplantation experiments performed in fish.



## 1. Introduction

Teleost primordial germ cells, as the embryonic precursors of gametes, have tremendous importance in the fields of developmental biology and aquaculture. From a basic science point of view, they offer, an ideal model to study cell migration mechanisms; from an applied science perspective relative to endangered species and high value culture species, they are an optimal cell type to be cryopreserved because they conserve both paternal and maternal genomes. Moreover, recent studies have demonstrated the competence and suitability of these cells for surrogate production. The implementation of these technologies provides precise control over many relevant reproductive aspects. For example, PGC or SG xenotransplantation could offer a solution for the management of species with reproductive failures, or for those species with long maturation periods. Model species have been relevant to develop basic knowledge and to make all of these applications accessible for commercially valuable species. As an example, zebrafish, has been essential for the discovery of important molecular aspects of teleost germline development, including specific germ cell molecular markers that have been crucial for the study of cell specification and migration. These markers allow the identification of PGCs and SGs, either to study their distribution in the genital ridge and gonad, their proliferation *in vitro*, or their migration after transplantation into a host.

The goal of this review is to provide a general picture of the scientific progress in germ cell studies, focusing on those achievements with special relevance for Aquaculture.

## 2. Germline development in male teleost fish

In fish, PGCs migrate during embryonic development from where they are formed toward the genital ridge. PGC migration is well studied in model fish species, such as zebrafish and medaka; primordial germ cell specification and development for these species have been thoroughly discussed (Raz 2004; Herpin et al., 2007; Dosch, 2015). However, the migratory pattern in fishes varies among species, in part because of variety of egg size, shape, and developmental specificities. For advanced PGC biotechnological applications, such as transplantation of PGCs for surrogate production, it is necessary to understand the difference of PGC development among fishes. In this section, the main focus will be to describe the PGC distinctive features, the different techniques

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http://dx.doi.org/10.1016/j.aquaculture.2016.03.004
0044-8486/

available for their identification and the migration process in model fish with new findings in non-model species.

### 2.1. Distinctive features in PGCs: key players in viability and migration

Some years ago, the absence of PGC markers limited their identification to simple morphological characteristics, using optical and electronic microscopy. These morphological studies indicated that, compared to somatic cells, PGCs are bigger (10–20 μm) and have a larger nucleus (6–10 μm). PGC ultrastructural revealed the presence of a structure called nuage or germplasm (Braat et al., 1999), which appears as an electrodense cytoplasmic inclusion, usually found in association with mitochondria (Eddy, 1975). Nuage has been described in at least eight animal phyla (Eddy, 1975), suggesting that this structure has a key role in germ line development. These electrodense structures store RNA and proteins that are needed for PGC differentiation and/or determination, probably regulating transcription and translation in germ line (Seydoux and Dunn, 1997; Seydoux et al., 1996; Williamson and Lehmann, 1996).

One of the main components of germplasm is *vasa*. Vasa is a RNA helicase, first identified in *Drosophila* (Hay et al., 1988). In this model species, it was demonstrated that this marker is maternally inherited. Only embryos from females with a *vasa* mutation (maternal-effect mutant) lack PGCs (Schupbach and Wieschaus, 1986). The identification of *vasa* as the first marker in the germ line of zebrafish (*Danio rerio*) (Olsen et al., 1997) provided a significant improvement for fish germ line evaluation from the molecular point of view (Braat et al., 2000; Knaut et al., 2000; Krøvel and Olsen, 2002; Yoon et al., 1997). There are several other genes relevant to PGC viability and migration. For example, knock down of genes such as *nanos3*, *dead end*, *sdf1* and *cxcr4* by antisense morpholino induces mismigration of PGCs during embryonic development (Doitsidou et al., 2002; Köprunner et al., 2001; Weidinger et al., 2003). It also has been demonstrated that Buc protein localizes in the germplasm during early embryogenesis of zebrafish, and its overexpression induces ectopic germ cell formation (Bontems et al. 2009). Knaut et al. (2002)proposed a mechanism of selective protection for these transcripts in germ cells. It is known that *vasa* mRNA (Wolke et al., 2002) and *nanos1* (Saito et al., 2006) are selectively degraded in somatic cells, but they are stable and therefore, translated in PGCs. There are regions in the 3′ extreme of their RNAs that are highly conserved among species, which are not translated and are considered fundamental for transcript stability (3′-untranslated region or 3′UTR). It is known that the stabilization relies on the interaction of mRNAs with certain proteins such as Dnd-1 that protects them from miRNA meditated repression (Kedde et al., 2007) and Dazl that prevents miRNA degradation by promoting polyadenylation (Giraldez et al., 2006; Mishima, 2012; Mishima et al., 2006).

### 2.2. Techniques and tools for PGC visualization

#### 2.2.1. In situ hybridization and immunohistochemistry

PGC migration in fish species can be studied by whole-mount *in situ* mRNA detection against germ line specific transcripts, such as *vasa* (Shinomiya et al., 2000; Yoon et al., 1997). In zebrafish, *vasa* transcript was identified by *in situ* hybridization in oocytes (Braat et al., 1999), and in fertilized eggs (Braat et al., 1999; Yoon et al., 1997). These analyses showed that at the four-cell stage, four positions along the dividing planes are enriched in *vasa* RNA. Apart from these points, *vasa* is also expressed in some embryonic cells. The *vasa* transcripts localized in the segmentation grooves are inherited by PGCs, while the rest of *vasa* transcripts disappear, showing lower stability.

The *vasa* gene has been cloned in different teleost species like medaka and rainbow trout (Shinomiya et al., 2000; Yoshizaki et al., 2000). The *vasa* sequence is highly conserved, not only among teleost fishes, but also in other vertebrates that are evolutionarily distant, like mouse (Chuma et al., 2003). This fact has allowed the creation of recombinant antibodies, which can recognize Vasa protein in different fish species such as the gibel carp (*Carassius auratus gibelio*) (Xu et al., 2005).

#### 2.2.2. Transgenic lines

As previously mentioned, the characterization of molecular markers in fish germ cells has developed progressively, and several markers in addition to *vasa*, such as *nanos* (Köprunner et al., 2001), *dnd-1* (Liu and Collodi, 2010), *dazl* (Kosaka et al., 2007) and *sdf1a*/*cxcr4b* (Knaut et al., 2003) have been discovered. These markers have been studied in some fishes of commercial value such as Atlantic salmon (*Salmo salar*) (Nagasawa et al., 2013), Atlantic cod (*Gadus morhua*) (Skugor et al., 2014), and turbot (*Scophthalmus maximus*) (Lin et al., 2013). The knowledge of these molecular markers allowed the generation of different transgenic lines for PGC detection *in vivo*. Creating transgenic lines in fish presents some difficulties. The introduction of a genetic construction by microinjection has a low integration in the host genome, which results in a low transgenic production efficiency. Consequently, although transgenic lines have been created in fish, these are primarily in model species: zebrafish and medaka (*Oryzias latipes*). In zebrafish, the generation of the transgenic *line vasa EGFP zf45 strain*, *tg*{*vas*: *egfp*} (Krøvel and Olsen, 2002) was created by cloning *vasa* locus and its regulatory regions attached to Enhanced Green Fluorescent Protein (EGFP) (Krøvel and Olsen, 2002). There are transgenic lines of rainbow trout (*Oncorhynchus mykiss*) (Yoshizaki et al., 2000) which have transgenic lines with *vasa* promoter (VAS) and *nanos* (NOS), both of them specific in the germ line. The combination between NOS and VAS and GFP or RFP (Red Fluorescent Protein) has generated transgenic constructs which have been used in this species, medaka and zebrafish (Li et al., 2009).

#### 2.2.3. Localized RNA expression (LRE)

This technique was initially developed in zebrafish; it allows *in vivo* PGC visualization during embryonic development. By injecting synthesized mRNA, which contain GFP and *nanos3* 3′UTR sequences (GFP-*nos3* 3′UTR mRNA), into fertilized eggs, it is possible to visualize PGCs in zebrafish (Köprunner et al., 2001) and tench (Linhartova et al., 2014) (Fig. 1). As previously mentioned, the function of the 3′UTR is conserved among many fish species. In fact, it has been shown that the GFP-zebrafish *nos3* 3′UTR mRNA can allow the visualization of PGCs of various species (Herpin et al., 2007; Nagasawa et al., 2013; Saito et al., 2006, 2011, 2014). This PGC localization technique does not allow the stable expression of EGFP but has numerous advantages: it is fast, simple and very versatile.

### 2.3. PGC specification and migration to the genital ridge

The knowledge of the molecular basis for PGC specification, survival and migration allowed the development of the techniques for their visualization. Undoubtedly, these tools have been necessary for in-depth study of PGC migration during embryonic development. In fish, PGCs are specified by inheritance of maternally supplied germplasm, cytoplasm determinants. During oogenesis, germplasm is accumulated in the Balbiani body at the vegetal hemisphere, although some germline specific protein, such as Vasa, do not localize in this aggregation (Knaut et al., 2000). In zebrafish, a *bucky ball* (*buc*) gene plays an important role for the germplasm assembly in the Balbiani body during oogenesis and early embryonic development (Bontems et al., 2009). In the absence of *buc*, the germplasm fails to aggregate and oocytes lose polarity (Marlow and Mullins, 2008; Bontems et al., 2009). By injection of *buc*-GFP mRNA into one-cell stage embryos, Buc-GFP fusion protein can visualize germplasm in embryos at the marginal region of the first two cleavage furrows. Moreover, it seems that the function of *buc* is widely conserved among fish species. The zebrafish Buc-GFP localized and visualized sturgeon germplasm at cleavage stages in the same way (Saito et al., 2014). Moreover, overexpression of *buc* mRNA increases the number of PGCs during embryonic development, probably