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Germ line development in fishes

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ABSTRACT Classical work on germ cells in fishes has dealt with three main issues; their embryonic origin, the proliferation, and migration pathway during embryonic and larval development. Until recently, primordial germ cells (PGCs) have been studied in a number of fishes using morphological criteria only. The identification of the *Drosophila vasa* homolog gene of zebrafish now allows comparison of these morphological data with *vasa* RNA expression patterns in zebrafish. Teleost PGCs can be distinguished from somatic cells by their distinct morphology, at the earliest during gastrulation, and in most fishes their number varies between 10 and 30 during pregonial development. Mitosis is generally not observed in PGCs at extragonadal locations, whereas they are mitotically active once at the gonadal ridges. During gastrulation, PGCs appear to translocate from the epiblast to the hypoblast and during somitogenesis they are found associated with the most peripheral yolk syncitial layer (YSL). From the peripheral YSL they migrate through the median mesoderm into the dorsal mesoderm and then to the dorsal mesentery, where they establish the gonad primordia with mesenchymal cells. *Vasa* RNA positive cells, the PGCs of the zebrafish conform to these general observations. Interestingly, classical descriptive and experimental data can now be reevaluated using *vasa* as a molecular marker of the fish germ line. The power of zebrafish genetics together with possibilities of experimental embryology should accelerate research on aspects of vertebrate germ line development such as PGC migration, division and apoptosis, as well as (in) fertility. The present review summarizes some of the classical data on germ line development in fishes in relation to recent data on *vasa* expression in zebrafish and compares these findings, where appropriate, with those in other model organisms. Special emphasis is placed on *vasa* gene expression as a potential universal germ line marker and suggestions are made for novel, zebrafish specific approaches to investigate the vertebrate germ line.

KEY WORDS: *fish, germ line, PGC, vasa, nuage, zebrafish*

Introduction: the unique properties of the germ line

In most metazoans, germ cell lineage is constituted by an originally small number of diploid cells, which possess unique proliferative and differentiative capacities and eventually give rise to male and female gametes. The haploid gametes are derived from their precursors through the special division process of meiosis, which is characteristic of germ line cells only. The germ line is endowed with the capacity to maintain developmental totipotency in spite of cycles of proliferation, differentiation and meiosis. Developmental totipotency is obviously a prerequisite for reproductive cells, so they can provide the genetic link between the generations. In contrast to differentiating somatic cell lineages, which are destined to die after undergoing a fixed number of mitotic divisions, the germ line is thus "immortal".

The segregation of the germ cell lineage from the soma occurs early during embryonic development. In organisms such as *Drosophila* (Illmensee *et al.*, 1976; Lehmann and Ephrussi, 1994; Williamson and Lehmann, 1996), anuran amphibians (Nieuwkoop and Sutasurya, 1979; Dixon, 1981; Ikenishi, 1998) and the nematode *Caenorhabditis elegans* (Wolf *et al.*, 1983; Strome, 1994), the germ line is segregated from the soma during the early cleavages through localization of specific cytoplasmic determinants present in the "pole plasm " or "germ plasm" into presumptive primordial germ cells (pPGCs). In amniotes, such as chick and mouse, cytoplasmic determinants associated with germ cells have not

Abbreviations used in this paper: pPGC, presumptive primordial germ cell; PGC, primordial germ cell; GC, germ cell; GDB, germinal dense bodies; EG, embryonic germ cell.

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been identified. However, microdissection and culturing experiments of avian embryos suggest a very early segregation of the germ line, i.e. after the first few cleavages, or even earlier (Ginsburg, 1994). In the mouse, however, clonal analysis has unequivocally established that lineage restriction of germ line does not occur until the midgastrula stage (Lawson and Hage, 1994). In urodele amphibians and fishes, primordial germ cells have first been identified by their characteristic morphology during gastrulation (Nieuwkoop and Sutasurya, 1979).

These different modes of origin of germ line cells in different organisms have led to the idea that two modes of germ line development may exist. In the so-called "preformistic" germ line development, readily identifiable germ line determinants have been demonstrated to associate with the germ cell lineage continuously, throughout development. An example is the germ plasm in frogs (Bounoure, 1934; Nieuwkoop and Sutasurya, 1979). In the so-called "epigenetic" germ line development, as observed in the mouse the generation of germ cells from somatic cells occurs at cyclical intervals during development, and germ line determinants have thus far not been found to associate with the germ cell lineage throughout the life cycle. It is questionable, however, whether the molecular mechanisms utilized in these two apparently different modes of germ line development are fundamentally different. Recently published data on the *vasa* gene as a marker for PGC development in the zebrafish have provided new insights into this issue (Olsen *et al.,* 1997; Yoon *et al*., 1997).

Although diverse organisms appear to specify their germ line through mechanisms appropriate to their specific mode of embryonic development, the resulting germ line cells have common characteristics across very diverse Phyla (Eddy, 1975). When compared to somatic cells PGCs are large, they contain electron dense granular material at some stage of development, and they display distinct division properties, with quiescence during a certain period of development and subsequent passage through meiosis. The number of identifiable PGCs is always limited from a few in nematodes to 20-60 in insects, amphibians, mammals and fishes. They arise at extragonadal locations and migrate to the gonadal anlage during specific developmental phases (Wei and Mahowald, 1994).

Analysis of the germ line in various animal groups has led to an understanding of different aspects of its development. For instance, segregation of germ line determinants has specifically been investigated in worm, fly and frog, whereas establishment of the pPGCs founder population and PGC migration has been studied in the mouse (Gomperts *et al.,* 1994; Lawson and Hage, 1994; McLaren, 1998). Specific knowledge on *in vitro* survival and proliferation requirements of PGCs and their reprogramming into totipotent embryonic germ cell lines under such conditions has come from experiments performed on mouse PGCs. The zebrafish, a rookie in the study of germ line development, could provide answers on questions concerning specific aspects of germ line development relevant not only for lower vertebrates, but also for mice and man. Large scale screens for mutants affecting embryonic and larval development in zebrafish have clearly shown the potential of the system (Driever, *et al.,* 1996; Haffter *et al.,* 1996), and provide novel opportunities to study genes involved in development of vertebrate germ line and aspects of vertebrate fertility.

The aim of the present article is to review some of the mainly descriptive data on germ line development in diverse fish species,

TABLE 1

CHARACTERISTICS AND PRIMARY IDENTIFICATION SITE OF PGCS IN SEVERAL FISH SPECIES

	primary identification site				
species	**stage**	**location**	**appearance**	**nr**	**mitosis**
Oryzias latipes (1)	Gastrula (st 14)	All germ layers	standard *	0-20	no
Oryzias latipes (2)	Early somitogenesis (st 20)	Peripheral endoderm/YSL	standard	8-15	no
Oryzias latipes (3)	Early somitogenesis (st 20)	Peripheral endoderm	standard & "strand like" nuage (type A)		
Cyprinus carpio (4)	90% epiboly	Posterior unsegregated mesoderm	standard & pseudopodia & nuage	~10	yes
Barbus conchonius (5)	100 % epiboly	Laterally within mesoderm	standard & nuage		
Barbus conchonius (6)	10 somite st				
	19 somite st	Between mesoderm and YSL	standard & nuage	14-23	yes, until late epiboly^{O}
Platipoecilus maculatris (7)	Mid/late somitogenesis	Peripheral mesoderm/endoderm	standard & amoeboid		
Fundulus heteroclitus (8)	Early somitogenesis	Contact with YSL	standard & "chromatin knots"		
Micropterus salmoides (9)	Late epiboly	Dorso/caudal between mesoderm and YSL, ventral to Kupffer's vesicle	standard		
Danio rerio (10)	5 somite stage	Peripheral endomesoderm close to YSL (Fig. 1A)	standard		
Danio rerio (11)	32-cell stage	Marginal blastomeres	vasa mRNA positive	4	
	shield stage	Near blastoderm margin	vasa mRNA positive	~30	

References: 1: Gamo, 1961; 2: Hamaguchi, 1982; 3: Hamaguchi, 1985 and Hogan, 1978; 4: Nedelae and Steopoe, 1970; 5: Gevers *et al*., 1992; 6: Timmermans and Taverne, 1989; Wolf, 1931; 8: Richards and Thompson, 1921; 9: Johnston, 1951; 10: Braat *et al*., 1999; 11: Yoon *et al*., 1997. Abbreviations: st: stage; nr: number of PGCs. Mitosis is indicated by "yes" or "no". *: "standard" morphology: large round cells, distinct outline, large nucleus, (amoeboid), sometimes nuage. ^{O}: as judged by ^{3}H thymidine incorporation.

to discuss these data in relation to recently published work on *vasa* gene as a marker of the germ cell lineage in the zebrafish and to compare, where appropriate, the mode of germ line development in fishes to other more extensively studied model systems. Finally, experimental approaches to studies of germ line development in the zebrafish will be discussed.

Germ cells in fishes

The Class *Pisces* consists of a heterogeneous group of fishes, which on basis of type of embryonic development can be divided into two groups. The embryos of the first group, the *Agnatha* and *Osteichtes*, display a hololecithal cleavage like amphibians. The embryos of the second group, the *Chondroichtes* and *Teleostei,* have a meroblastic cleavage in which only the cytoplasmic, embryo-proper part cleaves, whereas the yolk cell remains uncleaved. Most knowledge on germ line development has been obtained from studies on *Teleostei,* bony fishes. Classical work on germ cell development in fishes has mainly dealt with three issues: embryonic origin, proliferation and migration pathway of germ cells during embryonic and larval development (Wolf, 1931; Johnston, 1951; Vivien, 1964). Since the PGCs of fishes unlike those of mouse, frog and chick could not be identified using (molecular) markers, these descriptive studies relied on PGC morphology using both light- and electron microscopy.

In the present paper, we will use the terms precursor of primordial germ cell or "presumptive" primordial germ cell (pPGC), primordial germ cell (PGC) and germ cell (GC) as operational definitions. We consider pPGCs to be the germ line precursors that, during early cleavages, divide unequally as stem cells, to give rise to one daughter cell which maintains the germ line stem cell character and another daughter cell, which enters the somatic lineages. We consider PGCs to be the founders of the germ line that upon division give rise to daughter cells which both contribute to the germ cell lineage. We consider germ cells to be gonial cells that reside in the gonad and represent the stem cells of the germ line and give rise to gametes.

Morphology of primordial germ cells and presence of "nuage"

In species such as *Drosophila, C. elegans* and *Xenopus*, PGCs were initially identified by the presence of germ plasm (Mahowald, 1962; Czolowska, 1969; Strome and Wood, 1982). These observations were helpful in identifying PGCs in species without germ plasm such as for instance urodele amphibians and fishes (reviewed in Nieuwkoop and Sutasurya, 1979) since it appears that PGCs are very much alike morphologically across the species. In comparison with somatic cells, PGCs are large (10-20 μm) with large nuclei (6-10 μm) which contain one or two prominent nucleoli within the evenly distributed chromatin and possess a distinct nuclear membrane. Ultrastructural analysis of PGCs has revealed the presence of "nuage" (Andre and Rouiller, 1957) (see Tables 1 and 2), an aggregation of RNAs and proteins, which appears as discrete, electron dense cytoplasmic inclusions, often observed in association with mitochondria (Eddy, 1975). These electron dense materials have also been referred to as "germinal dense bodies", "nucleolus like bodies", "intermitochondrial cement", and "chromatoid bodies". In its fine structure and its distribution, the nuage has a striking similarity to the pole plasm of insects and the germ plasm of frogs (Eddy, 1975). Nuage has been observed not only in PGCs, but also in oogonia, oocytes, spermatogonia, spermatocytes and spermatids and has been documented in at least 8 animal phyla (Eddy, 1975), which suggests it has an essential role in some stage of germ line development. It has been shown that germ line-specific electron-dense structures represent a storage of RNA and proteins which may be necessary for differentiation and/or determination of PGCs (Williamson and Lehmann, 1996; Ikenishi, 1998) perhaps by regulating germ line-specific translation and/or transcription (Seydoux *et al.*, 1996; Williamson and Lehmann, 1996; Seydoux and Dunn, 1997; Van Doren *et al.,* 1998b; Seydoux and Strome, 1999).

The morphology of PGCs in various subclasses of fishes is similar to that in other organisms (Table 1 and Fig. 1) (Wolf, 1931; Johnston, 1951; Vivien, 1964; Nieuwkoop and Sutasurya, 1979; Gevers *et al.,* 1992; Timmermans, 1996). The presence of "nuage" is a general characteristic of fish PGCs and correlates in time with their becoming distinct from the somatic cells. Using light microscopy, nuage-like structures have been identified in medaka and carp embryos during gastrulation (Gamo, 1961; Nedelea and Steopoe, 1970). At the ultrastructural level nuage has been recognized in different teleosts from the tailbud stage onward: in *Barbus conchonius* nuage was first observed at 100% epiboly (Gevers *et al.,* 1992), in medaka during early somitogenesis (stage 20) (Hamaguchi, 1982). Experiments utilizing incorporation of radioactive uridine and amino acids carried out on cyprinids (Clérot, 1979) and on *Xiphophorus* (Azevedo, 1984) demonstrate, that in fishes too, "nuage"-like structures contain RNA and protein.

Interestingly, the nuage-like structures undergo remodeling at the ultrastructural level according to the differentiation stage of germ cells (Mahowald, 1968; Kalt, 1973; Ikenishi and Kotani, 1975). In medaka, the so-called germinal dense bodies (GDBs) of the A type consist of "strand-like structures". These structures are characteristic of PGCs that are associated with the peripheral endoderm at the time directly preceding the onset of migration to the gonadal anlage (Table 2) (Hamaguchi, 1985). When PGCs detach from the peripheral endoderm to migrate towards the gonadal anlage (Table 2) (Gamo, 1961), and during their migration through the medial mesoderm, the type A GDBs gradually change into type D GDBs, "amorphous fibrous bodies" (Hamaguchi, 1985). This resembles the changes that occur in the ultrastructure of *Xenopus* PGCs, where "irregular shaped-string like bodies" (ISBs) typical of PGCs residing in endoderm of the stage 40 tadpole, gradually change to granular materials by stage 46 when PGCs have reached the genital ridges (Ikenishi and Kotani, 1975). In medaka, the compacted D-type nuage material of the PGCs located in the gonadal anlage is often associated with nuclear pores (Table 2). In the gonadal ridges type D nuage, or here called perinuclear dense bodies, has been identified not only in germ cells of medaka (Satoh, 1974; Hogan, 1978; Hamaguchi, 1985), but also of *Poecilia reticulata* (Billard, 1984), and *Mugil auratus* (Bruslé and Bruslé, 1978). In *Serranus hepatus* (Bruslé, 1983) and carp (van Winkoop *et al.*, 1992) electron dense nuage, here called "cement", is associated with groups of mitochondria and is specific for PGCs, oogonia and oocytes at the gonadal location.

During germ line development in medaka, more GDBs are present in PGCs during late migration and at gonadal stages than during the early stages of embryogenesis, suggesting *de novo*

synthesis of this material (Hamaguchi, 1985). *In vitro* treatment of testes with puromycin or actinomycin D, inhibitors of protein- and RNA synthesis, respectively, resulted in a significant shift from "amorphous fibrous bodies", the prevalent nuage type in GCs, into "strand-like" type nuage, characteristic of younger PGCs (Hamaguchi, 1993). These data suggest that the absence of some *de novo* synthesized component(s) results in structural changes of the nuage, and that nuage polymorphism throughout development of the germ line may result from turnover of nuage components, in which strand-like GDBs are replaced by newly formed amorphous bodies. This is in accordance with the findings of Eddy and Ito (1971), who measured a rapid turnover of substances in the dense bodies of amphibian PGCs upon the incorporation of radioactive amino acids.

Most dramatic changes in the structure of nuage are coincident with the onset of PGC migration and may be indicative of cell determination. It has been postulated that in fishes, the differentiation of PGCs proceeds under the influence of the newly established contacts with surrounding mesodermal cells during their migratory stages (Gamo, 1961). Whether the process of nuage remodeling is indicative of PGC differentiation and whether the change is cell autonomous or dependent on inductive interactions has not been experimentally tested in vertebrates. The question whether morphological changes of the nuage, or its presence *per se*, reflect the irreversible commitment of germ cells to their specific fate may partly be answered by the data from experiments performed in frog and mouse.

Wylie *et al.* (1985) tested the commitment and restriction of developmental potency of PGCs by grafting labeled PGCs from stage 45 frog tadpole. PGCs of this developmental stage contain nuage and their normal fate is to migrate through the dorsal mesentery to colonize the gonadal anlage. After transplantation into blastocoels of stage 9 blastulae, they were clearly pluripotent and differentiated into a variety of cell types, except into the germ cells of the host. Transplantation of late PGCs from stages 33-34 which contain nuage, into their proper position in the gut cavity in the posterior region of the endoderm of stage 19 neurula hosts, resulted in differentiation of the transplanted PGCs into germ cells (Ikenishi and Tanaka, 1993), which demonstrates that late PGCs, if placed correctly in the host, can still contribute to the germ line. These experiments strongly suggest that in the frog, these PGCs are still totipotent and responsive to and dependent on the signals of the embryonic environment, although the morphological parameters, such as nuage, seem to indicate their further specialization and differentiation.

TABLE 2

CHARACTERISTICS AND MIGRATION ROUTE/SEQUENCE OF TRANSLOCATION OF PGCs IN SEVERAL FISH SPECIES

	migration route/sequence of translocation				
species	**stage**	**location**	**appearance**	**nr**	**mitosis**
Oryzias latipes (1)	Late neurula and early somitogenesis	Peripheral endoderm, in contact with YSL	standard * & nuage	~20	no
	Late somitogenesis (st 25)	Somatopleure of the lateral plate mesoderm	nuage	~40	no
	st 26		nuage	~80	no
	24 h after hatching	Gonad	–	~100	yes
Oryzias latipes (2)	Late somitogenesis/ early pharyngula		standard & gdb	30±10	no
	st 27-29	Somatic mesoderm dorsal mesentery	standard & gdb		
	st 29-33	& gonadal anlage	standard & gdb	40-75	yes
Oryzias latipes (3)		Gonadal anlage	standard & "electron-dense fibrils" (type D)		
Cyprinus carpio (4)	Hatching stage	Gonadal	standard	~30	
Barbus conchonius (5)	Late somitogenesis	Near pronephric tubules	nuage		
	Pharyngula	Dorsal to the gut	nuage	~27	no
Micropterus salmoides (6)		Subintestinal yolk sac extension into the mesoderm ventral to the gut From ventromedian mesoderm into splanchnic mesoderm, dorsalward around the gut to gonadal anlage	standard	~60	no
Danio rerio (7)	48-72 hpf	Ventral to pronephric ducts (Fig. 1C)	standard		
	4-5 dpf	Dorsal mesentery (Fig. 1E)	nuage		
	9 dpf	Gonadal anlage (Fig. 1 G)			
Danio rerio (8)	Late epiboly	Halfway germ ring/animal pole	vasa mRNA positive cells	25-35	no
	6 somite st	Peripheral endoderm, in contact with YSL	vasa mRNA positive cells	25-35	no
	10 day larvae	Dorsal to the gut, ventral to pronephric tubules at gonadal anlage	vasa mRNA positive cells	>>30	yes

References: 1: Gamo, 1961; 2: Hamaguchi, 1982; 3: Hamaguchi, 1985 and Hogan, 1978; 4: Nedelae and Steopoe, 1970; 5: Timmermans and Taverne, 1989; 6: Johnston, 1951; 7: Braat *et al.*, 1999; 8: Yoon *et al.*, 1997. Abbreviations: st: stage, hpf: hours post fertilization; dpf: days post fertilization; gdb: germinal dense bodies; nr: number of PGCs. Mitosis is indicated by "yes" or "no". *****: "standard" morphology: large round cells, distinct outline, large nucleus, (amoeboid), sometimes nuage.

In contrast, the results of transplantation of *Drosophila* PGCs (pole cells) and mouse PGCs suggest that the pregonial germ line of these organisms is not pluripotent (Underwood *et al.,* 1980; Technau and Campos-Ortega, 1986; Wylie and Heasman, 1993; Stewart *et al.,* 1994). However, under the appropriate *in vitro* culture conditions, mouse PGCs of 8.0-12.5 dpc are capable of generating totipotent EG cell lines (Matsui *et al*., 1992; Labosky *et al.,* 1994; reviewed in Donovan, 1998; Matsui, 1998), although the efficiency of establishing such cultures from the 12.5 dpc PGCs and later stages rapidly declines (Labosky *et al.,* 1994; Stewart *et al*., 1994). Ultrastructural analysis of mouse embryos at 9 days p.c. (Spiegelman and Benett, 1973) and rat embryos at 10 days p.c. (Eddy and Clark, 1975), i.e. the time PGCs migrate through the hind gut, revealed the presence of the nuage material in the germ lineage from that time onward. Thus it appears that the presence of nuage-like structures in PGCs, so characteristic for progressive germ line differentiation, is compatible with the maintenance of their capacity for totipotency. However, it is intriguing that when PGCs are exposed to *in vitro* conditions, their reprogramming into EG cells results in disappearance of nuage.

It appears that in different species the presence of nuage coincides with the initiation and execution of PGC migration. A role for germ plasm in PGC migration has been postulated based on UV irradiation experiments of *Xenopus* embryos designed to destroy the RNA in the germ plasm (Smith, 1966; Zust and Dixon, 1977; Kotani *et al.,* 1994). These experiments suggest that proper organization of the germ plasm is a prerequisite for the migration of PGCs to the gonadal anlage (Smith, 1966) and that specific UV irradiation could lead to the elimination of germ line cells from irradiated animals (Kotani *et al.,* 1994). In contrast, other reports have indicated that UV irradiation could only result in the delayed migration of PGCs to the gonadal primordium (Zust and Dixon, 1977). These experiments are not conclusive and not fully comparable due to the lack of standardization of UV technology. Still, they draw the attention to a possible, yet unresolved role of germ plasm-like structures in PGC migration. In vertebrates, only circumstantial evidence points to a possible regulatory activity of germ plasm-like structures in the processes of germ line differentiation, determination and migration. Thus, the question remains open, with an interesting possibility that the electron dense germ plasm-like structures may be involved in localizing regulatory molecules for the preservation of totipotency.

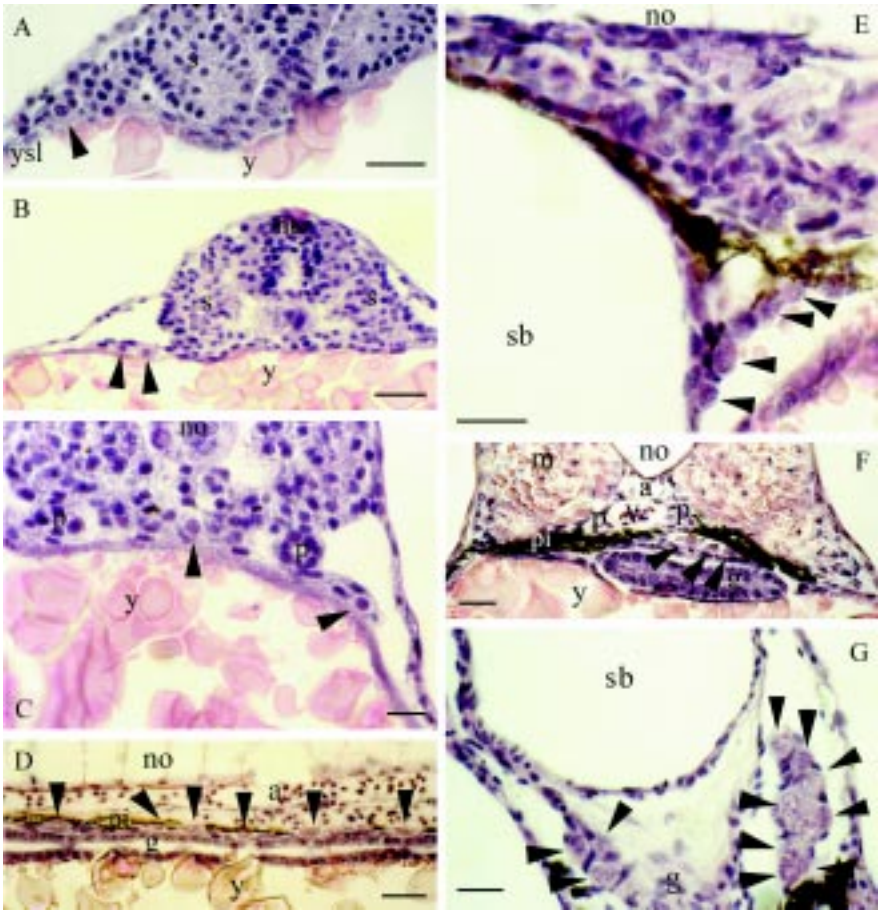

Fig. 1. Location of PGCs (arrowheads) in zebrafish embryos and larvae. (A) *Lateral position of PGC and its association with the ysl (5 somite stage).* **(B)** *Two PGCs reside closely to the ysl and lateral to the notochord (18 somite stage).* **(C)** *Two PGCs are close to the ysl and vary in their medio-lateral positions (20 somite stage).* **(D)** *At 72 hpf, the PGCs are dorsally of and close to the gut.* **(E)** *In 4 day old embryos, the PGCs are associated with the mesentery just posterior to the swim bladder and* **(F)** *in proximity to the gut.* **(G)** *Primordia of the gonads (9 day old larvae). Bars, 40 μm in A,B and F; 20 μm in C,D,E and G. Abbreviations: a, aorta; g, gut; no, notochord; m, muscular tissue; nt, neural tube; p, pronephric duct; pi, pigment cells; s, somite; v, vein; y, yolk; ysl, yolk syncytial layer. Transversal sections: A,B,C,F. Sagittal sections: D,E. Frontal section: G.*

Embryonic origin of PGCs in fishes

Due to the absence of markers for the early recognition of PGCs in fishes, analysis of their embryonic origin has been concerned with the first identification site rather than the actual site of PGC origin (Johnston, 1951; Vivien, 1964; Nieuwkoop and Sutasurya, 1979). The following identification data are all based on light microscopical observations of PGC morphology (Table 1). In medaka, PGCs were first recognized during early and mid gastrula stages within ectoderm and mesoderm and during neurula stages within endoderm (Table 1) (Gamo, 1961). In carp, PGCs were first recognized during late gastrulation among mesodermal derivatives (Nedelea and Steopoe**,** 1970). In *Micropterus salmoides*, Johnston (1951) observed PGCs in the posterior hypoblast during gastrulation. In *Barbus* c*onchonius,* PGCs were first identified at 100% epiboly, within the mesoderm. Subsequently, during early somitogenesis PGCs were found in the peripheral endoderm (Gevers *et al.*, 1992). Richards and Thompson (1921) also identified PGCs in the peripheral endoderm of *Fundulus heteroclitus* embryos during early somitogenesis. In zebrafish, we identified PGCs by their distinct morphology at the 5 somite stage in the peripheral endoderm in association with the YSL. (Braat *et al.,* 1999; Fig. 1A), whereas we identified PGCs within the hypoblast

already during mid gastrulation using *vasa* riboprobe (Fig. 2C,D; Braat *et al.,* 1999).

The only experimental evidence concerning the localization of PGCs in a specific region of the mid-gastrula fish embryo comes from Oppenheimers' work on organizational capacities of shield-grafts in *Fundulus* (Oppenheimer, 1959a,b). Shields originating from mid-gastrula (50% epiboly) donor embryos were transplanted onto the extra embryonic yolk sac of isochronic hosts. Prior to grafting, the shields were cut along the antero-posterior axis into 4 portions, (the portion nearest to the blastoderm margin designated the most posterior and the portion nearest to the animal pole the most anterior portion of the shield) and one of these portions was transplanted. The host embryos were analyzed for the development of embryonic structures between stages 30 and 33 when in *Fundulus* the formation of the swimming bladder has started (Oppenheimer, 1937). In subsequent experiments, the setup was identical to the first one except that the shields were fragmented into 9 pieces and subsequently reassociated, and transplanted as halves or thirds of the aggregates onto host yolk sacs (Oppenheimer, 1959b). Grafting of the posterior quarter of *Fundulus* shield yielded gonads and germ cells in 5 out of 13 cases whereas grafting of the quarter that situated more anterior i.e. further away from the blastoderm margin, resulted in induction of germ cells, in only 1 out of 12 grafts. Transplantation of the other two more anterior quarters of the shield yielded no germ cells. In transplantations with fragmented and reassociated shields, 9 out of 16 explants containing posterior shield fragments resulted in generation of germ cells and gonad, which indicates that progenitors of germ cells are localized in the posterior shield at the mid-gastrula stage. Interestingly, in another series of experiments on early-and mid-gastrula *Fundulus* embryos, Oppenheimer, grafted fragments of the germ ring being 90 or 180 degrees away from the shield onto extraembryonic yolk sac. These grafts yielded ahistogenic, non-differentiated material only (Oppenheimer, 1938) and therefore it is unclear whether lateral or ventral germring regions can generate PGCs. It would be interesting to study, using lineage-tracing techniques, whether PGCs at different locations in the gastrula are alike with respect to their developmental potency.

From which germ layer do PGCs in fishes originate?

The question from which germ layer pPGCs derive in fishes could be adequately addressed by dye-labeling a common set of progenitors that can still contribute to two differentiated cell types and subsequently identifying one labeled cell type as PGC and the other as a somatic cell. Such studies have been carried out in the

TABLE 3

VASA* AND *VASA-LIKE* RNA AND PROTEIN EXPRESSION IN *DROSOPHILA MELANOGASTER*, *CAENORHABDITIS ELEGANS*, MOUSE, *XENOPUS LAEVIS* AND *DANIO RERIO

		Drosophila (1, 2, 3, 4)		*C. elegans* (5, 6)		mouse (7)		*X. laevis* (8, 9)		*D. rerio* (10, 11)	
		tissue(dev stage)	**expression**	**tissue(dev stage)**	**expression**	**tissue(dev stage)**	**expression**	**tissue/dev stage**	**expression**	**tissue/dev stage**	**expression**
RNA	embryo	Preblastoderm Cellular blastoderm pole cells Pole cells (st 12 onward)	cytoplasm – +	All cells (until 8 cell stage) 16 cell - L3 stage L3 onward	 cytoplasm cytoplasm	NN	NN	NN	NN	1 cell 2-16 cell 32 –1000 cell 4000 cell onward	cytokinetic ring* cleavage furrows clumps in cyto- plasm of 4 cells cytoplasm
	ovary	Germarium: germ line stem cells and proliferating cysts Nurse cells (st 9-10) Nurse cells+oocyte (st 14)	 cytoplasm cytoplasm cytoplasm	Glh-1: throughout gonad Glh-2: prevalent in meiotic region of gonad	 + +	?	–	?	+	Previtellogenic oocytes Vitellogenic oocytes (Fig. 2A)	 cytoplasm cortex
	testis	Germ line stem cells Early spermatocytes	+ +	Glh-1: throughout gonad Glh-2: very low	 + +	Early spermatocytes Early round spermatids Later stages	 + + –	?	+	NN	
protein	embryo	Preblastoderm Pole cells (st 8-9) Pole cells (st 14)	post pole/pg pg pn/nuage	Early embryo (P1 onward) From P4 onward	 cytoplasm/Pg pn/Pg	PGC after colonization of the genital ridges (11.5 dpc)	 +	Animal (st 6) +Vegetal half pPGCs (st 12) pPGCs (tailbud onward)	cytoplasm islands of germ plasm pn pn	24 hpf 2 dpf PGC 3 dpf PGC 4 dpf PGC	+ + + +
	ovary	Germ line stem cells Nurse cells (vitellarium stage egg chamber) Nurse cells (st 10) Oocyte (st 10) Oocyte (st 14)	pn/nuage pn/nuage pn/nuage+low cytoplasm post pole/pg post pole/pg	Premature oocyte Mature oocyte	pn/Pg cytoplasm/Pg	PGC (11.5 dpc)	 cytoplasm	Oocytes (early stage) stage) Oocyte (st VI)	 pn+cytoplasm pn	Previtellogenic oocytes Early vitellogenic oocytes (Fig. 2B)	 + pn/cytoplasm
	testis	Germarium: germ line stem cells Apical and distal primary spermatocytes Later stages	 pn/nuage pn/nuage –	In all stages Spermatozoa	pn/Pg –	From 11.5 dpc in PGCs Spermatocytes to round spermatids	 cytoplasm cytoplasm+pn	Spermatogonia Spermatocytes Spermatids Spermatozoa	nuclear + pn cytoplasm cytoplasm –	Spermatogonia Spermatocytes Spermatozoa (Fig. 2C)	+ + –

Subcellular localization of RNA or protein is specified where possible.

Abbreviations: dev stage: developmental stage, st: stage, hpf: hours past fertilization, dpf: days post fertilization, pn: perinuclear, pg: polar granule, NN: not known, Pg: P granule. *: unpublished data. 1: Hay *et al.*, 1988a; 2: Hay *et al.*, 1988b; 3: Lasko and Ashburner, 1988; 4: Liang *et al.*, 1994; 5: Roussell and Bennett, 1993; 6: Gruidl *et al.*, 1996; 7: Fujiwara *et al.*, 1994; 8: Komiya *et al.*, 1994; 9: Ikenishi *et al.*, 1996; 10: Yoon *et al.*, 1997; 11: Braat *et al.*, 1999.

mouse embryo by Lawson and Hage (1994) and provided evidence for the origin of mouse PGCs from precursors of extraembryonic mesoderm.

The recent identification of the *vasa* gene in zebrafish brings the issue of allocation of PGCs to a specific germ layer in a novel perspective. *Vasa* is an RNA helicase, originally identified in *Drosophila*, whose homologs, are present as RNA or protein in the germ cell lineage of all organisms studied so far. Zebrafish *vasa* transcript was detected by *in situ* hybridization in oocytes (Braat *et al.*, 1999; Fig. 3A) and by Northern hybridization and whole-mount *in situ* hybridization in freshly fertilized eggs (Yoon *et al.,* 1997; Braat *et al.*, 1999), which demonstrates its maternal presence. During subsequent embryonic and larval development the message was identified using whole-mount *in situ* hybridization. At the 2-cell stage, *vasa* transcript is observed in short stretches of expression along the cleavage plane. At the 4-cell stage, the expression along the first cleavage plane becomes stronger and is followed by new transcript localized in the same manner along the second cleavage plane. During the 8-cell stage, the expression along the fist two cleavage planes condenses into clumps and eventually localizes to four cells at the 32-cell stage. Through the 1000-cell stage, the transcript remains in the four cells in which it is subcellularly localized, to be detected, by the 4000-cell stage (dome) in the cytoplasm of four to twelve cells. At the shield stage *vasa* is present in 16-25 cells per embryo organized as four evenly spaced clusters near the blastoderm margin. During epiboly these four clusters appear to concentrate to the dorsal side of the embryo to eventually form two groups each lateral to the midline. By this time their number does not exceed 30. During the somitogenesis these two groups remain clustered at the periphery of the embryo at the level of the third to fifth somite. During subsequent development the *vasa* positive cells become organized in two bilateral rows of cells dorsal to the gut. Once vasa expressing cells are found at the gonadal anlage, in 10 days old larvae, their number has markedly increased (Yoon *et al.,* 1997).

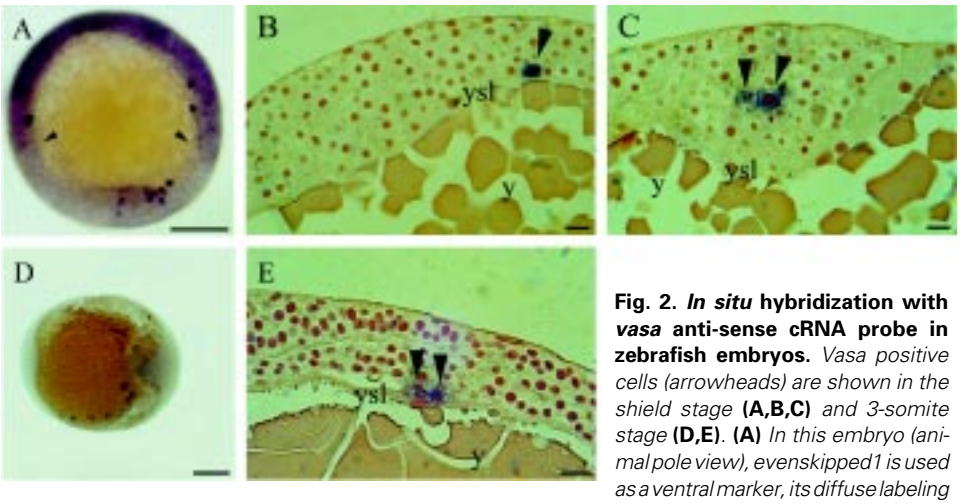

Fig. 2. *In situ* hybridization with *vasa* anti-sense cRNA probe in zebrafish embryos. *Vasa positive cells (arrowheads) are shown in the shield stage* **(A,B,C)** *and 3-somite stage* **(D,E)**. **(A)** *In this embryo (animal pole view), evenskipped1 is used as a ventral marker, its diffuse labeling being delineated by arrowheads. Most of the vasa positive cells are localized in the shield (pointing downward).* **(B)** *Section clearly shows vasa positive cells associated with the ysl.* **(C)** *The vasa positive cells in the shield area are surrounded by somatic cells.* **(D)** *Animal view (with anterior to the right) shows the vasa positive cells on both sides of the antero-posterior (A-P) axis, with the most anterior vasa positive cells being closest to the midline.* **(E)** *Section shows vasa positive cells in contact with the ysl. All sections are (semi) sagittal. Abbreviations: y, yolk; ysl, yolk syncytial layer. Bars, 200 μm in A and D; 20 μm in B,C and E.*

The restricted expression of *vasa* RNA in only four cells during the early cleavages in the zebrafish embryo proves that segregation of the germ cell lineage from the soma occurs very early in fishes. The following supports the assumption that these *vasa* RNA-positive cells represent pPGCs. Eigenmann (1981) was the first to propose a very early segregation of the PGC lineage from the soma of the fish *Micrometrus aggregatus,* and he claimed that the germ cells could be recognized from the soma by their larger size as early as 5th or 6th cleavage which corresponds to the time that 4 *vasa*-RNA-positive cells can be identified in the zebrafish embryo (Yoon *et al.*, 1997). Strikingly, this number, 4, is identical to the PGCs founder population containing germ plasm in anurans (Nieuwkoop and Sutasurya, 1979; Dixon, 1981; Ikenishi, 1998). In addition, Walker and Streisinger (1983) mutagenized zebrafish embryos using γ-rays at 66 min, 180 min, and 240 min post fertilization (p.f) and subsequently evaluated mutant clone size at the golden locus. They calculated that the number of PGCs should be approximately 5, which corresponds to the number of *vasa* positive cells. Yoon *et al.* (1997) refer to experiments carried out by Lin *et al.* (1992), in which chimeric zebrafish embryos were generated with an efficiency of 20% by transplanting about 50 blastula cells from pigmented donors into albino hosts at about the 1000-cell stage. When taking into account the number of transplanted cells and the frequency of germ line chimerism, it appears that only four founders of the germ line are present at about the 1000 cell stage, which equals the number of *vasa* positive cells at that stage.

During later development, the number of *vasa* positive cells at extragonadal locations does not exceed 30 to 40 in the zebrafish. This is in agreement with the number of morphologically recognizable PGCs observed in different fish species at different times of development (Table 1), as well as with the numbers of PGCs in anurans and urodeles (Nieuwkoop and Sutasurya, 1979). It is difficult to decide how the distribution of morphologically recognizable PGCs during development of different fish species (Tables 1 and 2) correlates with the position of *vasa* positive cells in the zebrafish embryo. For instance, during the early- and mid-shield stage, 15-25 *vasa*-positive cells are arranged in 4 clusters evenly spaced and located near the blastoderm margin (Yoon *et al.*, 1997). At this point in development, morphologically detectable PGCs have rarely been observed (Gamo, 1961). The grafting experiments carried out by Oppenheimer (1959a,b) suggest that during the mid-shield stage, the posterior shield may be the source of PGCs and the ventral and lateral germ ring may not give rise to PGCs, although, *vasa* positive cells are present at all these locations. During subsequent gastrulation, however, PGCs have been recognized mostly in the posterior shield region (Johnston, 1951; Nedelea and Steopoe, 1970). We have carried out whole-mount *in situ* hybridization on zebrafish embryos at the shield stages using a *vasa* riboprobe to detect the PGCs and zebrafish

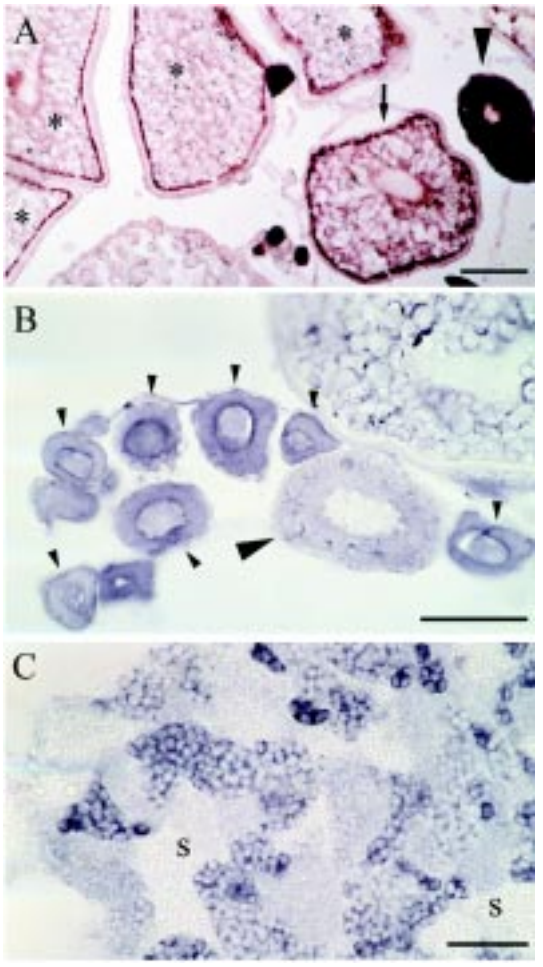

Fig. 3. *Vasa* RNA and protein expression in male and female gonads of zebrafish *(stages adapted from Van Ree, 1976, 1977a,b).* **(A)** *Vasa RNA expression is shown in the ovary; the intensely stained small (about 20 μm) cells are probably oogonia or early oocytes. The larger, pre-vitellogenic oocyte (large arrowhead) displays intense cytoplasmic staining. Early-vitellogenic (endogenous vitellogenic) oocytes show vasa RNA throughout the cytoplasm as well as concentrated in the cortex (arrow). In vitellogenic oocytes (*****) vasa RNA is localized to the oocyte cortex only.* **(B)** *Immunohistochemistry using polyclonal antibody to vasa (Braat et al., unpublished) reveals cytoplasmic and perinuclear labeling of pre-vitellogenic oocytes (small arrowheads). The larger (late) pre-vitellogenic oocyte (large arrowhead) is only weakly stained.* **(C)** *Immunohistochemistry of testis reveals labeling in germ cells of several stages. Spermatozoa (s) are not stained. Bars, 100 μm in A and B; 50 μm in C.*

evenskipped1 (Joly *et al.*, 1993) as a marker for ventral gastrula and found that *vasa* positive cells were also localized to the shield, which is in agreement with the classical studies (Fig. 2A; Braat *et al.*, 1999).

Although the presence of *vasa* RNA most likely marks the PGCs, it is not certain that all of these *vasa*-positive cells would differentiate, migrate properly to the gonad and be functional germ cells once in the gonad. Some of the PGCs may not accomplish differentiation into PGC fate or may take a "wrong" migration route or they may differentiate into somatic cells or die (Gamo, 1961). In *Drosophila* only a subset of the pole cells (PGCs) reaches the gonads (Technau and Campos-Ortega, 1986). Thus v*asa* may be necessary, but perhaps not sufficient for progressive determination of PGCs. Secondary inductive or guidance signals may be required to establish their definitive fate as has been postulated for urodele amphibians (Nieuwkoop and Sutasurya, 1979) and the mouse (Lawson *et al.,* 1999).

Putative differentiation signals and localized factors acting upon *vasa* expressing cells in the zebrafish embryo

The location of *vasa*-positive cells in the 32-cell stage zebrafish embryos places them among the descendants of the marginal blastomeres. The descendants of these blastomeres reproducibly remain in their original position at the blastoderm margin and maintain a fixed orientation relative to their neighbors at the fate map stage and undergo almost no cell mixing during epiboly (Strehlow and Gilbert, 1993; Helde *et al.*, 1994; Wilson *et al.,* 1995). At the shield stage, these cells contribute to the regions of the blastula that would give rise to ventral and dorsal mesoderm. Their more or less fixed position during epiboly makes these cells possible candidates for the inheritance of maternal cytoplasmic determinants that obviously would have to be distributed accurately. The yolk cell (Oppenheimer, 1936; Tung *et al.,* 1945) has been postulated to be a source of such determinants and the descendants of marginal blastomeres are in a position to receive these putative determinants. It is tempting to speculate that germ cell specific maternal (transcription) factor(s) could become localized into the marginal blastomeres. The regulation of such putative maternal (transcription) factor(s) by *vasa* protein would abolish the requirement for its specific localization within a subset of marginal blastomeres. Instead, they could be localized in all marginal blastomeres, but be regulated by *vasa* in *vasa* containing cells, thus endowing these cells with germ line-specificity. However, the *vasa* positive cells are positive at the RNA level and the presence of *vasa* protein still has to be clarified.

In addition to early localization of germ line specific determinants, putative signals may pass from the yolk to the blastoderm until late in gastrulation. This possibility is supported by the results obtained from analysis of gap-junctional communication in *Barbus conchonius.* Dye-communication between the YSL and the hypoblast appears to occur until about 60% epiboly, at which time the hypoblast and the YSL become uncoupled (Gevers and Timmermans, 1991). This means that small signaling molecules such as cAMP can pass the gap junctions between the YSL and the hypoblast and establish one gap junctional communication compartment until that time in development (Wolpert, 1978; Lo and Gilula, 1979). Interestingly, v*asa* positive cells are in the marginal blastoderm position until about the time when in *Barbus* the hypoblast and the yolk cell become uncoupled, and in addition to early localized maternal factors, may receive additional yolk derived signals until that time. It has been demonstrated for trout embryos that maternal factors from the yolk pass developmentally required information into the blastomeres during early cleavages but also later when the YSL appears to be crucial for the imprinting of axial polarity (Long, 1983).

In addition to maternal components inherited by pPGCs, an inductive signal may be required for fish cells to make the transition from pPGCs into PGCs. pPGCs may be specifically competent to respond to some of the inductive signals. Such an inductive signal

has been postulated in the mouse (Lawson *et al*., 1999), but also in *Urodeles* (Nieuwkoop and Sutasurya, 1979; Wakahara, 1996). In the urodele embryo PGCs can be formed from any part of the animal half of the blastula under the influence from the ventral endoderm, the competence being highest in the ectoderm near the equator and lowest at the animal pole (Wakahara, 1996). The equatorial region of urodele blastula may be considered homologous to the marginal blastomere region in the zebrafish in which the *vasa* positive cells reside. If so, then in the fish signals from the yolk cell may induce *vasa* positive cells to express their further fate. It would be interesting to assess the expression of the urodele homolog of *vasa* in these embryos and in classical induction experiments as carried out by Nieuwkoop and Sutasurya (1979).

Secondary inductive signals may also originate from the cells surrounding PGCs in the embryo. In the fish embryo such signals may originate from the shield and would be received by all PGCs that come into its region of influence through the convergence and extension movements during gastrulation. Perhaps ventrally located *vasa* positive cells, whose equivalents in *Fundulus* in grafting assays during mid-gastrula stages did not give rise to PGCs (Oppenheimer, 1938), acquire differentiative capacity later during epiboly. These ventrally and laterally located putative PGCs could be exposed to shield-derived signals later in gastrulation, as a result of posterior-dorsal convergence. Another source of inductive molecules may be the YSL. Obviously, cell-labeling experiments in conjunction with functional analyses such as extirpation and grafting of target regions, as well as gain of function studies using the *vasa* gene, will be required to confirm the identity of *vasa* RNA-positive cells as functional germ cells.

An interesting finding of the shield-grafting experiments (Oppenheimer, 1959a,b) is that in a number of cases germ cells had differentiated in the absence of any endodermal structures, indicating that they did not segregate from a common endodermal precursor nor that they required signals from endoderm in order to differentiate. This contrasted with previously published reports on PGCs development in fish (Richards and Thompson, 1921), which suggested that PGCs derive from endoderm. This idea was based on observations from many fish species that during somitogenesis PGCs reside in the peripheral endoderm in close association with the YSL (Tables 1 and 2; Figs. 1,2). Gamo (1961) and Johnston (1951) even postulated -although this was not supported by experimental data- that only those PGCs, which could establish contact with YSL could further differentiate as germ cells. Gamo (1961) suggested that uptake of components from the yolk cell could be considered as an inductive signal. Interestingly, when Oppenheimer (1959a,b) grafted shields or shield fragments of *Fundulus* onto the extraembryonic yolk sac of hosts, they yielded differentiated PGCs with or without gonads. Since the grafting sites were on the extraembryonic yolk and the grafts were not in contact with the YSL this may suggest that contact of PGCs with YSL is not a prerequisite for their subsequent differentiation or that the cells in the graft had already received the signal prior to the extirpation. However, the morphology of the GCs arising from the graft was not analyzed for the presence of nuage and since their functionality as germ cells was not tested by transplantations, it is difficult to draw conclusions about their definitive fate. It would be interesting to see whether blastoderms isolated from zebrafish blastula stages and cultured *in vitro* without the YSL would have the capacity to generate functional GCs. In Oppenheimers' work (1936) on isolated and *in vitro* cultured blastoderms of *Fundulus* there is no mention of differentiation into PGCs and/or gonads. Although the YSL may not be the source of differentiation-inductive signal(s), it may prove to be the source of differentiation-restrictive signal(s) or a substrate for the migration of PGCs to the gonad primordia.

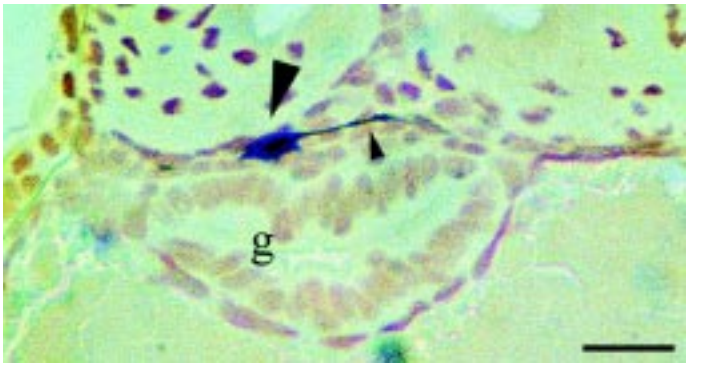

Fig. 4. Cytoplasmic extension of a PGC in a four day old larva. *A transversal section shows a PGC, in close vicinity of the gut (arrowhead), projecting a cytoplasmic extension (small arrowhead). The extension is 2.5 times longer than the PGC cell body. Abbreviations: g, gut. Bar, 20 µm.*

Migration of fish PGCs

In most animals, PGCs are set apart early during development and migrate to the genital ridges later in development. In species in which PGC migration has been studied, elements of a common pattern can be discerned. In *Drosophila*, frog and mouse, PGCs first associate with the hindgut at the onset of migration and then migrate through the hindgut to the dorsal mesentery and further to the primordial gonad.

Because histological techniques were used, only distinct time points during the migration of PGCs could be distinguished, either if cells were identified by their morphology or by *vasa* expression (Table 1). Therefore, their migration pathway can only be reconstructed by inference. It is also difficult to determine which parts of the presumed migration route are taken by actively motile, solitary PGCs and which parts they cover by joining, albeit actively, other cells in their morphogenetic movements. In the zebrafish blastula and early-gastrula v*asa* positive cells are associated with the blastoderm margin and subsequently remain in the vicinity of the germ ring. Later during gastrulation, their position is variable "being halfway between the leading edge of the blastoderm and the anterior half of the embryo" (Yoon *et al*., 1997). Although the variability in the distribution of PGCs between individual embryos is high, there seems to be a trend for concentration of *vasa* positive cells in the shield region during mid-gastrula and later (Fig. 2A). Most likely, the PGCs translocate, upon involution, by convergence and extension movements towards the body axis of the embryo during gastrulation, and thus the most anterior located PGCs would have involuted earlier than the more posterior located ones. (Braat *et al*., 1999 and Fig. 2). Studies on late gastrula medaka (Gamo, 1961) and carp embryos (Nedelea and Steopoe, 1970) identified pseudopodia on PGCs, supporting the idea of active locomotion at this stage. Lack of such findings in other studies, including our own, may be related to the type of fixation used or to variability in the PGC population and their low number within one embryo. Using

mild fixation in whole-mount *in situ* hybridization experiment we identified pseudopodia on *vasa* positive cells (Fig. 4; Braat *et al.*, 1999).

It has generally been assumed that during translocation from the peripheral endoderm to the axial mesoderm and further to the gonads, PGC migration occurs as single cells. In medaka this migration pathway has been studied thoroughly (Table 2). PGCs depart from the peripheral region of the "subendodermal" space on the YSL into the cavity between the lateral plate mesoderm and the ectoderm, thereafter into the somatic mesoderm and to the dorsal mesentery, where they form the primordial gonad with the mesenchymal cells (Gamo, 1961; Hamaguchi, 1982). Similarly, in *Micropterus salmoides*, during the so-called yolk-extension phase, PGCs were found to migrate from the subintestinal yolk sac-extension into the mesoderm ventral to the gut and to proceed around the gut to the dorsal mesentery. Subsequently, PGCs are found dorsal to the gut and to shift ventrolateraly toward the pronephric ducts (Johnston, 1951). In *Barbus conchonius*, at the end of somitogenesis, PGCs reside between the pronephric ducts and subsequently localize dorsally to the gut (Gevers *et al.,* 1992). In zebrafish, like in medaka, the PGCs translocate to the mesentery and later contribute to the primordia of the gonads, lateral and posterior to the swim bladder (Braat *et al.*, 1999; Fig. 1E,G).

The position of *vasa*-positive cells generally agrees with the localization of PGCs as described for other fishes (Yoon *et al.*, 1997). During somitogenesis, vasa- positive cells appear to be close to the YSL in the peripheral endoderm (Fig. 2F). At 24 h p.f. *vasa*-positive cells (PGCs) are localized ventral to the anlage of pronephric ducts and at 48 h p.f. they are situated dorsal to the gut anlage, and medial and ventral to the pronephric duct. At 10 days p.f., *vasa* positive cells are localized in the gonadal anlage, which is already apparent at 9 days p.f. (Fig. 1G). In contrast to some other studied organisms, fish PGCs do not enter the gut endoderm during migration (Johnston, 1951), although they are closely associated with it (Fig. 1D,F; Braat *et al.*, 1999). In relation to the antero-posterior axis of polarity, *vasa* positive cells are found at the prospective hind gut region during somitogenesis (20 somites) (Yoon *et al.*, 1997; Braat *et al.*, 1999) and eventually all end up at the boundary between the yolk ball and yolk extension. This means that *vasa* positive cells, which were previously localized posterior to the boundary will have to migrate anteriorward, and those positioned anteriorly at the level of about the 3rd somite will have to execute posteriorward migration. Gamo (1961) counted morphologically recognizable PGCs in medaka embryos in relation to their cephalo-caudal position and the data (Fig. 2.2 in his paper) indicate a strong preference of PGCs for the posterior half of the body throughout development, with almost complete absence of PGCs anteriorly. The discrepancy between his data and those in zebrafish, where anterior PGC locations are common, (Yoon *et al.,* 1997; Braat *et al.*, 1999) may be the result of species-specific differences. It is also possible that *vasa* positive cells identified anteriorly do not differentiate further and do not display their characteristic morphology. However, this hypothesis is not supported by our data which show that at the *5* somite stage, anteriorly positioned PGCs (between somite 2 and 3) are recognizable by their distinct morphology (Fig. 1A).

Based on the data on *vasa* positive cells in zebrafish, in conjunction with the data from other fish species, the hypothesis could be put forward that PGCs may take separate migration routes. Those PGCs localized further away from the germ ring, anteriorly, move medially to the embryonic axis at the end of gastrulation and subsequently move into posterior direction, whereas those localized in the vicinity of the germ ring during late gastrulation would end up near and ventral to the tail bud and would have to move only slightly to the anterior toward the yolk extension/ yolk ball boundary. This hypothesis could be tested by analysis of *vasa* expression in combination with lineage tracing experiments. An exciting option would be to prepare transgenic zebrafish for *vasa* promoter-GFP fusion constructs and analyze GFP expression *in vivo* (E. Raz, personal communication). Such experiments should provide the first definitive insight into the exact path of migration of vertebrate PGCs as well as into their mitotic properties.

Proliferation of fish PGCs

In all systems studied, the founding population of PGCs appears to be limited, ranging from only a few cells in nematodes to a few dozen or less in insects, fish, amphibians, birds and mammals. The founding population of the germ line arises from a stem cell population of presumptive primordial germ cells (pPGCs). In *Xenopus*, the first four cleavage blastomeres that contain the germ plasm divide it unequally, each giving rise to one somatic daughter cell without germ plasm and one daughter stem cell containing the germ plasm. The four germplasm-containing cells thus formed represent the founder cells of the germ line, the PGCs. During subsequent divisions of the PGCs, both daughter cells inherit the germ plasm (Dixon, 1981). It is at this point during development i.e. gastrulation, that the PGCs become morphologically distinct (Nieuwkoop and Satusurya, 1979; Dixon, 1981).

Due to the absence of germ plasm as a germ lineage marker in fish embryos, PGCs have been identified by their morphology only. In medaka, for instance, the number of PGCs during gastrulation and early somitogenesis was thus determined to vary between 0 and 20. During mid and late somitogenesis this number increases about twofold, up to 40, whereas this number doubles at the pharyngula stage to reach about 80 cells (Gamo, 1961). Assuming a founding population of about 20 PGCs, two cell divisions would then establish about 80 PGCs. Hamaguchi (1982) reported for medaka that the number of PGCs increased from about 15±5 cells at the end of gastrulation to about 30±10 during the first half of somitogenesis (stage 25; ca.18 somites), with no further increase observed later in development (Table 2). These numbers found by Gamo and Hamaguchi agree up to stages 25/26 (18-23 somites), but during stage 26, Gamo observed that the number of PGCs rose from ca. 30 to 70-80, whereas Hamaguchi did not detect any increase in cell numbers during migration of the PGCs from stage 26 onward. This discrepancy in data for the same species illustrates the difficulty in assessing cells as PGCs. However, both authors reported the absence of mitotic figures in the PGCs at all extragonadal locations, whereas the mitotic activity of PGCs resumed upon their arrival into the gonadal anlage, with a higher mitotic activity in the female lineage (Hamaguchi, 1982). Similarly, in carp about 10 PGC were recognized at late gastrula, whereas at the hatching stage about 30 cells were detected (Nedelea and Steopoe, 1970). These authors documented mitotic activity of PGCs during late gastrulation. Mitotic activity of PGCs was studied in *Barbus conchonius* using autoradiography after 3H thymidine incorporation as a marker for DNA synthesis and thus cell division. Embryos were injected at successive stages of development from 1 to 11 h p.f. and radioactive labeling of cells was analyzed at 48 h p.f. (Note that embryonic development of *Barbus*

conchonius is closely comparable in time to that of zebrafish). During embryonic and early larval development the authors counted a total of 14-23 PGCs with an increase during subsequent larval development to about 40 cells. No mitoses were observed, until rapid proliferation began in the gonad after about three weeks of development (Timmermans and Taverne, 1989). Identification of DNA synthesizing cells revealed that at the blastula stage, 100% of the PGCs divided, which supports the idea that these cells are pPGCs that still divide synchronously with blastula cells. During the early- and mid-gastrula stages, 80±16 % of the PGCs divided, whereas during late gastrulation until the end of epiboly 45% of the PGCs divided. During early somitogenesis only 1-2% of the PGCs divided, indicating that PGCs terminated their mitotic activity towards the end of epiboly.

The analysis of *vasa*-positive cells in zebrafish now allows accurate assessment of the number of putative PGCs during development. During the early cleavages, the number of *vasa*-positive cells remains four. It is tempting to speculate that subcellularly localized maternal *vasa*-RNA is unequally distributed to the daughter cells before midblastula transition (Kane and Kimmel, 1993), similarly to the localization of germ plasm in *Xenopus*. In this manner the stem cell character of pPGCs is preserved during early cleavages and a limited number of early founders of the germ line is maintained. An increase in the number of *vasa* RNA positive cells from 4 at the 1000 cell stage to about 12 at the 4000 cell stage (Table 3) takes place before the shield stage, which appears to be in agreement with results reported for *Barbus conchonius* that 100% of PGCs divide during these stages (Timmermans and Taverne, 1989). During the remainder of epiboly, the total number of *vasa* positive cells reaches about 25 to 30, which is the number of PGCs that will eventually populate the gonad. The number of PGCs reported for different fishes at extragonadal locations is similar, about 30 (Table 2). It appears that the mitotic activity of PGCs in most fishes ceases by mid-somitogenesis, and that they remain mitotically quiescent during migration (Timmermans and Taverne, 1989). The increase in numbers recorded in different species and at various successive times of development such as, for instance, from 16 to 25 cells in zebrafish and 17 to 27 in medaka suggests that not all PGCs undergo the same number of cell cycli, and if they do, that their cycli may not be synchronous. Generally, the number of PGCs in the fish agree with those in *Xenopus* which suggests that PGCs undergo two to three mitotic divisions before arrival into the genital ridges (Dziadek and Dixon, 1975; Whitington and Dixon, 1975).

Upon establishment of the gonad intensive mitotic activity in the fish germ line is initiated. In zebrafish, for instance the number of *vasa*-positive cells has increased greatly by 10 days of development (Yoon *et al.,* 1997). In other fish species the mitotic activity of gonadal PGCs is preceded by a quiescence period of several weeks (Timmermans and Taverne, 1989; van Winkoop *et al*., 1992). It is interesting to note that whereas in frog and fish the mitotic activity of PGCs is low during embryogenesis and rapid proliferation is reinitiated in the gonad, in the mouse PGCs divide vigorously during migration. The population of PGCs in the mouse rises from about 150 at 8.5 days p.c. prior to their entry into the hindgut, to about 25000 at 13.5 d p.c in fully colonized gonads (Tam and Snow, 1981). Thus the timing of proliferation of mammalian PGCs differs greatly from frog or fish (Donovan, 1998). These different division dynamics may correlate to the different expression patterns of germ line-specific genes such as *vasa*.

***Vasa*: the germ line marker from *Drosophila* to zebrafish**

Among the molecules localized to the germ line, only the protein product of the *vasa* gene has been detected throughout the life cycle in germ lineages of *Drosophila* (Hay *et al*., 1988a,b; Liang *et al.,* 1994), *C. elegans* (Gruidl *et al*., 1996) and *Xenopus* (Komiya *et al*., 1994; Ikenishi *et al*., 1996; and see Table 3). *Vasa* belongs to the family of RNA helicases, constituted by a large group of proteins that are present in a wide range of organisms and are involved in processes such as nuclear and mitochondrial splicing, RNA editing, rRNA processing, translation initiation, nuclear RNA export and mRNA degradation. These proteins are characterized by a core region of 290-360 amino acids that show a high sequence homology to the eukaryotic translation initiation factor eIF-4A known to exhibit ATP dependent RNA helicase activity (Schmid and Linder, 1992). This core region consists of eight highly conserved domains, which are also conserved, in respect to their interspacing (Luking *et al.,* 1998). A subfamily of the RNA helicase family, the so-called DEAD (Asp-Glu-Ala-Asp) box proteins, is characterized by strong homology in the fifth of the eight structural elements, the so-called DEAD motif (Linder *et al*., 1989). The *vasa* gene in *Drosophila* encodes a member of the DEAD box protein family of putative RNA helicases, which was shown to function as an ATP dependent RNA helicase *in vitro* (Liang *et al*., 1994). *Vasa* protein represents one of the components of both nuage in germ cells and polar granules at the posterior pole of the oocyte and the early embryo of *Drosophila.*

In *Drosophila*, a number of maternal effect mutants have identified genes, which are required for specific steps of pole cell (PGC) formation and further germ line development. *Oskar*, *vasa* and *tudor* are required for establishment of the polar granules. During oocyte development, *oskar* mRNA is supplied to the stage 9 oocyte by the nurse cells and is anchored to its posterior pole. Translation of *oskar* at this site nucleates the formation of posterior polar granules and polar plasm (Ephrussi *et al*., 1991; Ephrussi and Lehmann, 1992; Smith *et al*., 1992). In addition to *oskar* mRNA, vasa and tudor proteins, also supplied by the nurse cells, are required. Mislocalization of *oskar* mRNA to the anterior of the oocyte under regulation of the *bicoid* 3' untranslated region, results in recruitment of both vasa and tudor proteins to the anterior of the oocyte and subsequent ectopic pole cell formation (Ephrussi and Lehmann, 1992). The localization of *vasa* in *Drosophila* operates at the level of protein and not at the level of mRNA. *Vasa* protein is localized to the posterior pole of the oocyte while maternal *vasa* mRNA is distributed throughout the oocyte with no apparent localization. In early oogenesis, the *Drosophila* oocyte does not yet contain maternal vasa protein, which is deposited later into the oocyte by the nurse cells and can therefore be specifically localized to the posterior RNA/protein complex nucleated by *oskar*.

Surprisingly, in zebrafish, it is *vasa* mRNA that is localized to the cortex of the oocyte (Braat *et al*., 1999; Fig. 3A). During the first 3 cleavages of the zygote, the mRNA is localized to the cleavage furrows of the blastomeres and eventually becomes segregated into four cells at the 32-cell stage (Yoon *et al*., 1997). Maternal v*asa* message remains subcellularly localized from the 32 cell stage until a time point between the midblastula transition (Kane and Kimmel, 1993) and the dome stage, whereafter it becomes cytoplasmic (Table 3). Most likely, this cytoplasmic *vasa* RNA

represents the zygotic transcript (Yoon *et al.,* 1997). It is essential that future studies of the zebrafish *vasa* gene resolve the mode of localization of the maternal transcript as well as the onset of zygotic *vasa* transcription in relation to nuage formation. The information for intracellular targeting of the *vasa* RNAs may be conserved from yeast to mammals and therefore cis acting localization elements may be revealed in the 3' untranslated region of *vasa* similar to those in other localized maternally supplied RNAs (reviewed by St. Johnston, 1995). Regarding the protein product of *vasa,* it is plausible that maternal *vasa* protein is distributed at low levels throughout the early zebrafish embryo as has been shown in *Xenopus* (Ikenishi *et al.*, 1996). This is supported by our findings that *vasa* protein is expressed in zebrafish oocytes, most likely resulting in a maternal contribution of the protein in the zygote (Fig. 3B). Therefore, it would be difficult to establish a local and functional enrichment of *vasa* protein, in a limited region of the oocyte or limited number of cells of the zygote against this high background of maternal *vasa* protein. Instead, localization of maternal *vasa* RNA to a limited number of cells and subsequent *de novo* translation of the message against the decaying maternal protein would increase the level of localized *vasa* protein, reaching the threshold for execution of its RNA regulatory functions. Moreover, this would also allow the regulation of *vasa* RNA itself by vasa maternal protein.

Vasa protein is a component of *Drosophila* germ line specific structures such as dense cytoplasmic masses, polar granules, nuclear bodies and nuage, demonstrating their biochemical relatedness and the molecular continuity of the germ line (Hay *et al.*, 1988a,b, 1990; Lasko and Ashburner, 1988; Liang *et al.*, 1994). The physical continuity of germ plasm-like structures is demonstrated by the posterior localization of *vasa*, which involves the translocation of perinuclear localized *vasa*-containing nuage particles from the nurse cells to the posterior pole of the oocyte. Moreover, all mutants in *vasa* that have been reported to interfere with pole plasm assembly (Liang *et al.*, 1994) also abolish the perinuclear localization of the *vasa* protein. For instance, the *vas* PD23 mutation shows that this gene is indispensable for germ plasm assembly and its continuity, since in this mutant polar granules as well as *vasa* protein in oocytes and early embryos are absent (Schupbach and Wieschaus, 1986). Together, these data show that *vasa* is essential for the formation and/or structural integrity of the perinuclear nuage in *Drosophila* and perhaps in all species containing nuage as a universal germ line marker (Eddy, 1975). Interesting in this respect is the fact that perinuclear localization of *vasa* in *Drosophila* is independent of *cappuccino (capu), spire (spir), oskar (osk)* or *staufen (stau),* genes required upstream of *vasa* in the pole plasm assembly pathway. This indicates the independence of *vasa* function in the formation of nuage which is a universal germ-line specific structure in all studied organisms, as opposed to pole plasm assembly which is unique to *Drosophila* (Lehmann, 1992; Liang *et al.*, 1994).

The segregation of germ plasm into four founder cells of the germ line at gastrulation in *Xenopus* and the formation of P-granules in *C. elegans* (Strome and Wood, 1982; Hill and Strome, 1988), are thought to be structurally and functionally similar to the localization of pole plasm in *Drosophila* (Mahowald, 1968; Illmensee and Mahowald 1974, 1976; Illmensee, 1976). Continuity of the electron dense germ line specific material has also been postulated in *Xenopus* development. It has been suggested that the germinal granules of the germ plasm in cleavage stage embryos, which contain *Xenopus vasa* homolog (XVLG1) (Komiya *et al.,* 1994; Ikenishi *et al.*, 1996) later in development give rise to the granular material of the mitochondrial cloud in the PGCs, oocytes and early zygotes (Watanabe *et al.*, 1992). The significance of the *vasa*-like gene(s) in *Xenopus* and *C. elegans* has been studied by loss-of-function experiments, and as in *Drosophila*, where *vas* mutants do not form pole plasm and eventually lack pole cells, these confirm the essential role of *vasa* in germ line development. In *C. elegans*, the protein products of the GLH-1 and GLH-2 genes, putative germ line helicases of the DEAD box family, are components of the germ line P-granules (Gruidl *et al.*, 1996). Either one or both genes appear to be required for germ line development, since injection of anti-sense GLH-1 or GLH-2 RNA into wild-type nematodes resulted in a decreased number of pole cells and the disappearance of the P-granules, resulting in sterile nematodes. In *Xenopus*, a monoclonal antibody directed against XVLG1 injected together with a lineage tracer into one of the four germ plasm containing vegetal blastomeres at the 32 cell stage, resulted in the absence of dye labeled PGCs that would otherwise have been derived from this blastomere (Ikenishi and Tanaka, 1997). Surprisingly, the effect of antibody injection on the PGC population was only apparent after stage 37/38 of development, while before stage 38 a normal number of dye-labeled PGCs was observed, which indicates the requirement for *vasa* protein in late phases of *Xenopus* germ line determination and/or maintenance.

In the mouse, the homolog of the *Drosophila vasa* gene (mvh) is expressed as protein in PGCs after 11.5 days of development at the onset of gonad colonization (Fujiwara *et al.,* 1994) (Table 3). This relatively late protein expression in the mouse together with late effects of anti-vasa antibody injections on PGCs in *Xenopus* suggest a role for *vasa* protein in determination, specification or maintenance of the germ line. Moreover, the time of appearance of *vasa* protein in mouse PGCs correlates with a gradual loss of capacity of these cells to generate totipotent EG cell lines (Matsui, 1998). It may be that *vasa* and its target genes initiate a differentiation cascade in mouse gonadal PGCs restricting their developmental potency. Although we know that zebrafish oogonia and oocytes express *vasa* protein (Fig. 3B, Braat *et al.*, unpublished) more detailed data on the temporal pattern of zebrafish *vasa* protein expression are required to draw a general conclusion about the role of *vasa* protein and/or RNA in vertebrate germ line determination.

The germ plasm of *Drosophila* must contain a combination of factors controlling both the cellular behavior of the early germ cells as well as the activation of germ cell specific gene expression. *Vasa* may play a role in both steps. Although the role of *vasa* in germ line development is all but clear, it is striking that at some point in time both *vasa* RNA and protein overlap in the cells of the germ lineage (see Table 3). Consistently, cytoplasmic *vasa* RNA appears only in those cells in zebrafish embryo that already contain localized *vasa* RNA (Yoon *et al.*, 1997) and presumably high levels of its as nuage- localized translational product. It is not known what the significance of this zygotic *vasa* transcription is, but it is plausible that this cell specific expression may cause a substantial rise in *vasa* protein content of these cells, resulting in the maintenance of their identity as germ lineage derivatives. In *Drosophila*, zygotic *vasa* transcription is restricted to the germ lineage, just as we postulated for zebrafish and starts at stage 12 only in pole cells

which contain *vasa* protein in polar granules derived from stages 8 and 9 onwards. It appears that only after zygotic transcription of *vasa* has been initiated at stage 12, nuage in pole cells is formed at stage 14 (Table 3), indicating that zygotic *vasa* RNA or its protein product somehow organizes the perinuclear nuage (Liang *et al.*, 1994).

The fact that *vasa* zygotic RNA precedes nuage formation in *Drosophila* pole cells may also be true in fishes. In zebrafish PGCs, cytoplasmic, presumably zygotic *vasa* RNA, is already present long before the tail bud stage (Yoon *et al.,* 1997, Table 3) when in *Barbus conchonius,* PGCs become morphologically recognizable and concomitantly contain nuage (Gevers *et al.*, 1992, see Table 1). If one proposes that zygotic transcription of *vasa* in cells in which *vasa* protein is present at a certain level and/or in a certain structure leads to nuage formation than a testable prediction would be that in all species containing nuage, zygotic *vasa* expression precedes formation of nuage. It would be interesting to assess the presence and subcellular localization of *vasa* message in cleavage stage embryos of urodeles, chick and the mouse, since it is likely that *vasa* maternal mRNA will also be localized to pPGCs in these species.

It is tempting to speculate that genes regulated by localized *vasa* protein may be required for *de novo* zygotic transcription of *vasa* gene, which ensures maintenance of the identity of the germ line cells in both *Drosophila* and zebrafish. Van Doren and colleagues (1998b) speculated that in *Drosophila* at least one maternally supplied transcription factor localized to the germ cells would activate germ line specific targets once the germ line is competent for gene expression. *Vasa* would be the target for responsiveness to such a transcription factor, which in its turn may be under translational regulation of *vasa* protein. A similar cascade of events may be postulated for zebrafish, but its demonstration will have to await new experiments.

Concluding remarks

Here we have addressed the establishment of the germ line in fishes, considering classical data from several fish species. These merely descriptive data have been complemented with data obtained from other extensively studied species, such as *Drosophila*, *C. elegans*, *Xenopus laevis* and the mouse. More importantly, we have also considered the recent cloning of the germ line marker *vasa* in zebrafish, which has opened new possibilities to study germ line development in fishes.

All descriptive data deal with origin, migration route, the number and proliferation of PGCs (see Table 1 and 2). Since these data are based on microscopical observations, the origin of PGCs is difficult to establish with certainty. Also, the number of PGCs in a particular species is difficult to assess. Gamo (1961) reported 70-80 PGCs whereas Hamaguchi (1982) reported only 40 PGCs in medaka at comparable developmental stages, which stresses the difficulty of these studies based on morphology only. The cloning of *vasa* in zebrafish is relevant to both issues, and has revealed that PGCs do not seem to originate from a particular germ layer but are already separated from the soma at the 32 cell stage. However, they participate in the morphogenetic movements with the endoderm (Warga and Nüsslein-Volhard, 1999) and may be derived from a common precursor. The number of PGCs can now also be easily determined using this molecular marker. However, the question is whether all *vasa* RNA positive cells have the potential to differentiate into PGCs with a characteristic PGC morphology and whether they all finally end up in the gonad. Or is the position in the gastrulating embryo in relation to the shield or other region/structure, as source of inductive signals, also of importance? Classical transplantation experiments, in which labeled PGCs are grafted into a “neutral” environment, could provide an answer.

The mechanism of *vasa* mRNA localization and distribution in the embryo via the cleavage furrows into the most marginal blastomeres of the 32-cell stage embryo is most intriguing. As already mentioned, these blastomeres are in closest contact to the YSL which might contribute specific maternal factors to the *vasa* positive cells. To assess the role of the YSL in the determination of the PGCs, isolated blastoderms should be cultured in absence of the yolk and YSL, and the presence of (functional) PGCs should be evaluated by transplantations. One such maternal factor could be transcriptional regulator of the *vasa* gene that would activate the zygotic *vasa* transcription. Subsequently this would lead to expression of zygotic vasa protein and the possible determination of the germ line. It would be of great interest to determine when zygotic *vasa* transcription starts and how these new transcripts are distributed within a cell. Moreover, the distribution of vasa protein has to be determined, since in other studied organisms, it is vasa protein that is expressed in the germ line throughout the lifecycle.

One of the characteristics of PGCs is the presence of nuage. In *Drosophila*, nuage is dependent on the expression of vasa since vasa mutants fail to form nuage. It will be informative to determine whether *vasa* RNA and/or protein is a component of the nuage in zebrafish. The presence of nuage correlates in time with the PGCs becoming distinct from the somatic lineage. Is this nuage a result of zygotic *vasa* expression and would these cells lose their PGC identity if vasa expression was blocked? Loss-of-function experiments using anti-sense technology would, if the stated were true, result in loss of nuage and loss of PGCs. In *Drosophila*, it has been shown that vasa protein is necessary for pole cell development, but not sufficient. Moreover, nuage formation is dependent on vasa but is independent of four genes required upstream for its pole plasm localization. Interestingly, in fish no germ plasm has been observed so far, suggesting an independent and dominant role for vasa in PGC determination in fish.

Not only the intrinsic properties of the PGCs are important for their behavior in the embryo. Most likely, tissues surrounding them at certain time points during development are necessary for their survival and migration, but perhaps also for their further determination. Studies on developmental mutants could provide answers regarding the role of the environment of the PGCs in their survival, migration and proliferation. Preliminary results from our laboratory indicate that the gut might be involved in proper guidance of PGCs towards the gonad. New PGC guiding signals like those recently found in the somatic gonad of *Drosophila* (Van Doren *et al.*, 1998a) might also play a role in the migration of fish PGCs.

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