Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 305 (2007) 589–598
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

nanos1 is required to maintain oocyte production in adult zebrafish

Bruce W. Draper [a,c,*], Claire M. McCallum [b], Cecilia B. Moens [a]

[a] *Howard Hughes Medical Institute and Fred Hutchinson Cancer Research Center, P.O. Box 19024, Seattle, WA 98109, USA*
[b] *Arcadia Biosciences, 1102 Columbia St., Suite 600, Seattle, WA 98104, USA*
[c] *Section of Molecular and Cellular Biology, University of California, Davis One Shields Ave., Davis, CA 95616, USA*

Received for publication 29 April 2006; revised 6 March 2007; accepted 6 March 2007
Available online 13 March 2007

Abstract

Development of the germline requires the specification and survival of primordial germ cells (PGCs) in the embryo as well as the maintenance of gamete production during the reproductive life of the adult. These processes appear to be fundamental to all Metazoans, and some components of the genetic pathway regulating germ cell development and function are evolutionarily conserved. In both vertebrates and invertebrates, *nanos*-related genes, which encode RNA-binding zinc finger proteins, have been shown to play essential and conserved roles during germ cell formation. In *Drosophila*, maternally supplied *nanos* is required for survival of PGCs in the embryo, while in adults, *nanos* is required for the continued production of oocytes by maintaining germline stem cells self-renewal. In mice and zebrafish, *nanos* orthologs are required for PGC survival during embryogenesis, but a role in adults has not been explored. We show here that *nanos1* in zebrafish is expressed in early stage oocytes in the adult female germline. We have identified a mutation in *nanos1* using a reverse genetics method and show that young female *nanos* mutants contain oocytes, but fail to maintain oocyte production. This progressive loss of fertility in homozygous females is not a phenotype that has been described previously in the zebrafish and underlines the value of a reverse genetics approach in this model system.

Keywords: Development; Zebrafish; *Nanos*; *Vasa*; *Ziwi*; Germline stem cells; Primordial germ cells; Ovary; Oogenesis; TILLING

Introduction

Eggs and sperm are derivatives of embryonic germ cells called primordial germ cells (PGC). In most, if not all animals, PGCs are specified at extragonadal locations and proliferate en route to and within the embryonic somatic gonad. In males of most species, PGCs give to germline stem cells that are the source of continual sperm production throughout the reproductive life of the adult (reviewed in Lin, 2002). In females the fate of embryonic germ cells appears to be species-dependent. In mammals it is generally accepted that a limited expansion of germ cell numbers occurs during embryogenesis and prior to birth all germ cells enter meiosis and arrest in the diplotene stage of the first meiotic prophase. As such, the ovaries of juvenile and adult mammals contain only post-mitotic germ cells and therefore have a determinate number of mature eggs they can produce (Franchi et al., 1962).

In contrast to mammals, there is histological evidence that the adult ovaries of many non-mammalian vertebrates, such as bony-fish (teleost) and amphibians, contain both mitotic germ cells, called oogonia, as well as maturing oocytes (reviewed in Wallace and Selman, 1990; Chaves-Pozo et al., 2005). Early germ cells can readily be identified from other cell types in electron micrographs based on their size, localization within a germinal epithelium, and because they uniquely contain germ cell-specific cytoplasmic granules, called nuage (Grier, 2000). It is hypothesized, though not directly proven, that in these species oocytes are either cyclically or continually produced in adults by oogonial stem cells. A stem cell-based mechanism for oocyte production could readily explain the extraordinary fecundity of fish such as the halibut, which can produce several hundred thousand eggs per spawning season (Norberg et al., 1991), or for other species, such as the zebrafish, that can produce eggs year-round (Selman et al., 1993). Thus, in contrast to mammals, the ovaries of some species of fish and amphibians appear to

* Corresponding author. Section of Molecular and Cellular Biology, University of California, Davis One Shields Ave., Davis, CA 95616, USA.

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.03.007

have an indeterminate number of eggs they can produce. However little is known about the genetic regulation of oocyte production in any vertebrate.

In *Drosophila*, oocyte production is known to be a stem cell-driven process and mutational analysis has identified several germ cell autonomous factors that are required for continued oocyte production in adults (reviewed in Gilboa and Lehmann, 2004b). Among these, mutations in the *nanos* locus result in females that produce a limited number of functional oocytes, but fail to maintain oocyte production (Forbes and Lehmann, 1998; Bhat, 1999). *nanos* encodes an RNA-binding protein that, together with Pumilio, negatively regulates the translation of *hunchback* mRNA in the posterior of the early embryo (Barker et al., 1992; Tautz and Pfeifle, 1989). In addition, *nanos* is also required cell-autonomously in PGCs for their maintenance in early embryos (Kobayashi et al., 1996; Forbes and Lehmann, 1998; Deshpande et al., 1999) and in adult ovaries is expressed in the germline stem cells (Forbes and Lehmann, 1998). It has therefore been proposed that, in adult ovaries, *nanos* functions to maintain GSCs, and thus oocyte production, by negatively regulating genes that promote GSCs to differentiate into oocytes (Gilboa and Lehmann, 2004a; Wang and Lin, 2004). The role of *nanos* in regulating PGC survival appears to be evolutionarily conserved as *nanos* orthologs in both mice (Tsuda et al., 2003) and zebrafish (Köprunner et al., 2001) are required for survival of PGCs during early embryogenesis. By contrast, a role for *nanos* orthologs in adult gonads in an organism other than *Drosophila* has not been described.

We show here that, in zebrafish, *nanos1*(*nos1*) is expressed in early stage germ cells in larval and adult ovaries. To address the function of *nos1* in germline development and function, we used the TILLING reverse genetics technique to identify an ENU-induced point mutation that results in a probable loss-of-function allele (Draper et al., 2004). We determined that *nos1* homozygous mutants derived from heterozygous parents are viable and have normal PGC survival in early embryos, indicating that maternally provided *nos1* function is sufficient for this process. In contrast the ovaries of 2.5-month-old *nos1* mutant females contain late-stage oocytes, but are devoid of early stage oocytes, and the gonads of 6-month-old adult females are devoid of all oocytes. Thus, similar to *Drosophila*, *nos1* in zebrafish is required to maintain oocyte production in adult ovaries.

Materials and methods

Fish strains

The wild-type strain used is *AB. Prior to characterizing the mutant phenotype, *nanos1*(*fh49*) carrier fish were outcrossed four times to wild-type fish, before crossing heterozygous parents to generate homozygous mutants.

Genotyping

The *fh49* allele eliminates an *Mse*I site in the wild-type *nanos1* sequence. This polymorphism was used to genotype all fish used in this study prior to analysis as follows: DNA was extracted from caudal fin tissue amputated from anesthetized fish by incubating tissue in 50 µl DNA extraction buffer (10 mM Tris pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3% Tween-20, 0.3% NP-40) for 20 min at 95 °C, cooled to 55 °C prior to adding 5 µl of 10 mg/ml Proteinase K, incubated for 1 h at 55 °C followed by 20 min at 95 °C. A 157 base pair fragment of the *nanos1* gene containing the *Mse*I polymorphisms was amplified by polymerase chain reaction (PCR) from individuals using the following primer pair (forward/reverse): AGACTGAGGCCGTGTACACCTCTCACTACT/GAGCAGTAGTTCTTGTCCACCATCG, in a 10 µl PCR reaction using the following amplification parameters: 95 °C 1 min, followed by 35 cycles of 95 °C, 10 s; 60 °C, 10 s; 72 °C, 15 s. Following PCR, 20 µl of *Mse*I digestion buffer (1× *Mse*I buffer supplied by manufacturer, 0.5 u *Mse*I/µl, 1 mg/ml bovine serum albumin) was incubated at 37 °C for a minimum of 4 h. *Mse*I digest the wild-type fragment into 125 bp and 32 bp fragments. Fragments were then separated by gel electrophoresis through 2% agarose. Post-*in situ* embryos were genotyped as above, except DNA extraction was preformed with 1/5 the volumes.

Whole-mount RNA in situ hybridization and immunostaining

Gonads for RNA *in situ* and immunostaining were prepared as follows: adult fish were euthanized with an overdose of Tricaine (Sigma) followed by submersion in ice water for 10 min. Prior to overnight fixation at 4 °C in 4% paraformaldehyde/1× phosphate buffered saline the heads were removed and the body cavities were opened along the ventral midline. After fixation, intact fixed gonads were dissected from the fish. RNA *in situ* hybridization was preformed as described previously (see http://zfin.org/zf_info/zfbook/chapt9/9.82.html) except that gonads were treated with 50 µg/ml Proteinase K, 15 min at 37 °C prior to hybridization as described in Onichtchouk et al. (2003). Riboprobes for *in situ* hybridization were generated as previously described: *nanos1* (Köprunner et al., 2001); *vasa* (Yoon et al., 1997); *egr2b* (Oxtoby and Jowett, 1993); *ziwi* (Tan et al., 2002); and *myod* (Weinberg et al., 1996). Embryonic staging was according to Kimmel et al. (1995). For double-labeling for *nos1* RNA and Vasa protein, antibody staining was carried out after RNA *in situ* hybridization essentially as described previously (Amacher et al., 2002). Anti-Vasa antibody (Knaut et al., 2000) was used at 1:5000.

Reverse-transcription polymerase chain reaction (RT-PCR)

Tissue was dissected from adult zebrafish and RNA was isolated as previously described (Draper et al., 2001). RT-PCR was as previously described (Draper et al., 2001) using the following primer pair for *nos1* (forward/reverse): AGACTGAGGCCGTGTACACCTCTCACTACT/GAGCAG-TAGTTCTTGTCCACCATCG, and *odc* (forward/reverse): ACACTAT-GACGGCTTGCACCG/CCCACTGACTGCACGATCTGG.

Microinjection into zebrafish embryos

Templates for synthesis of *nos1(wt)* and *nos1(fh49)* mRNA were generated by PCR and cloned into the CS2+ plasmid. Synthetic *nos1(wt)* and *nos1(fh49)* mRNA was then prepared using the mMessage mMachine kit (Ambion) and was diluted in 100 mM KCl prior to injection into 1-cell stage embryos (quantity specified in Fig. 3 legend). For experiment shown in Figs. 3G and H, embryos were obtained from a female with the genotype *nos1(fh49);*Tg[vas:EGFP], which expresses EGFP in germ cells (Krøvel and Olsen, 2002).

Histology

Adult zebrafish were sacrificed with an overdose of Tricaine (Sigma), their heads and tails were removed and their torsos fixed for 2 days at room temperature in either Bouin's Solution (Sigma) or Dietrich's Fixative (30% EtOH, 10% formalin, 2% glacial acetic acid), as previously described (Moore et al., 2002). Fish were then paraffin embedded following standard protocols. Seven-micrometer-thin sections were cut, deparaffinized in Histo-Clear (National Diagnostics) and stained with hematoxylin and eosin. For *in situ*