Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 301 (2007) 417–431
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Intermolecular interactions of homologs of germ plasm components in mammalian germ cells

Mark S. Fox [a,b], Amander T. Clark [a,b], Mohammed El Majdoubi [a,b], Jean-Louis Vigne [a,b], Jun Urano [a,b], Chris E. Hostetler [c], Michael D. Griswold [c], Richard I. Weiner [a,b], Renee A. Reijo Pera [a,b,*]

[a] *Center for Reproductive Sciences, Human Embryonic Stem Cell Research Center, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California at San Francisco, San Francisco, CA 94143-0556, USA*
[b] *Institute for Stem Cell and Tissue Biology, Departments of Physiology and Urology, University of California at San Francisco, San Francisco, CA 94143-0556, USA*
[c] *School of Molecular Biosciences, Washington State University, Pullman, WA 99163-3520, USA*

Received for publication 12 May 2006; revised 2 August 2006; accepted 18 August 2006
Available online 24 August 2006

Abstract

In some species such as flies, worms, frogs and fish, the key to forming and maintaining early germ cell populations is the assembly of germ plasm, microscopically distinct egg cytoplasm that is rich in RNAs, RNA-binding proteins and ribosomes. Cells which inherit germ plasm are destined for the germ cell lineage. In contrast, in mammals, germ cells are formed and maintained later in development as a result of inductive signaling from one embryonic cell type to another. Research advances, using complementary approaches, including identification of key signaling factors that act during the initial stages of germ cell development, differentiation of germ cells *in vitro* from mouse and human embryonic stem cells and the demonstration that homologs of germ plasm components are conserved in mammals, have shed light on key elements in the early development of mammalian germ cells. Here, we use FRET (Fluorescence Resonance Energy Transfer) to demonstrate that living mammalian germ cells possess specific RNA/protein complexes that contain germ plasm homologs, beginning in the earliest stages of development examined. Moreover, we demonstrate that, although both human and mouse germ cells and embryonic stem cells express the same proteins, germ cell-specific protein/protein interactions distinguish germ cells from precursor embryonic stem cells *in vitro*; interactions also determine sub-cellular localization of complex components. Finally, we suggest that assembly of similar protein complexes may be central to differentiation of diverse cell lineages and provide useful diagnostic tools for isolation of specific cell types from the assorted types differentiated from embryonic stem cells.

Keywords: Germ cells; Germ cell development; *DAZ*; *DAZL*; *PUM2*; *PUM1*; *NANOS*; Germ plasm; RNA-binding proteins

Introduction

In most organisms, germ cells are set aside from the somatic cells that form the rest of the organism, early in embryonic development (reviewed by Ikenishi, 1998; Wylie, 2000; Santos and Lehmann, 2004; Zhou and King, 2004). Elegant studies dating from the early- to mid-20th century have demonstrated that, in organisms such as flies, worms, frogs and fish, the germ cell lineage is established by the assembly of microscopically detectable germ plasm present in the oocyte and the subsequent segregation of germ plasm to cells which are destined to be germ cells (Ikenishi, 1998; Wylie, 2000; Santos and Lehmann, 2004; Zhou and King, 2004). In contrast, in mammals, it is clear that microscopically distinct germ plasm is not assembled in the oocyte and that germ cells are set aside later in development by inductive signaling from extraembryonic cells to pluripotent precursors in the proximal epiblast (Wylie, 2000; Santos and Lehmann, 2004). Yet, recently, it has been shown that many genes that encode homologs of germ plasm components are conserved in mammals, including genes such as *DAZ* (*Deleted*

* Corresponding author. Center for Reproductive Sciences, Human Embryonic Stem Cell Research Center, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California at San Francisco, San Francisco, CA 94143-0556, USA.
E-mail address: reijoperar@obgyn.ucsf.edu (R.A. Reijo Pera).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.08.047

in AZoospermia), *DAZL* (*DAZ-Like*), *Nanos*, *Pumilio* and *Vasa* homologs (Reijo et al., 1995; Lin and Spradling, 1997; Forbes and Lehmann, 1998; Parisi and Lin, 1999; Castrillon et al., 2000; Tanaka et al., 2000; Mochizuki et al., 2001; Jaruzelska et al., 2003; Moore et al., 2003; Tsuda et al., 2003). Indeed, where functional data are available, these genes are required for establishing, maintaining and differentiating germ cell populations (Reijo et al., 1995; Eberhart et al., 1996; Reijo et al., 1996; Ruggiu et al., 1997; Maegawa et al., 1999; Houston and King, 2000; Karashima et al., 2000; Tsuda et al., 2003; Tung et al., 2006; Lin and Page, 2005). For example, in humans, deletions and variants of *DAZ* homologs are associated with the production of very few or no germ cells (Reijo et al., 1995, 1996), whereas, in *Xenopus*, *xDazl* encodes a component of germ plasm that is required for primordial germ cell development initially and subsequent development of mature germ cell types (Houston and King, 2000; Padmanabhan and Richter, 2006). In other organisms, such as zebrafish and salamanders, it has also been shown that *DAZL* homologs encode germ plasm homologs (Howley and Ho, 2000; Johnson et al., 2001). In addition, the localization and conservation of germ plasm components such as *Pumilio* and *Nanos* homologs across diverse species are also well-documented (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Asaoka-Taguchi et al., 1999; Parisi and Lin, 1999; Subramaniam and Seydoux, 1999; Koprunner et al., 2001; Nakahata et al., 2001; Jaruzelska et al., 2003; Tsuda et al., 2003; D'Agostino et al., 2006).

Recent studies demonstrated that mouse embryonic stem cells (mESCs) are capable of differentiating into female and male germ cells *in vitro* (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004; Nayernia et al., 2006). Hubner and colleagues noted that oocyte differentiation from mESCs was obtained via spontaneous differentiation of adherent cultures, as indicated by expression of genes such as *Vasa*, *Gdf9* and *Scp3*, and corroborated by morphological evidence and production of follicular steroids (Hubner et al., 2003). Two other studies reported the differentiation of spermatogenic cells from mESCs (Toyooka et al., 2003; Geijsen et al., 2004). Toyooka and colleagues differentiated mESCs to embryoid bodies and analyzed expression of germ cell-specific markers including a reporter GFP integrated into the mouse *Vasa* locus (Toyooka et al., 2003). Initial differentiation *in vitro* was then followed by transplantation studies, in which the authors observed that transplanted primordial germ cells readily formed sperm, whereas transplantation of undifferentiated mESCs resulted in teratoma formation (Toyooka et al., 2003). Geijsen and colleagues extended these studies with analysis of imprinting and further evidence that haploid male gametes form *in vitro* and are capable of promoting development to blastocyst stage, when injected into oocytes (Geijsen et al., 2004). Finally, most recently, another group demonstrated that mESC-derived male gametes can generate offspring in mice, thus bringing the work full circle to the ultimate proof of functional gametogenesis *in vitro* (Nayernia et al., 2006).

Concurrent with studies in mice, human embryonic stem cells (hESCs) were shown to differentiate to germ cells (Clark et al., 2004a,b). Three independently derived hESC lines were differentiated to embryoid bodies and assayed for germ cell development *in vitro* (Clark et al., 2004a,b). Markers examined included those that were used to assay mouse germ cell differentiation *in vitro* as well as others diagnostic of different stages of germ cell development. It was shown that the earliest steps of human germ cell development, including expression of *VASA* and meiotic synaptonemal components, occurred *in vitro* (Clark et al., 2004a, b). A caveat, however, in these studies in both mice and humans is the common expression of protein and mRNA markers in both primordial germ cells and ESCs. This observation has in fact led to the hypothesis that hESCs are closely related, or even identical, to early germ cell precursors (Clark et al., 2004a,b; Zwaka and Thomson, 2005).

Here we sought to address the hypothesis that specific interactions of mammalian homologs of germ plasm components accompany formation and/or maintenance of early germ cell populations, in a process analogous to the assembly of germ plasm in lower organisms. Thus, we tracked the intermolecular interactions, in living germ cells and embryonic stem cells, of two proteins that are homologs of germ plasm components: DAZL and PUM2. Mouse, human and frog homologs of these proteins, and other germ plasm homologs, were previously shown to interact specifically *in vitro* (Moore et al., 2003; Fox et al., 2005; Urano et al., 2005; Padmanabhan and Richter, 2006).

Materials and methods

Preparation of protein homogenates of mouse testis

Murine testis was dissected and homogenized with a Dounce homogenizer in buffer containing 60 mM KCl, 150 mM or 500 mM NaCl, 15 mM HEPES, pH 7.8, 0.3 M sucrose, 14 mM β-mercaptoethanol, Complete (Protease inhibitor cocktail; Roche Diagnostics Corporation) and 300 μg/ml of RNAse A (Sigma-Aldrich) or RNAsin (Promega) as per manufacturer's instructions. Extract treated with RNAse A or RNasin was incubated at room temperature for 5 min (Tsui et al., 2000b). The crude tissue homogenate was then centrifuged at 3000×*g* for 5 min to remove large tissue debris and supernatant was collected.

Size exclusion chromatography

Murine testis and ESC extracts were fractionated by size exclusion chromatography on a 30 ml S-300-HR column (Sigma-Aldrich) by low-pressure chromatography (Biologic LP system; BioRad). The column was equilibrated in 10 bed volumes of either physiological salt (150 mM NaCl, 16 mM $Na_2HPO_4{\cdot}2H_2O$, 4 mM $NaH_2PO_4{\cdot}2H_2O$, 4 mM KCl, pH 7.2) or high salt buffer (500 mM NaCl, 16 mM $Na_2HPO_4{\cdot}2H_2O$, 4 mM $NaH_2PO_4{\cdot}2H_2O$, 4 mM KCl, pH 7.2) prior to sample loading. Gel Filtration Standards (BioRad) were used to approximate the column resolution. Prior to loading, the supernatant was centrifuged at 100,000×*g* for 1 h; the sample was concentrated using Ultrafree Centrifugal Filter 30,000 (Millipore). Three milliliters of extract was loaded onto the column and collected at a flow rate of one 500 μl fraction per minute; protein peaks were detected by UV monitoring at 280 nm.

Immunoblotting

Western blotting was essentially as described (Moore et al., 2003). Antisera dilutions were: DAZL (1:700), BOULE (1:500), PUM2 (1:500), NANOS1 (1:1000), VASA (1:500), DZIP1 (1:500), DAZAP1 (1:500; from Dr. P Yen, University of California Los Angeles) and Ribosomal P (1:500; Immovision). HRP-conjugated secondary antibodies were used as per manufacturer's instructions (Amersham Biosciences, and Calbiochem). Western blots were visualized by chemiluminescence as described (Amersham Biosciences).