Develop. Growth Differ. (2007) **49**, 561–570 doi: 10.1111/j.1440-169x.2007.00944.x

Ectopic germline cells in embryos of *Xenopus laevis*

Kohji Ikenishi,* Takeshi Ohno and Tohru Komiya

Department of Biology, Graduate School of Science, Osaka City University, 3-3-138, Sugimoto, Sumiyoshi, Osaka 558-8585, Japan

Whether all descendants of germline founder cells inheriting the germ plasm can migrate correctly to the genital ridges and differentiate into primordial germ cells (PGCs) at tadpole stage has not been elucidated in *Xenopus*. We investigated precisely the location of descendant cells, presumptive primordial germ cells (pPGCs) and PGCs, in embryos at stages 23–48 by whole-mount *in situ* hybridization with the antisense probe for *Xpat* RNA specific to pPGCs and whole-mount immunostaining with the 2L-13 antibody specific to *Xenopus* Vasa protein in PGCs. Small numbers of pPGCs and PGCs, which were positively stained with the probe and the antibody, respectively, were observed in ectopic locations in a significant number of embryos at those stages. A few of the ectopic PGCs in tadpoles at stages 44–47 were positive in TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) staining. By contrast, pPGCs in the embryos until stage 40, irrespective of their location and PGCs in the genital ridges of the tadpoles at stages 43–48 were negative in TUNEL staining. Therefore, it is evident that a portion of the descendants of germline founder cells cannot migrate correctly to the genital ridges, and that a few ectopic PGCs are eliminated by apoptosis or necrosis at tadpole stages.

Key words: apoptosis, ectopic primordial germ cell, TUNEL staining, *Xenopus vasa*, *Xpat*.

Introduction

It is well known in various animal species that germline founder cells arise at a site far from the future gonadal region in embryos, then proliferate, migrate with development and finally reach the genital ridges or gonadal rudiment in the larvae (for review, see Nieuwkoop & Sutasurya 1979). However, whether all descendants of germline founder cells can reach the genital ridges and differentiate into primordial germ cells (PGCs) in the following developmental stages has not been fully elucidated in almost all animal species, except for *Caenorhabditis elegans* (*C. elegans*). In *C. elegans*, the founder cell or P4 cell, which is formed at the 16–24 cell stage by four successive unequal divisions of fertilized eggs, divides once during embryogenesis into Z2 and Z3, which become the predecessor of germ cells in the larval gonad (Strome & Wood 1983). In *Xenopus*, the germline founder cells or presumptive primordial germ cells (pPGCs) inheriting the germ plasm at the end of the cleavage stage are thought to migrate toward the uppermost dorsal part of the endoderm cell mass, proliferating two to three times during the stages from gastrula to young tadpole, and finally reach the genital ridges where they are recognized as PGCs at the feeding tadpole stage (Ikenishi & Kotani 1975; Whitington & Dixon 1975; Kamimura *et al*. 1976, 1980; Dziadek & Dixon 1977; Heasman & Wylie 1983; Nishiumi *et al*. 2005). The terminology that we use in the present study for descendants of germline founder cells in *Xenopus* follows the nomenclature of Whitington and Dixon (1975); pPGCs are the cells inheriting the germ plasm in the endoderm cell mass of embryos until stage 40 and PGCs are those in tadpoles at stages 43–48.

It has been reported in *Drosophila* embryos that PGCs in an ectopic location to the gonadal rudiment are rapidly eliminated by apoptosis or autophagy, and that genes involved in the migration of germline cells could also affect programmed cell death (Coffman 2003). Similarly, a significant number of PGCs have been found in ectopic locations of mouse embryos during the embryonic day 10.5 (E10.5) to E11 period, implying a failure in the migration to the genital ridges (Stallock *et al*. 2003). This was suggested to be disintegrated by apoptosis by E15.5. We have also noticed in *Xenopus* that PGC-like cells, being similar in morphological features to PGCs in the genital

*Author to whom all correspondence should be addressed.
Email: Ikenishi@sci.osaka-cu.ac.jp
Received 17 April 2007; revised 25 April 2007; accepted 1 May 2007.

ridges, have frequently been observed in an ectopic location, mainly in the intestine of the tadpoles. The cells have been roundish in shape, still having many yolk platelets inside, and had an intercellular space to the surrounding, columnar shaped intestinal cells. Apoptosis was reported to occur in some of the cells that were involved, mainly, in neural induction, neural plate patterning and the development of the central nervous system at stages later than the gastrula in *Xenopus* embryos (Hensey & Gautier 1998). However, apoptosis has not been studied in the precursors of germ cells in *Xenopus* embryos so far because of their small number, basically. The maximum number of PGCs reported in *Xenopus* tadpoles at stages 45–47 was at most, 60 (Dixon 1981), meaning that the number of pPGCs in the preceding stages was probably fewer.

In the present study, we investigated whether PGCs are found in a location ectopic to the genital ridges in *Xenopus* tadpoles at stages 43–48, at which almost all PGCs are considered to colonize in the genital ridges (Ijiri & Egami 1975; Züst & Dixon 1977). We then studied when ectopic pPGCs, possibly forerunners of ectopic PGCs, are first noticed in embryos, and what the fates of ectopic, pPGCs and PGCs are. First, PGCs in and ectopic to the genital ridges of tadpoles at the above-mentioned stages were examined by whole-mount immunostaining with the 2L-13 antibody specific to the protein of *Xenopus vasa* homolog (*Xenopus vasa-like gene 1*, *XVLG1*) (Komiya *et al*. 1994). Second, the location of pPGCs was examined in embryos at stages 23–40 by whole-mount *in situ* hybridization with the antisense probe for *Xpat* (Hudson & Woodland 1998). Finally, pPGCs and PGCs that were found not only in ectopic but also in proper locations were studied in embryos at stages 18–48 by immunostaining with the 2L-13 antibody and TUNEL (TdT-mediated dUTP digoxigenin nick end labeling) staining (Gavrieli *et al*. 1992) in combination.

Materials and methods

Embryos

Fertilized eggs were obtained by the mating of mature, *Xenopus* males and females as previously described (Kotani *et al*. 1973). Eggs were dejellied with 1.5% Cysteine-HCl (Wako pure Chemical Industries, Osaka, Japan) in 1/10 Holtfreter's solution (60 mM NaCl, 0.67 mM KCl, 0.9 mM $CaCl_2$ and 2.4 mM NaHCO3; pH 8.0) and allowed to develop in the Holtfreter's solution thereafter. Embryos were staged after Nieuwkoop & Faber (1967). For whole-mount immunocytology, embryos at stages 18–48 were fixed with Dent's fixative (Dent & Klymkowsky 1989) at –20°C overnight. For whole-mount *in situ* hybridization, embryos at stages 23, 28, 33/34 and 40 were fixed with 4% paraformaldehyde at room temperature (22–24°C) for 2 h, essentially as previously described (Ikenishi & Tanaka 2000). Fixed embryos were kept in methanol until the following use.

Whole-mount immunocytology with the 2L-13 antibody

To examine PGCs in and ectopic to the genital ridges, whole-mount immunocytology was carried out on tadpoles at stages 43–48, according to the method of Dent and Klymkowsky (1989). The tadpoles were reacted with the 2L-13 antibody (2 μg/mL) (Komiya *et al*. 1994), followed with horseradish peroxidase (HRP)-conjugated, goat antimouse IgG + IgM (1 : 100 in dilution; Biosource International, Camarillo, CA, USA) and colored with diaminobenzidine (DAB) as a substrate. Serial sections of the colored tadpoles embedded in paraplast (Oxford Labware, St Louis, MO, USA) were cut transversely at 8 μM thick, essentially as previously described (Ikenishi *et al*. 1996). After removing the wax with xylene, they were immersed with Entellan (Merck, Darmstadt, Germany) and examined under a light microscope (Olympus BH2, Olympus Kogaku, Tokyo, Japan). PGCs stained dark brown with DAB in and ectopic to the genital ridges were counted and photographed as usual.

Whole-mount in situ *hybridization with antisense probe for* Xpat

To investigate precisely the location of pPGCs in the endoderm cell mass, whole-mount *in situ* hybridization was carried out with antisense probe for *Xpat* RNA because the RNA was specifically expressed in pPGCs until the stage at which the dorsal mesentery forms (Hudson & Woodland 1998). The hybridization was carried out on embryos at stages 23, 28, 33/34 and 40 of the same egg batch as used in Series III of Table 1 in the same manner as previously described (Ikenishi & Tanaka 2000), i.e. the embryos were hybridized with the antisense probe, then reacted with alkaline phosphatase-conjugated antidigoxigenin antibody and colored with NBT (4-nitro blue tetrazolium chloride) (Boehringer, Ingelheim, Germany) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (Boehringer) as the substrate.

Serial sections of the colored embryos embedded in paraplast were cut transversely at 10 μM thick, dewaxed with xylene, immersed with Entellan and

Table 1. Ectopic primordial germ cells (PGCs) and PGCs in the genital ridges of *Xenopus* tadpoles at stages 43–48

Experiment	Stage	No. tadpoles examined	No. tadpoles with ectopic PGCs	No. ectopic PGCs in a tadpole	Average no. ectopic PGCs†	Average no. PGCs in the genital ridges‡
Series I	44	13	9 (69%)	1, 1, 1, 2, 2, 3, 3, 4, 5	2.4 ± 0.4	7.8 ± 9.2
	46	15	4 (27%)	1, 2, 3, 3	2.3 ± 1.0	14.1 ± 9.1
	47	18	9 (50%)	1, 1, 1, 1, 1, 1, 2, 3, 3	1.6 ± 0.9	12.5 ± 7.0
	48	18	2 (11%)	1, 1	1.0 ± 0	13.6 ± 7.7
Series II	43	17	17 (100%)	4, 5, 5, 6, 7, 7, 9, 9, 11, 12, 13, 14, 14, 14, 19, 20, 23	11.3 ± 5.6	44.7 ± 14.9
	44	19	19 (100%)	2, 2, 3, 4, 4, 5, 9, 10, 10, 11, 12, 12, 12, 14, 17, 18, 19, 26, 45	12.4 ± 10.2	53.3 ± 10.9
	46	14	14 (100%)	1, 2, 3, 5, 5, 5, 5, 10, 11, 12, 13, 14, 17, 19	8.7 ± 5.8	49.2 ± 10.2
	48	15	0 (0%)		0	38.4 ± 10.3
Series III	44	17	6 (35%)	1, 1, 1, 1, 1, 2	0.4 ± 0.6	29.7 ± 15.5
	45	20	13 (65%)	1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 3, 4, 5	1.3 ± 1.4	37.1 ± 13.3
	46	20	9 (45%)	1, 1, 1, 1, 2, 2, 2, 3, 3	0.8 ± 1.1	24.5 ± 11.8
	47	20	13 (65%)	1, 1, 1, 1, 1, 1, 2, 2, 2, 2, 3, 3, 5	1.3 ± 1.3	36.5 ± 8.3
	48	14	7 (50%)	1, 1, 1, 1, 1, 2, 2	0.6 ± 0.7	30.4 ± 14.4

Experiment	Stage	No. tadpoles examined	No. tadpoles with ectopic PGCs	No. ectopic PGCs in a tadpole	Average no. ectopic PGCs‡		Average no. PGCs in the genital ridges
					anterior	genital ridges	
Series IV	43	15	12 (80%)	1, 1, 2, 2, 2, 2, 3, 3, 3, 4, 5	1.3 ± 1.2 (0–3)	1.2 ± 1.0 (0–3)	27.9 ± 7.5
	44	16	14 (88%)	1, 1, 1, 1, 1, 1, 2, 2, 3, 3, 4, 4, 5, 7	1.8 ± 1.4 (0–4)	0.8 ± 0.9 (0–3)	34.6 ± 10.3
	45	17	15 (88%)	1, 1, 2, 2, 3, 3, 3, 3, 3, 4, 4, 5, 5, 6, 10	2.4 ± 1.9 (0–7)	1.3 ± 1.2 (0–3)	29.5 ± 8.4
	46	16	15 (94%)	1, 1, 2, 2, 3, 3, 4, 4, 4, 5, 6, 6, 8, 10, 11	3.7 ± 2.7 (0–9)	1.0 ± 1.2 (0–4)	36.5 ± 15.2
	47	17	17 (100%)	2, 2, 2, 3, 4, 4, 4, 5, 5, 6, 6, 6, 6, 7, 8, 11, 12	4.2 ± 2.8 (1–11)	1.2 ± 1.1 (0–3)	39.6 ± 9.3
	48	17	15 (88%)	1, 1, 1, 1, 2, 2, 3, 3, 4, 5, 5, 6, 6, 6, 8	2.5 ± 2.4 (0–8)	1.1 ± 1.3 (0–5)	36.9 ± 12.0

†Average number of ectopic primordial germ cells (PGCs) in tadpoles with ectopic PGCs. Ectopic PGCs in Series IV were subdivided into two groups, 'anterior' and 'genital ridges', according to their location in the antero-posterior direction of the tadpoles. PGCs found at a more anterior level than the genital ridges in transverse sections (region 'a' in Fig. 2C) were classified as the 'anterior' group and those at the level of the genital ridges as the 'genital ridges' (region 'b' in Fig. 2C). There are significant differences in the average numbers between both groups at all stages, except for 43 by *t*-test ($P < 0.05$). Numbers within parentheses are the range in numbers of ectopic PGCs. ‡Average number of PGCs in the genital ridges of tadpoles, including those with and without ectopic PGCs.

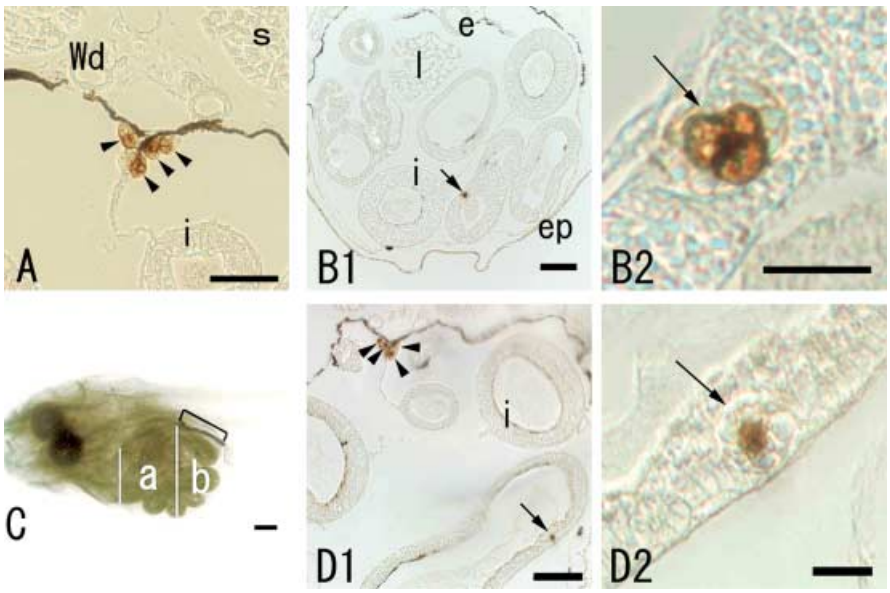

Fig. 1. Whole-mount immunostaining for tadpoles with the 2L-13 antibody against XVLG1 (Xenopus Vasa) protein. Positive cells with the antibody or primordial germ cells (PGCs) were stained dark brown with diaminobenzidine (DAB). The top of all figures is dorsal and the bottom is ventral. e, esophagus; ep, epidermis; i, intestine; l, liver; s, somite; Wd, Wolffian duct. (A) A transverse section of a stage-47 tadpole. PGCs in the genital ridges (arrowheads) or a proper location are heavily stained. Bar, 50 μM. (B1) A transverse section of the same tadpole in A at a more anterior level than the genital ridges (corresponding to region 'a' in Fig. 2C). A positively stained cell, probably an ectopic PGC (arrow), is seen in the intestine at the ventral side of the tadpole. Bar, 50 μM. (B2) A higher magnification of the cell (arrow) in B1. It is strongly stained with the antibody like PGCs in the genital ridges, indicating it is an ectopic PGC. Bar, 25 μM. (C) Lateral view of a stage-46 tadpole. Heavily stained PGCs lining the genital ridges (bracket) are externally visible because of the bleaching and clearing agent. Ecotopic PGCs in region 'a' was defined as 'anterior' and those in region 'b' as 'genital ridges' in Series IV of Table 1. Bar, 200 μM. (D1) A transverse section of a stage-47 tadpole at the level of the genital ridges (corresponding to region 'b' in Fig. 2C). Positively stained PGCs are seen in the genital ridges (arrowheads) and the intestine at the ventral side (arrow) of the tadpole. Bar, 100 μM. (D2) A higher magnification of the ectopic PGC in the intestine (arrow). Bar, 25 μM.

examined with a light microscope. The locations of positive pPGCs in antero-posterior directions in the endoderm cell mass were calculated by the number of sections commencing with the first section in which the anterior end of the endoderm cell mass appeared. As there was a significant difference in the lengths of endoderm cells in the antero-posterior direction even in embryos at the same stages, the location of pPGCs at the direction was normalized by assuming that the anterior and posterior ends of the endoderm mass were 0 and 1 at the horizontal line, respectively. On the other hand, dorso-ventral direction was measured with a micrometer in the ocular. In effect, the distance of pPGCs from the bottom of the endoderm cell mass was measured (d1 in Fig. 2A2). As there was not much difference in the height of the endoderm cells of the embryos at the same stages, the distance of pPGCs in the dorso-ventral direction was not normalized. Lateral profiles of masses at each stage were drawn based on the average values of the top of the endoderm cell mass (d2 in Fig. 2A2) at every 10 sections from the above-mentioned first section in all embryos examined.

Whole-mount immunostaining with the 2L-13 antibody, followed by TUNEL staining

In order to know the fates of ectopic pPGCs and PGCs, embryos were treated with a combined method of immunostaining with the 2L-13 antibody and TUNEL staining (Gavrieli *et al.* 1992). TUNEL staining is widely accepted as the most specific and sensitive technique for *in situ* detection of DNA fragments specific to apoptotic or necrotic cells.

To identify pPGCs and PGCs in embryos, whole-mount immunocytology with the 2L-13 antibody, followed by Cy3-conjugated antimouse IgG (1: 100 in dilution; Chemicon International, Temecula, CA, USA) was carried out on fixed embryos at stages 18–48, essentially as mentioned above. Sections of the embryos embedded in polyester wax were cut 8 μM-thick and mounted on a glass slide according to the method as previously described (Nishiumi *et al.* 2005). After removing polyester wax with 95% ethanol, they were postfixed with 4% paraformaldehyde in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 8.1 mMNa_2HPO_4, 2.7 mM KCl and 136 mM NaCl) for 5 min at room

temperature. After washing with PBS, the sections were stained with a TUNEL staining kit (Wako), essentially according to the protocol of the manufacturer. Sections were reacted with terminal deoxynucleotidyl transferrase (TdT) and dUTP, and then with HRP-conjugated antibody for dUTP, followed with Alexa 488-labeled tyramide (Invitrogen, Paisley, UK) as a substrate. After washing with PBS, sections were immersed with a mixture of glycerine and PBS (9 : 1), and examined with a confocal microscope (Olympus Flouview). The cytoplasm of pPGCs or PGCs was stained red with Cy3, and the cytoplasm and the nucleus of PGCs in apoptosis or necrosis were stained red with Cy3 and green with Alexa 488, respectively.

Results

PGCs in and ectopic to the genital ridges at tadpole stages

Owing to the 2L-13 antibody specific to the protein of *Xenopus vasa* or XVLG1 protein, PGCs in the genital ridges or a proper location were strongly stained dark brown with DAB (Fig. 1A, D1) because the protein was restricted to PGCs in tadpoles at stages later than 43 (Ikenishi *et al.* 1996). Accordingly, it is reasonable to believe that cells in a location other than the genital ridges, which were positively stained with the antibody and had similar morphological features to PGCs in the genital ridges, were ectopic PGCs (Fig. 1B, D). That is, they were roundish in shape, still having many yolk platelets inside, and had an intercellular space to the surrounding cells of the intestine, being columnar in shape (Fig. 1B2, D2).

As shown in Table 1, PGCs were observed not only in the genital ridges but also in an ectopic location. Ectopic PGCs were present in a significant number of tadpoles at stages 44–48 in Series I and III and in all tadpoles at stages 43–46 in Series II. However, they were not observed in any tadpole at stage 48 in Series II. In all series, they were observed mostly in the intestine (Fig. 1B, D) and rarely in the stomach, both of which are of endodermal cell origin like PGCs, but never observed in the derivatives of ectoderm and mesoderm. They were variable in number among tadpoles from the same batch and among those from different batches; the number of ectopic PGCs per tadpole ranged from 1–5 in Series I, 1–45 in Series II, 1–5 in Series III and 1–12 in Series IV (Table 1). Similarly, there were considerable differences in the average numbers of PGCs in the genital ridges among these series. Nevertheless, there was no distinctive relationship between the numbers of ectopic PGCs and PGCs in the genital ridges among the series.

As for the location of ectopic PGCs in Series II, a tendency that a majority were observed in the intestine at a more anterior level than the genital ridges in the antero-posterior direction of tadpoles was noticed. In order to confirm the tendency, ectopic PGCs were subdivided into two groups, 'anterior' (corresponding to region 'a' in Fig. 1C) (Fig. 1B) and 'genital ridges' (corresponding to region 'b' in Fig. 1C) (Fig. 1D), according to their locations in tadpoles of Series IV. There were significant differences in the average numbers between both groups at all stages, except for 43, by *t*-test ($P < 0.05$) (Table 1). This means that a majority of ectopic PGCs were situated at a more anterior level than the genital ridges in the anterior-posterior direction of the tadpoles.

Location of pPGCs

In order to know precise locations of pPGCs or possibly, forerunners of PGCs, whole-mount *in situ* hybridization with the antisense probe for *Xpat* (Hudson & Woodland 1998) was carried out on embryos at stages 23, 28, 33/34 and 40. pPGCs were easily identified on serial sections by the positive staining in the *in situ* hybridization (Fig. 2A2). Locations of all positive pPGCs in embryos at those stages from the same egg batch as in Series III of Table 1 were examined as shown in Figure 2A2. Two-hundred and sixteen pPGCs of nine stage-23 embryos, 404 of 12 stage-28 embryos, 563 of 12 stage-33/34 embryos and 131 of 10 stage-40 embryos (Table 2) were plotted on schematic drawings of the endoderm cell mass

Table 2. Presumptive primordial germ cells (pPGCs) in embryos that were identified with the antisense probe for Xpat RNA

Stage	No. embryos	Total no. pPGCs	'Proper' location†	'Ectopic' location‡
23	9	216	211	5 (2.3%)
28	12	404	388	16 (4.0%)
33/34	12	563	550	13 (2.3%)
40	10	131	119	12 (9.2%)

†pPGCs forming a cluster in the endoderm cell mass shown in Figure 1 (blue dots). ‡pPGCs apart from the cluster of pPGCs shown in Figure 1 (red dots).

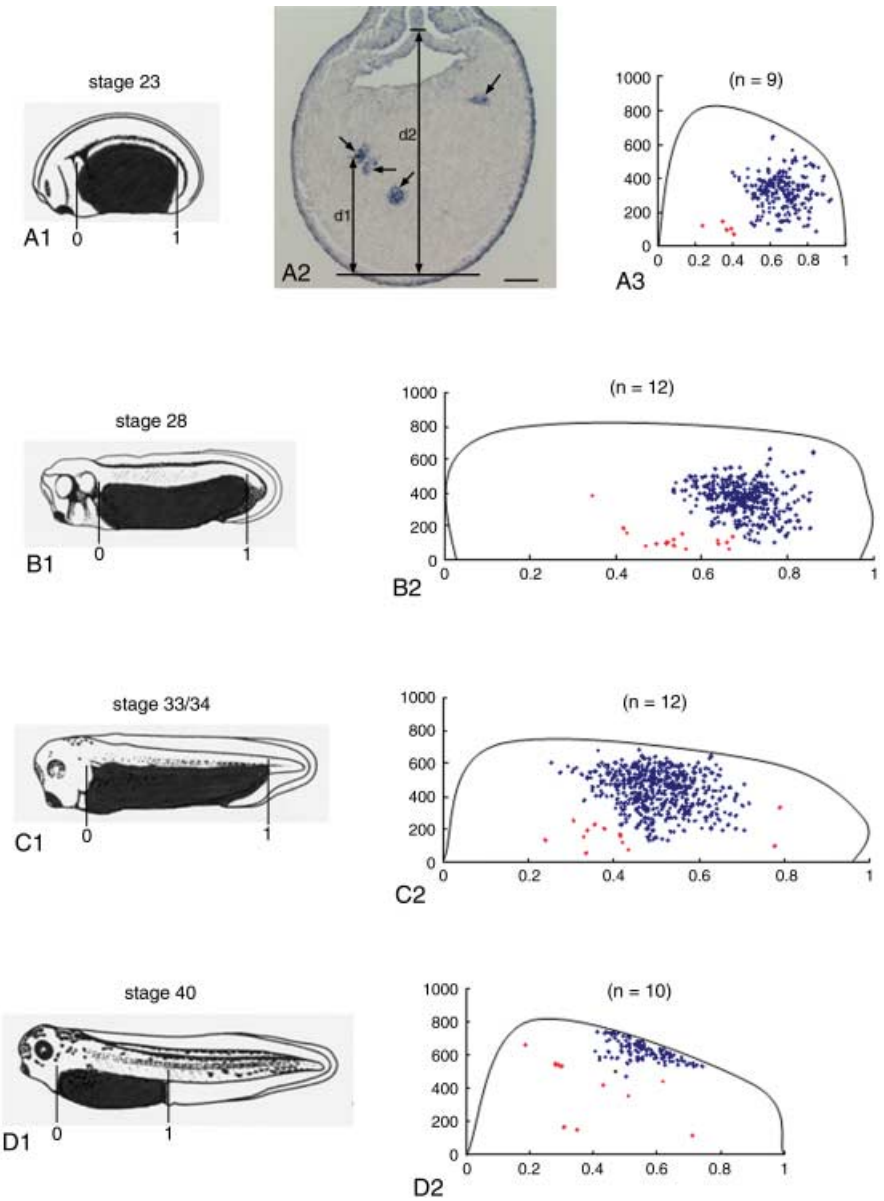

Fig. 2. Distribution of presumptive primordial germ cells (pPGCs) in the endoderm cell mass (a part stained black) of embryos at stages 23 (A), 28 (B), 33/34 (C) and 40 (D) in Series III. Location of pPGCs, being positive by whole-mount *in situ* hybridization with the antisense probe for *Xpat* RNA (arrows in A2) (216, 404, 563 and 131 in total at stages 23 [A3], 28 [B2], 33/34 [C2] and 40 [D2], respectively), was plotted on schematic drawings of the lateral profile of the endoderm cell mass of the embryos. It was difficult to distinguish accurately ectopic pPGCs from pPGCs in their proper location in embryos at stages 23–40. Therefore, pPGCs forming a cluster (blue dots) are defined as pPGCs in a 'proper' location and those distinctly apart from the cluster (red dots) as pPGC in an 'ectopic' location for convenience. Each dot represents the location of a single pPGC. 0 and 1 on the horizontal line represent the most anterior and posterior ends of the mass, respectively, to normalize the location of pPGCs in the antero-posterior direction of all embryos at each stage. The values on the vertical line (μM) represent the distance of each pPGC (d1 in A2) from the bottom of the mass. Lateral profile of the mass at each stage was drawn based on the average values of the top of the endoderm cell mass (d2 in A2) at every 10 sections from the first section of all embryos examined, in which the anterior end of the endoderm cell mass appeared. See Methods for details. The top and the bottom of A2 are dorsal and ventral sides, respectively. Bar in A2, 100 μM.

in embryos at those stages (Fig. 2). A majority of the pPGCs, forming a cluster, were found in the central part of the posterior endoderm cell mass at stages 23 and 28, in the centro-lateral part at stage 33/34 and in the centro-dorsal part at stage 40 (Table 2; blue dots in Fig. 2A3, B2, C2, D2), as reported earlier (Ikenishi & Kotani 1975). Also, a small number of pPGCs were distinctly observed in more anterior or more ventral parts (defined as an 'ectopic' location for convenience) than the part of the cluster ('proper' location) in the endoderm cell mass at all stages examined (Table 2; red dots in Fig. 2). Rarely, a few 'ectopic' pPGCs were recognized in more posterior parts than the cluster (Fig. 2C2)

The number of positive pPGCs in embryos at stage 40 was considerably small as compared with that at the preceding stages. This may be caused not by the decrease of pPGCs with development but by the disappearance of the signal with the antisense probe from pPGCs because the number of pPGCs identified with the 2L-13 antibody in embryos at stage 40 in the same egg batch was significantly larger than that with the antisense probe (average number per embryo in the former and the latter were 33.4 and 13.1,

Table 3. Presumptive primordial germ cells (pPGCs) or PGCs in *Xenopus* embryos that were positive or negative in TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) staining[†]

				Ectopic location[§]	
Stage	No. embryos examined	No. pPGCs or PGCs examined	Proper location[‡]	TUNEL-positive	TUNEL-negative
18	7	89	89		–[¶]
24	8	114	114		–[¶]
28	8	127	124	0	3
33/34	9	228	215	0	13
40	11	541	505	0	36
44	12	510	432	4	74
45	10	490	431	0	59
46	12	575	541	1	33
47	11	539	506	1	32
48	10	427	411	0	16

[†]Embryos were obtained from the same single batch. All sections of embryos were not always examined because of the removal or damage of sections in the process of TUNEL staining. [‡]pPGCs forming a cluster in the endoderm cell mass in embryos at stages 28–40 as shown in Figure 1 (blue dots) and PGCs in the genital ridges at stages 44–48 were defined as pPGCs and PGCs in proper locations. All pPGCs and PGCs in the proper location were negative in TUNEL staining. [§]pPGCs apart from the cluster as shown in Figure 1 (red dots) were assumed to be ectopic. Similarly, PGCs not in the genital ridges at stages 44–48 were ectopic. [¶]It was difficult to distinguish ectopic pPGCs from pPGCs in proper location in embryos at 18 and 24 of this series, so all pPGCsat in those stages were regarded as pPGCs in proper locations.

respectively [$P < 0.001$]). This is also confirmed on pPGCs identified with the antibody in Table 3; the average number of pPGCs in stage-40 embryos was significantly larger than that in embryos at the preceding stages in the same batch.

Apoptotic pPGCs and PGCs

To investigate the fates of ectopic pPGCs and PGCs mentioned above, a combined method of immunostaining with the 2L-13 antibody and TUNEL staining was applied to embryos at stages 18–48. This was because it is not technically possible to apply both *in situ* hybridization and TUNEL staining for the same embryos. In addition, *Xpat* RNA was no longer present in PGCs in embryos at stages later than 40 (Hudson & Woodland 1998). Fortunately, it was sufficient for the 2L-13 antibody to identify pPGCs as well as PGCs throughout the stages examined, as described previously (Ikenishi *et al*. 1996, 2006); in effect, the location of pPGCs identified with the antibody was almost similar to that with the antisense probe for *Xpat* in embryos until stage 33/34.

TUNEL-positive pPGCs were scarcely detected in the endoderm cell mass of embryos at stages 18–40, irrespective of an ectopic or a proper location (Table 3), though a lot of pPGCs were identified with the antibody. By contrast, TUNEL-positive cells were certainly recognized, mainly in the neural tube, epidermis, optic vesicles, tailbud and their derivatives at those stages (Fig. 3A), as reported previously (Hensey & Gautier 1998). The positive PGCs, being a few in number, were observed in the intestine of the ventral region of some of the tadpoles at stages 44, 46 and 47 (Table 3; Fig. 3B), while any positive cell was not recognized in intestinal cells adjacent to the PGCs. On the other hand, all PGCs in the genital ridges of tadpoles at stages 44–48 examined (Fig. 3C) were negative.

Discussion

By precise investigation on serial sections of tadpoles treated with whole-mount immunocytology, a significant number of PGCs were identified in an ectopic location, mostly in the intestine, of a majority of tadpoles at stages 43–48, owing to the marker protein for PGCs or the XVLG1 protein recognized with the 2L-13 antibody (Table 1; Fig. 1). A small number of pPGCs were also noticed in an 'ectopic' location obviously forward from the cluster of pPGCs in a 'proper' location of the endoderm cell mass and in the vicinity of the bottom of the mass in embryos at stages 23–40 by *in situ* hybridization with the antisense probe for *Xpat* RNA (Table 2; Fig. 2, red dots). It was demonstrated in an earlier study (Ikenishi & Tsuzaki 1988) that the location of pPGCs in the endoderm cell mass affected their successful migration to the genital ridges. That is, when [^{3}H]thymidine-labeled pPGCs in the explants were implanted into the anterior (ectopic) or posterior (proper) halves of the endoderm of unlabeled host neurulae (stage 20), the percentage of tadpoles with labeled PGCs in the genital ridges in the former experimental group was significantly lower than that

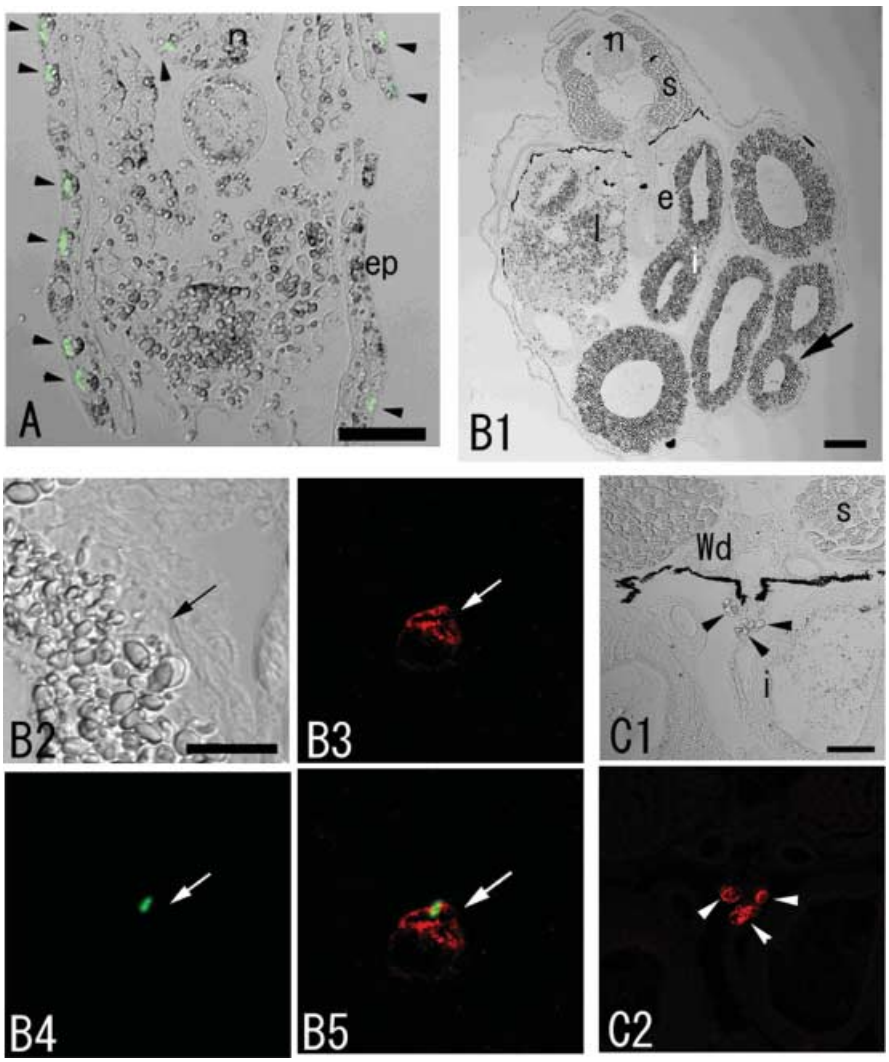

Fig. 3. Immunostaining with the 2L-13 antibody and TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) staining in combination. The cytoplasm of primordial germ cells (PGCs) reacted with the antibody was stained red with Cy3, and the cytoplasm and the nucleus of PGCs in apoptosis or necrosis were stained red with Cy3 and green with Alexa 488, respectively. Cells, except for PGCs, in apoptosis or necrosis were stained green only. The top of all figures is dorsal and the bottom is ventral. e, esophagus; ep, epidermis; i, intestine; l, liver; n, neural tube; s, somite; Wd, Wolffian duct. (A) A merged image of transmission and Alexa 488 of a transverse section of a stage-33/34 embryo at the level of the tail. Many epidermal cells and a cell in the neural tube are stained with Alexa 488 (arrowheads), indicating that they are apoptotic or necrotic cells. Bar, 50 μm. (B1) A transverse section of a stage-46 tadpole. An ectopic PGC (arrow) which is clarified in B3 is present in the intestine situated in the ventral side of the tadpole. Bar, 100 μm. (B2) A higher magnification of the cell in B1 (arrow). Bar, 25 μm. (B3) The same view of B2 with Cy3. The fluorescence of Cy3 for the marker protein or XVLG1 protein in PGCs is detected on the cell (arrow), indicating that the cell is an ectopic PGC. (B4) The same view of B2 with Alexa 488. The fluorescence of Alexa 488 seems to be detected on the nucleus of the cell (arrow). (B5) A merged image of B3 and B4. This clearly shows the PGC being in apoptosis or necrosis. (C1) A transverse section of a stage-47 tadpole. PGCs (arrowheads) are seen in the genital ridges. Bar, 50 μm. (C2) A merged image of the same section in C1 with Cy3 and Alexa 488. PGCs (arrowheads) are stained with Cy3 but not with Alexa 488. Likewise, PGCs in the genital ridges of tadpoles at stages 44–48 were negative in TUNEL staining.

in the latter. Accordingly, it is likely that ectopic pPGCs at stages 23–40 would not migrate correctly to the genital ridges at tadpole stages.

Recently, a cytokine or stroma cell-derived factor-1 (SDF-1) in the environment and its receptor or CXCR4 on the cell membrane of germline cells are reported to be involved in directed migration of those cells to the genital ridges in zebrafish, chick and mouse (Doitsidou *et al*. 2002; Ara *et al*. 2003; Molyneaux *et al*. 2003; Dumstrei *et al*. 2004; Stebler *et al*. 2004). In *Xenopus*, the protein of *xCXCR4* or *Xenopus CXCR4* homolog (Moepps *et al*. 2000) was detected in pPGCs of the 'proper' location mentioned above (Nishiumi *et al*. 2005). However, the protein was undetectable in the 'ectopic' locations during the active migratory phase from stage 24 onward (K. Ikenishi, unpubl. obs). *Xenopus sdf-1* homolog or *xsdf-1* was also expressed at about the same time in embryos (Braun *et al*. 2002). Assuming that directed migration of *Xenopus* pPGCs to the genital ridges via the uppermost dorsal part of the endoderm cell mass is also controlled by such signal(s) and receptor(s), including xSDF-1 and xCXCR4, the inability of 'ectopic' pPGCs in the migration can be interpreted in two ways. One is that the 'ectopic' pPGCs would not get the signals, possibly emanating from the posterior axial mesoderm (Gipouloux 1970; Gipouloux *et al*. 1979; Delbos *et al*. 1980), as suggested earlier (Ikenishi & Tsuzaki 1988)

because the pPGCs were distant from the posterior axial mesoderm. This is in good agreement with the suggestion made by Stallock *et al*. 2003 that if the range of survival signals released by the genital ridges stays constant, it restricts the survival of PGCs to those within a proper location of the embryos with development. The other interpretation is that the 'ectopic' pPGCs, not expressing the receptors like the xCXCR4 protein as mentioned above, would fail in the signal transduction that is necessary for migration, even if the signals could reach the pPGCs. Consequently, it is likely that 'ectopic' pPGCs that could not receive the signal wouldn't migrate to the genital ridges, resulting in the ectopic PGCs at tadpole stages.

In the present study, a small number of ectopic PGCs in some tadpoles were positive in TUNEL staining (Table 3; Fig. 3B), while all pPGCs in an ectopic or proper location of embryos at stages 28–40 and all PGCs in the genital ridges of the tadpoles were negative (Table 3; Fig. 3C). Thus, a small portion of the ectopic PGCs in *Xenopus* was demonstrated to be eliminated by apoptosis or necrosis at the tadpole stage, expressing the XVLG1 protein, as reported in Drosophila and mouse (Coffman *et al*. 2002; Stallock *et al*. 2003). In effect, germ cells ectopic to the gonads that should undergo programmed cell death persisted and continued to express germ cell markers.

As for the fate of ectopic PGCs that were negative in TUNEL staining, there are two possibilities. One is that the PGCs might be eliminated by apoptosis or necrosis in the following stages because no ectopic PGC expressing the XVLG1 protein was observed in the tadpoles at stage 48 in Series II (Table 1), despite the fact that 9–12 ectopic PGCs per tadpole on average were found in the tadpoles at the preceding stages of the series. The other is that they would differentiate into somatic cells in an ectopic location. Because when PGCs isolated from stage-45 tadpoles and were labeled with fluorescein and transplanted into an ectopic location or the blastocoel of host blastulae (stage 9), they differentiated into a variety of somatic cells according to their new location, such as myotome cells, notochord cells, skin cells and gut lining cells, in host tadpoles at stage 46 (Wylie *et al*. 1985).

In the present study, TUNEL-positive PGCs were never observed in the genital ridges of tadpoles until stage 48, though a considerable number of gonadal germ cells in embryos of mammals were eliminated by apoptosis (Coucouvanis *et al*. 1993; Ratts *et al*. 1995; Wang *et al*. 1998). Further study on apoptosis in *Xenopus* gonadal PGCs and their descendants at stages later than 49 may be helpful for thinking about the similarity or diversity in germ cell differentiation among animal species.

Acknowledgements

We are grateful to Dr Shigenobu Tone, Kawasaki Medical School, Okayama Prefecture for advice for TUNEL staining. Thanks also to Miss Yohko Tanigawa, a colleague of our Laboratory, for advice on light microscopy.

References

Ara, T., Nakamura, Y., Egawa, T. *et al*. 2003. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proc. Natl. Acad. Sci. USA* **100**, 5319–5323.

Braun, M., Wunderlin, M., Spieth, K., Knochel, W., Gierschik, P. & Moepps, B. 2002. *Xenopus laevis* stromal cell-derived factor 1: conservation of structure and function during vertebrate development. *J. Immunol*. **168**, 2340–2347.

Coffman, C. R. 2003. Cell migration and programmed cell death of *Drosophila* germ cells. *Ann. N. Y. Acad. Sci*. **995**, 117–126.

Coffman, C. R., Strohm, R. C., Oakley, F. D., Yamada, Y., Przychodzin, D. & Boswell, R. E. 2002. Identification of X-linked genes required for migration and programmed cell death of *Drosophila melanogaster* germ cells. *Genetics* **162**, 273–284.

Coucouvanis, E. C., Sherwood, S. W., Carswell-Crumpton, C., Spack, E. G. & Jones, P. P. 1993. Evidence that the mechanism of prenatal germ cell death in the mouse is apoptosis. *Exp. Cell Res*. **209**, 238–247.

Delbos, M., Lestage, J. & Gipouloux, J. D. 1980. Localisation cytoplasmique d'une importante activite adenylate cyclasique dans le cordo-mesoderme embryonnaire des amphibiens anoures. *Arch. Anat. Microsc. Morph. Exp*. **69**, 47–56.

Dent, J. A. & Klymkowsky, M. W. 1989. Whole-mount analyses of cytoskeletal reorganization and function during oogenesis and early embryogenesis in *Xenopus*. In *The Cell Biology of Fertilization* (eds H. Schatten & G. Schatten), pp. 63–103. Academic Press, San Diego.

Dixon, K. E. 1981. The origin of the primordial germ cells in the amphibia. *Neth. J. Zool*. **31**, 5–37.

Doitsidou, M., Reichman-Fried, M., Stebler, J. *et al*. 2002. Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell* **111**, 647–659.

Dumstrei, K., Mennecke, R. & Raz, E. 2004. Signaling pathways controlling primordial germ cells migration in zebrafish. *J. Cell Sci*. **117**, 4787–4795.

Dziadek, M. & Dixon, K. E. 1977. An autoradiographic analysis of nucleic synthesis in the presumptive primordial germ cells of *Xenopus laevis*. *J. Embryol. Exp. Morph*. **37**, 13–31.

Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. 1992. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol*. **119**, 493–501.

Gipouloux, J. D. 1970. Recherches experimentales sur l'origine, la migration des cellules, et l'edification des cretes genitals cheZ. les amphibiens anoures. *Bull. Boil. Fr. Belg*. **104**, 21–93.

Gipouloux, J. D., Delbos, M. & Girard, C. 1979. Influence de l'adenosine monophosphate cyclique sur la migration des cellules germinales primordiales des amphibiens anoures. Arch. *Anat. Microsc. Morph. Exp*. **68**, 61–71.

Heasman, J. & Wylie, C. C. 1983. Amphibian primordial germ cells-what can they tell us about directed cell migration? In *Current Problems in Germ Cell Differentiation* (eds

A. McLaren, & C. C. Wylie), pp. 73–90. Cambridge University Press, Cambridge.

Hensey, C. & Gautier, J. 1998. Programmed cell death during *Xenopus* development: a spatio-temporal analysis. *Dev. Biol.* **203**, 36–48.

Hudson, C. & Woodland, H. R. 1998. *Xpat*, a gene expressed specifically in germ plasm and primordial germ cells of *Xenopus laevis*. *Mech. Dev.* **73**, 159–168.

Ijiri, K. & Egami, N. 1975. Mitotic activity of germ cells during normal development of *Xenopus laevis* tadpoles. *J. Embryol. Exp. Morph.* **34**, 687–694.

Ikenishi, K. & Kotani, M. 1975. Ultrastructure of the 'germinal plasm' in *Xenopus* embryos after cleavage. *Dev. Growth Differ.* **17**, 101–110.

Ikenishi, K. & Tanaka, T. S. 2000. Spatio-temporal expression of *Xenopus vasa* homolog: the presence of *XVLG1* RNA in somatic cells as well as germline cells. *Dev. Growth Differ.* **42**, 95–103.

Ikenishi, K. & Tsuzaki, Y. 1988. The positional effect of presumptive primordial germ cells (pPGCs) on their differentiation into PGCs in *Xenopus*. *Development* **102**, 527–535.

Ikenishi, K., Tanaka, T. S. & Komiya, T. 1996. Spatio-temporal distribution of the protein of *Xenopus vasa* homologue (*Xenopus vasa-like gene 1*, *XVLG1*) in embryos. *Dev. Growth Differ.* **38**, 527–535.

Ikenishi, K., Nishiumi, F. & Komiya, T. 2006. The Xdsg protein in presumptive primordial germ cells (pPGCs) is essential to their differentiation into PGCs in *Xenopus*. *Dev. Biol.* **297**, 483–492.

Kamimura, M., Ikenishi, K., Kotani, M. & Matsuno, T. 1976. Observations on the migration of gonocytes in *Xenopus laevis*. *J. Embryol. Exp. Morph.* **36**, 197–207.

Kamimura, M., Kotani, M. & Yamagata, K. 1980. The migration of presumptive primordial germ cells through the endodermal cell mass in *Xenopus laevis*: a light and electron microscopic study. *J. Embryol. Exp. Morph.* **59**, 1–17.

Komiya, T., Itoh, K., Ikenishi, K. & Furusawa, M. 1994. Isolation and characterization of a novel gene of the DEAD box protein family which is specifically expressed in germ cells in *Xenopus laevis*. *Dev. Biol.* **162**, 354–363.

Kotani, M., Ikenishi, K. & Tanabe, K. 1973. Cortical granules remaining after fertilization in *Xenopus laevis*. *Dev. Biol.* **30**, 228–232.

Moepps, B., Braun, M., Knöpfle, K., Dillinger, K., Knöchel, W. & Gierschik, P. 2000. Characterization of a *Xenopus laevis* CXC chemokine receptor 4: implications for hematopoietic cell development in the vertebrate embryo. *Eur. J. Immunol.* **30**, 2924–2934.

Molyneaux, K. A., Zinszner, H., Kunwar, P. S. *et al.* 2003. The chemokine SDF-1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development* **130**, 4279–4286.

Nieuwkoop, P. D. & Faber, J. 1967. *Normal Table of Xenopus Laevis (Daudin)*. North-Holland Publishers, Amsterdam.

Nieuwkoop, P. D. & Sutasurya, L. A. 1979. *Primordial Germ Cells in the Chordates; Embryogenesis and Phylogenesis*. Cambridge University Press, Cambridge.

Nishiumi, F., Komiya, T. & Ikenishi, K. 2005. The mode and molecular mechanisms of the migration of presumptive PGC in the endoderm cell mass of *Xenopus* embryos. *Dev. Growth Differ.* **47**, 37–48.

Ratts, V. S., Flaws, J. A., Kolp, K., Sorensen, C. M. & Tilly, J. L. 1995. Ablation of *Bcl-2* gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. *Endocrinology* **136**, 3665–3668.

Stallock, J., Molyneaux, K., Schaible, K., Knudson, C. M. & Wylie, C. 2003. The pro-apoptotic gene *Bax* is required for the death of ectopic primordial germ cells during their migration in the mouse embryo. *Development* **130**, 6589–6597.

Stebler, J., Spieler, D., Slanchev, K. *et al.* 2004. Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Dev. Biol.* **272**, 351–361.

Strome, S. & Wood, W. B. 1983. Generation of asymmetry and segregation of germ-like granules in early *Caenorhabditis elegans* embryos. *Cell* **35**, 15–25.

Wang, R. A., Nakane, P. K. & Koji, T. 1998. Autonomous cell death of mouse male germ cells during fetal and postnatal period. *Bio. Reprod.* **58**, 1250–1256.

Whitington, P. M. C. D. & Dixon, K. E. 1975. Quantitative studies of germ plasm and germ cells during early embryogenesis of *Xenopus laevis*. *J. Embryol. Exp. Morph.* **33**, 57–74.

Wylie, C. C., Heasman, J., Snape, A., O'Driscoll, M. & Holwill, S. 1985. Primordial germ cells of *Xenopus laevis* are not irreversibly determined early in development. *Dev. Biol.* **112**, 66–72.

Züst, B. & Dixon, K. E. 1977. Events in the germ cell lineage after entry of the primordial germ cells into the genital ridges in normal and u.v.-irradiated *Xenopus laevis*. *J. Embryol. Exp. Morph.* **41**, 33–46.