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Expression of *vasa* and *nanos3* during primordial germ cell formation and migration in Atlantic cod (*Gadus morhua* L.)

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Abstract

Primordial germ cells (PGCs), progenitors of gametes, are specified very early in embryonic development and undergo an active migration to the site where the future gonads will form. While the developmental pattern of PGCs during embryogenesis has been documented in few model teleost fishes, there is currently no information available for any representative of Superorder Paracanthopterygii. This includes Atlantic cod (*Gadus morhua*), which is a historically important food fish in both fisheries and aquaculture industries. In the present study, we cloned and characterized *vasa* and *nanos3* and used them as germ cell markers in Atlantic cod. Sequencing results showed prospective *vasa* and *nanos3* mRNA contained the domains used to describe their respective protein family. Furthermore, phylogenetic analysis using the amino acid sequence placed Atlantic cod Vasa distinct from representatives of three other taxonomic Superorders. Atlantic cod Nanos3 was placed with other homologues from the Nanos3 subfamily. Expression of both genes was detected from the first cleavage division; both were specifically expressed in Atlantic cod PGCs from the 32-cell stage. While *nanos3* expression ceased during early somitogenesis, *vasa* was strongly expressed throughout embryonic development. Using *vasa* as a marker, we described the Atlantic cod PGC migration pattern. We demonstrated that Atlantic cod PGCs migrate ventral to the trunk mesoderm. With the exception of Pacific herring (*Clupea pallasii*), PGCs in other described teleost fishes migrate lateral to the trunk. The results from this study are the first step toward understanding germ line formation in Atlantic cod.

Keywords: Embryogenesis; mRNA; Nanos; ISH; primordial germ cells; Vasa

1. Introduction

In teleost fishes, the differentiation between the somatic cells and the germ cells begins early in embryogenesis with the specification of the primordial germ cells (PGCs) [1]. In teleost species studied so far, the specification of PGCs is controlled by a process referred to as preformation. Under this mechanism cell fate is determined by inheriting maternally supplied determinants, such as mRNA, noncoding RNA, and proteins which are stored in the oocyte during the early stages of oogenesis in a cytoplasmic structure called the germ plasm [2,3]. In zebrafish (*Danio rerio*), at the 32-cell stage, four cells inherit these components and become fated as PGCs [4]. After PGC formation, the number of PGCs remains constant until the maternal to zygotic transition during early gastrulation when the PGCs are specified [1,4]. After specification, PGCs multiply and begin an active migration to the site of the forming gonad [1]. While PGCs in teleost species studied so far appear to be specified in similar positions, the migration routes have been shown to vary between species [5,6].

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In order for PGCs to maintain a developmental pattern independent of somatic cells they retain a specific control over RNA transcription [1]. This regulation is provided through the inheritance of the maternally supplied regulatory mRNA and proteins. *Vasa* and *nanos* are two key germ cell specific genes that play crucial roles in PGC development. *Vasa* was first identified in genetic screens for *Drosophila melanogaster* [7]. It was demonstrated that females with homozygous *vasa* mutations produced sterile offspring lacking germ cells. In teleost fishes, *vasa* was first identified in *D. rerio* where it was expressed specifically in germ cells throughout development [4,8]. The transcript codes for an ATP-dependent RNA helicase of the Dead-box family [4]. While the biological function of the gene product in teleost fishes is yet to be determined, gene knockdown experiments in medaka (*Oryzias latipes*) resulted in abnormal PGC migration patterns [9].

In vertebrates there are three *nanos* gene subfamilies which are identified by the conserved *zf-nanos* zinc-finger domain. This domain is characterized by cysteine and histidine residues in a repeated CCHC CCHC pattern used to bind zinc ions to stabilize the protein structure [10,11]. In *D. rerio*, maternal *nanos1* was shown to be expressed specifically in PGCs [12]. Knockdown experiments of *D. rerio nanos1* results in defects in PGC migration followed by cell apoptosis [12]. In *O. latipes*, maternal *nanos3* shows PGC-specific expression. Recently it was shown that *D. rerio* Nanos1 interacts and regulates phosphorylation of myosin light chain II (Myzl2) [13]. Myosin II is the main component of the muscle thick filament and it is through phosphorylation that contractile or motile events can be initiated [13]. Because PGC migration during gastrulation was shown to be driven by formation of pseudopodia, which rely on the contraction of myosin [14], the function of Nanos1 in regulating the phosphorylation of myosin light chain II would explain its importance to PGC migration.

The study of germ line development may lead to practical applications for commercial aquaculture. In *D. rerio*, knockdown of *dead end* resulted in 100% sterile phenotypic males [15]. One of the predominant constraints in Atlantic cod aquaculture is the precocious sexual maturation of fish before reaching a harvestable size. In addition to economic consequences of slow growth because of sexual maturation, uncontrolled and unwanted gamete production creates a threat of genetic pollution to natural populations [16]. The present study seeks to establish a knowledge base for PGC formation in Atlantic cod in order to develop an alternative technology to produce sterile cod. We hypothesize that the answer to precocious maturation in Atlantic cod may be found in ways to prevent the development of primordial germ cells through methods, such as targeted cell ablation.

In this study, we have cloned and characterized homologues of the germ cell markers *vasa* and *nanos3* in Atlantic cod; we have determined their suitability as a molecular marker for PGC development in Atlantic cod; and we have described PGC formation and patterning throughout embryonic development and early larval stages in Atlantic cod. To our knowledge, this is the first time such a study has been undertaken in a representative species of the teleost Superorder Paracanthopterygii and a marine cold water aquaculture species. The anticipated result is to provide the necessary groundwork for future studies on Atlantic cod PGC development, which may lead to biotechnology applications in aquaculture.

2. Materials and methods

2.1. Fish

The adult Atlantic cod sampled were 2 years old or older farmed fish provided from CodJuveniles ASA in Bodø, Norway. Fish were kept at Mørkvedbukta Research Station (University of Nordland, Norway) in indoor tanks. Sea water for the flow-through system was pumped from a depth of 200 m and had a mean temperature of 7.4 ± 0.4 °C. Fish were fed a commercial diet daily using automated belt feeders.

Atlantic cod embryos were collected from the F1 generation spawners of a MarinBreed AS (Bodø, Norway) selective breeding program. The 2-year-old fish were maintained at CodJuveniles.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted from various adult Atlantic cod tissues and unfertilized eggs. The tissue samplings took place November 2009 (F1 and F2) and May 2011 (M1, M2; Table 1). In compliance with the Norwegian regulation for animal experimentation act, fish were euthanized using MS222 (Sigma, Oslo, Norway) at a concentration of 0.01 g/L. Immediately post mortem the fish were sampled for the brain, gill, heart, head-kidney, kidney, liver, spleen, stomach, midgut, gonad, muscle, skin, and blood. All samples were immediately frozen using liquid nitrogen and stored at −80 °C. The cDNA was transcribed using QuantiTect Reverse Transcription