

Development of Primordial Germ Cells (PGCs) in an Indian Freshwater Teleost, *Labeo rohita* (Hamilton)

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ABSTRACT

Labeo rohita is one of the Indian major carps present in all the freshwater ecosystems of India and is an economically important fish. Though primordial germ cells (PGCs) are biologically important as founders of germ cell lineage, no work has been carried out on PGCs of any of the Indian major carp. Therefore, the present work was undertaken to investigate the localization and migration of PGCs in *L. rohita*. PGCs are studied by collecting different stages of *L. rohita* by employing simple Hematoxyline-Eosine (HE) staining technique and were identified on the basis of their characteristic shape and size. Morphologically, they were elliptical as well as oval to spherical with clear cytoplasm. 24hrs (5mm) after hatching (ah), PGCs were identified and by 50mm stage of development, they were located on the gonadal ridge. These cells were bigger than the somatic or cyst cells which were present in their vicinity. Migrating germ cells were generally elliptical in shape and produced cytoplasmic extensions called fillopodia which help them in migration and adhesion. PGCs seem to have originated from the gut endoderm and started descending along the wall of intestine. From 24 hrs onwards, pronephric ducts are pushed upwards due to subsequent development of air sacs between the kidney and alimentary canal. Gonads were seen suspended in the coelomic cavity from 8 mm stage onwards from the dorsal body wall by a mesentery. Oogenic development was observed in 50 mm stage under light microscope in some specimens.

Key words: Development, PGCs, Teleost, *Labeo rohita*.

INTROCUTION

Germs cells prior to sex differentiation are called primordial germ cells (PGCs). They are the progenitor cells of the germ cell lineage and possess the ability to differentiate either into spermatogonia or oogonia. Embrogenic gonads of vertebrates develop from the genital ridge, a region that has the biopotential capacity to form either a testis or an ovary. The direction for ovary or testis deep in determined by the chromosomal composition of the individuals in most of the vertebrates [7]. A genetic factor probably determines the sexual fate of initially biopotential gonad in amphibians as in mammals, birds and fish [20]. However the differentiation of gonads is influenced by steroid hormones in some species of fish [30, 18], amphibious and reptiles [5] and birds [10, 25]. Thus it seems probable that endogenous sex steroids can act as natural inducers of gonadal differentiation.

In all the animals examined to date, PGCs are reported to set aside from somatic cell lineages during early embryonic development [31]. Development of the germ line is essential for survival of the species, as this provides continuity of life between generations. Despite this importance, relatively little is known about the piscine germ line development. Development of PGCs is a very classic phenomenon and therefore, investigators are curious to know at what development stage they will be irreversibly determined, whether specific inducing factors exist to make them germ cells etc. In the present work, identification, migration and differentiation of primordial germ cells is determined during development in the Indian fresh water major carp *Labeo rohita*.

MATERIALS AND METHODS

Healthy developmental stages of *Labeo rohita* were collected from the Pench Fish Seed Center (Maharashtra State Govt.), Pench, located near Nagpur city. All the stages were fixed in aqueous Bouin's fixative for 24 hours. From 30 mm onwards, longitudinal incision was given on the abdomen for proper fixation. Decalcification of these stages was carried out in 15% formic acid solution containing 5% formalin. 5% ammonium oxalate was used to assess the decalcification test for 5 minutes. Later, it was washed and dehydrated in series of ethyl alcohol grades, cleared in xylene, infiltrated in paraffin wax (58° - 60°C) and blocks were prepared.

Both the transverse and saggital sections were cut at 6-8 µm on rocking microtome (Cambridge) and were mounted serially. Sections were stained with Haematoxyline-Eosine and microphotographs were taken with Nikon camera.

RESULTS

Development of PGCs was studied from 24 hrs ah (5mm sac fry) up to 50 mm juvenile stage in *L. rohita*. Both saggital and transverse serial sections were considered for locating the PGCs. Identification was made on the basis of their characteristic shape and size.

24 hrs ah the fry attains the length of about 5mm. In the saggital section, PGCs, both spherical and elliptical were found scattered among the somatic or cyst cells, posterior to the yolk sac (Fig. 1) above the intestine and below the pronephric duct. Their cytoplasm was lightly stained and contained large sized nucleus with spherical nucleolus. Elliptical germ cells were presumed to be in the process of migration. In transverse section, PGCs appeared positioned dorso-lateral to the intestine on the gonadal ridge and ventral to a pair of pronephric ducts. (Fig. 2).

Three days ah (72 hrs ah), larva measured about 7 mm in length with almost completely absorbed yolk sac. In transverse and saggital section, PGCs were located at the anterior end of pancreatic streak with variations in their shapes and sizes. Some cells were oblong and others appeared elliptical (Fig. 3 & 4). Both the large and small sized PGCs were located above the dorsal side of the alimentary canal over the gonadal ridge. Kidney tissue was still not visible.

At 8 mm stage, yolk sac was completely absorbed, mouth opened and larva started feeding actively. All the systems were well developed. Kidney showed glomeruli and tubules. Pair of mesonephric ducts ran antero-posteriorly and lied at the ventral surface of the kidney. Intestinal folds increased and were coiled. Darkly stained peritoneum membrane was visible just ventral to the kidney and air bladder, alongside which, the PGCs were present (Fig. 5, 6).

At 10 mm stage, in the transverse section, PGCs were observed below the kidney along the peritoneum membrane which lies over the intestine (Fig. 7). In the sagittal section, they were seen in the posterior region of the abdominal cavity just posterior to the air sac. These were oval or spherical with clear cytoplasm and darkly stained nuclei. At this stage, PGCs were arranged in their form of string of beads (Fig. 8). These strings were not in contact with one another but were distributed along the peritoneum, spaced at a distance from each other.

By 15 mm stage, liver occupied almost the entire abdominal cavity like in the adults. PGCs were located caudally along the peritoneum membrane (Fig. 9). Larger PGCs were located at the surface of the pancreatic rim with clear cytoplasm and lightly stained nuclei, while smaller PGCs with clear cytoplasm and darkly stained nuclei were observed at the caudal region.

In saggital section in 20 mm stage, the gonadal ridge was seen for the first time along which both large and small PGCs were present (Fig.10). Darkly stained peritoneum membrane was present along the margin of gonadal ridge.

Nucleus was eccentric in position. However in transverse section, from the wall of alimentary canal an extension of somatic cells (cyst cells) appeared to arise, on which numerous PGCs were present. Both large and small PGCs were compactly arranged on the gonadal ridge (Fig.11) at this stage.

In 30 mm stage also, the large and small PGCs were observed. The germ cells at this stage appeared oval with darkly stained nuclei and clear cytoplasm. The nucleus was eccentrically placed in the cytoplasm (Fig 12 to 13).

By 40mm stage in transverse section, air sac was seen in between the kidney, pancreas and alimentary canal. The alimentary canal underwent prolific folding (Fig. 14). In saggital section, PGCs were present between the darkly stained pancreatic cells and alimentary canal (Fig.15). Differentiation in testes or ovaries could not be observed.

In transverse section at 50 mm stage, PGCs were found suspended in the body cavity just beneath the air sac along with the blood vessels. They were surrounded by few cyst cells. In some specimens only, ovary was seen with immature oocytes in the nest at the periphery of pancreas near the intestine (Fig.16). In majority of the specimens sex could not be differentiated even at this stage.

DISCUSSION

Primordial germ cells (PGCs) in *Labeo rohita* are distinguished as early as 24 hrs ah both in the transverse and saggital sections. These are placed below the pronephric duct and above the yolk sac in caudal region and above the dorsal mesentery in anterior region of the body cavity. In freshwater teleost *Cyprinus carpio*, PGCs are reported at the sites of gonadal ridges in the newly hatched larva at day 3 after fertilization [21]. In *L. rohita*, the mesonephric ducts are slowly pushed upwards, the yolk sac which is large initially in a newly hatched larva later slowly gets absorbed and by 72 hrs it is very much reduced. The mouth opens and larva starts feeding actively. In the 8 mm stage, glomeruli in the kidney are seen indicating its mesonephric status.

In *C. carpio*, gonadal primordia start to increase in size at about 3 weeks after fertilization [21]. In this carp, PGCs appear ovoid under light microscope and are easily recognized by their large size and irregular nucleus. Despite their mitotic resting phase, the average size of PGCs however increased during the development from week four and at each development stages, PGCs of different sizes are distributed randomly along the long axis of gonadal areas in individual animals [26]. In the major carp *L. rohita*, the primordial germ cells are very large initially which are called 'giant cells' at early stage of development but in later stages, the size of PGCs becomes comparatively smaller. In *Cottus bairdii*; [15] PGCs are first found before the gut and later along the ventral and lateral margins of the gut. In *L. rohita* also, these cells appear descending along the dorsal and lateral margins of gut from 24 hrs onwards. Similar findings are recorded for Zebra fish, *Danio rerio* [19]. In *L. rohita* PGCs are located mostly in the posterior half of the body.

As regards to their final destination, [1] investigated in mice that PGCs which appeared at the gonadal ridge at 24 hrs stage, after specification, follow the guidance cues that will ultimately lead them to the developing genital or gonadal ridge. In addition, between the time of specification and arrival in the genital ridges, the PGCs must not only survive, but proliferate. Finally, during this period, the PGCs must not respond to any inappropriate positional cues that would compromise their totipotency. Several evidences suggest that the PGCs are guided to the developing gonadal ridge, at least in part, by chemotropic means in chick embryo [17] and in mouse [12, 13]. During this migratory phase in mouse PGCs extend long filopodial processes that bind them together in extensive arrays [14]. Such a situation is seen in saggital section of *L. rohita* at 24 hrs a h and in some other subsequent stages. PGCs are somewhat elliptical in shape with long processes extending from both the terminals which must be helping them in adhering while migrating.

Different parameters are reported to characterize the PGCs in different fishes. In *C. bairdii* it was impossible to distinguish clearly between the cells which were to form the gut and those which were to become germ cells but in embryo of 2.77 mm, the germs cells could be identified with certainty. They are round or oval, larger than surrounding cells and stain less deeply, lying dorsolateral to the gut between the gut and wolffian duct [15]. In *Salmoidea salmoides*, early germ cells are characterized by yolk granules, presence of attraction spheres in their cytoplasm and cellular membranes by their darkly stained nuclei and hyaline cytoplasm and by their ability to migrate independently [16]. In *L. rohita*, such granular bodies are observed in the cytoplasm of 'giant cell' during

early period of development. Supporting cells are visible from 24 hrs onwards and are differentiated from PGCs by their comparatively dark staining.

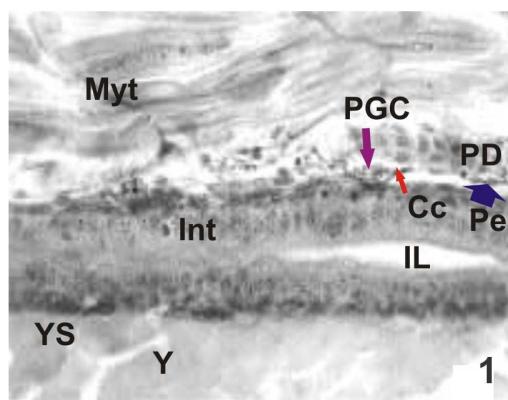
Among the mammals, in mouse embryos, on the basis of morphology in sectioned material (Clark and Eddy, 1975) and their behavior in culture (Donovan et al., 1986), PGCs seem to actively migrate from the hindgut to the genital ridge. Once they were immigrated from the gut, PGCs extended a long process which was used to associate with one another and by aggregation they arrive at the genital ridges (Gompert et al., 1994). In *L. rohita*, however, they descend in coelomic cavity along the gut. Coelomic cavity in this fish is quite distinct in 24 hrs which subsequently increased from 72 hrs stage; darkly stained pancreatic cells are very distinctly noticed. These are placed in close association with PGCs above the alimentary canal.

In the amphibian *Xenopus laevis*, PGCs were found in dorsal mesentery, clumped at the root of the mesentery between stages 43-45mm. At this stage PGCs bulged outward from the dorsal mesentery at or near its junction with posterior body wall [28]. Such bulging is seen in 20mm stage in *L. rohita*. Size of PGCs increased, they are elongated and 2 nuclei are noticed in the cytoplasm of PGCs in 48 hrs and 60hrs stages. PGCs do not divide until they reach the region of genital ridge in medaka, *Oryzias latipes* [11]. However the division of PGCs is observed in *L. rohita* at 48 hrs and 60 hrs stage post-hatching, which is designated as sac fry stage.

Sex differentiation is another feature which shows large variation in different fish species. In *Micropterus salmoides* (Black bass), sex was first microscopically distinguishable in the gonads of fingerlings of 3cm length. In 11-13 mm fry, gonad was not a continuous strand of germ cells but composed of discontinuous aggregations with intervening spaces, resembling chain of beads [16]. Such chain of beads in *L. rohita* is observed in 10mm stage sagittal section. Actually, single isolated primordial germ cells are seen placed on the ridge at this stage. In Black bass, between 20mm to 3cm stage, gonads pass through an indifferent period, marked increase in size by an increase in stroma and by multiplication of germ cells [16]. In *L. rohita*, however there is no consistency as far as the increase in size and number of PGCs is concerned.

In Japanese Eel (*Anguilla japonica*), sex differentiation occurs during late juvenile period i.e. at the time of pigmentation and upstream migration [6]. In *C. punctatus*, sex was differentiated suddenly between 5.4 mm to 12 mm stage [2]. In *L. rohita*, sex is differentiated at 50 mm stage only in some specimens.

Fig.1. Sagittal section of 24 hrs after hatching (a.h.) showing the myotomes (Myt), intestine (Int), yolk sac (YS), pronephric duct (PD) and primordial germ cells (PGCs) and cyst cells (Cc). X200.



Concomitant with the movement of PGCs in an amphibian *X. laevis*, a population of somatic cells possibly arising from the coelomic lining differentiates to form a highly organized band of tissues which completely covers the PGCs and was probably the precursor cells of germinal epithelium of the developing gonads. The somatic cells differentiate themselves without any influence from PGCs and they come to lie beneath them [29]. In *L. rohita*, PGCs appear to have originated from gut endoderm which is clearly visible in sagittal sections and also in the transverse sections of early stages. They get enveloped by the lining of coelomic cells and these cells may form somatic components of the gonads. The primordial germ cells (PGCs) in mice formed during embryogenesis ultimately give rise in the male to spermatogonia, the only self-renewing cell type in an adult which is capable of

making a genetic contribution to the next generation. Such a contemporary germ cell research in fish is important to both basic and applied biological sciences because of renewed interest in the origin and fate of PGCs with the aim to produce transgenic lines.

Fig.2. Transverse section of 24 hrs stage of *Labeo rohita* showing YS, Int, and Myt. X36.

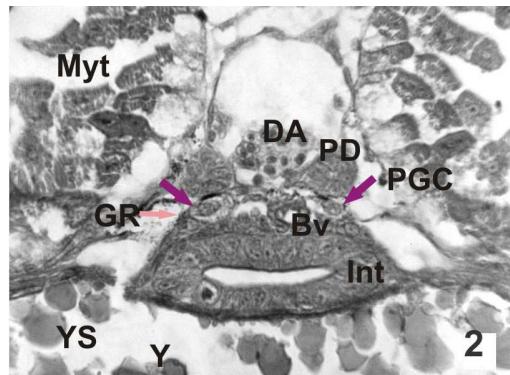


Fig.3. Magnified view of T.S. of 72 hrs stage (a.h.) showing PGCs (arrow) on gonadal ridge, cyst cells (Cc), intestine (Int) and pronephric duct (PD). X160.

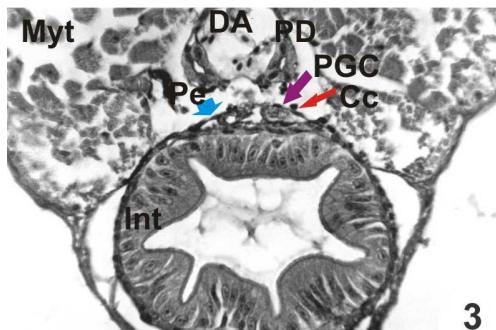


Fig.4. Magnified view of 72 hrs (a.h.) showing PGCs and intestine (Int). X400.

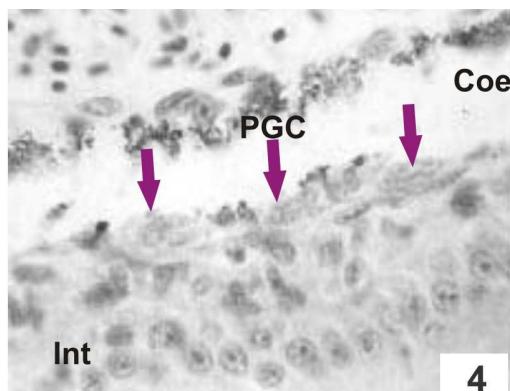


Fig.5. T.S. of 8 mm stage (a.h.) showing PGCs, kidney (K), mesonephric duct (MD) and intestine (Int). X200.

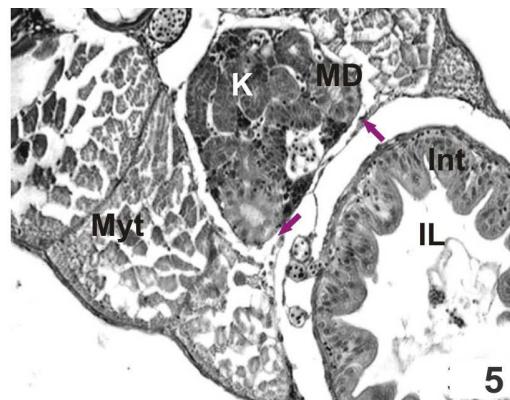


Fig.6. Magnified view of T.S. of 8 mm stage (a.h.) showing blood vessels (Bv), and elliptical PGCs (arrow) below the kidney. X400.

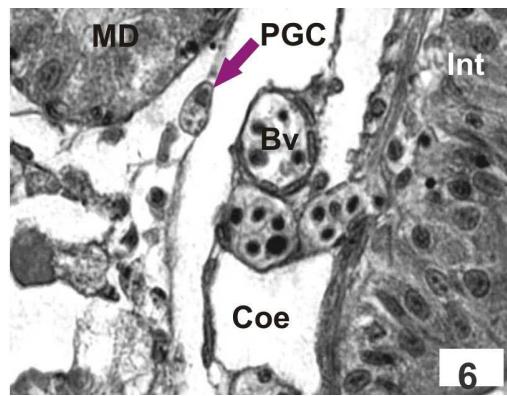


Fig.7. T.S. of 10mm stage (a.h.) showing PGCs, peritoneum (Pe), mesonephric duct (MD) and intestine (Int). X100

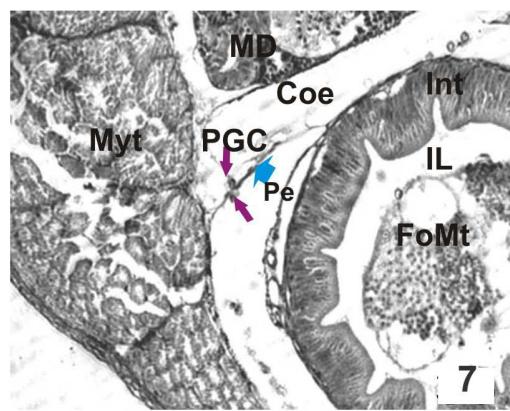


Fig.8. Magnified view of 10mm stage (a.h.) showing both large and small sized PGCs on the gonadal ridge (GR), peritoneum (Pe) and intestine (Int). X400.

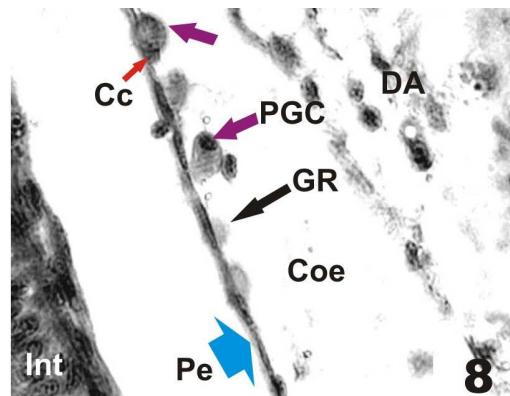


Fig.9. Magnified view of 15mm stage (a.h.) showing both large and small sized PGCs with filopodia, cyst cells (Cc), peritoneum (Pe), Pancreas (P) and intestine (Int). X400.

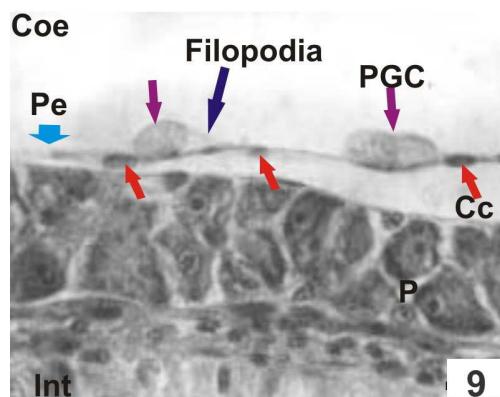


Fig.10. Sagittal section of 20 mm stage (a.h.) showing PGCs, air bladder (Ab), pancreas (P) and intestine (Int). X160.

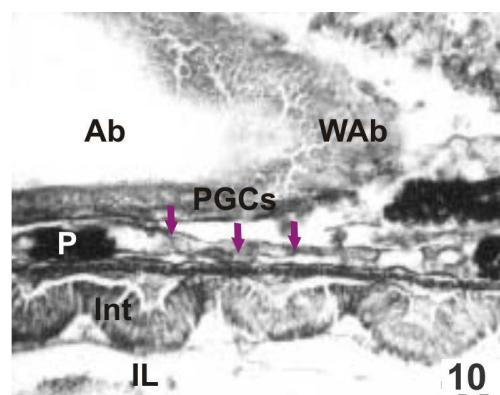


Fig.11. Magnified view of 20 mm stage (a.h.) showing large and small sized PGCs (arrow) near the intestine (Int) X400.

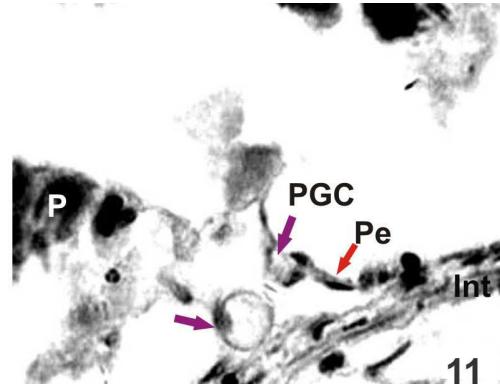


Fig.12. T.S. of 30 mm stage (a.h.) showing PGCs, peritoneum (Pe) and intestine (Int). X100.

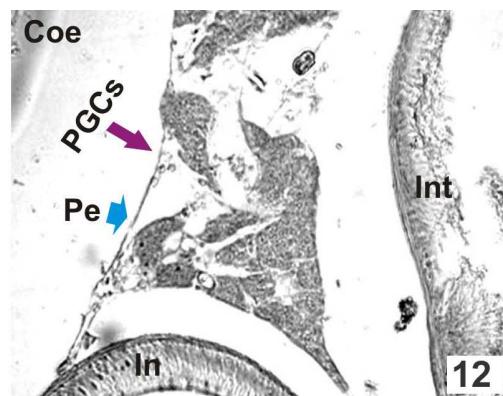


Fig.13. Magnified view of 30 mm stage (a.h.) showing PGCs, cyst cells (Cc), Blood vessel (Bv) and pancreas (P). X400.

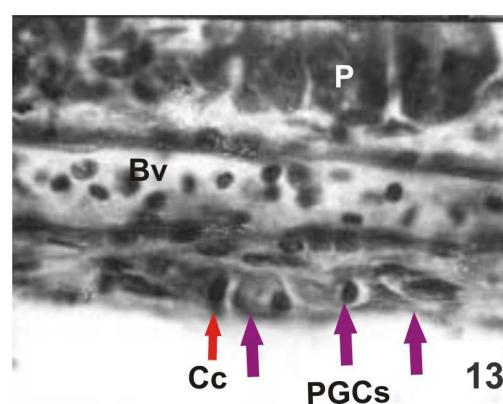


Fig.14. Magnified view of T.S. of 40 mm stage (a.h.) showing PGCs, cyst cells (Cc), peritoneum (Pe), pancreas (P) and intestine (In). X400.

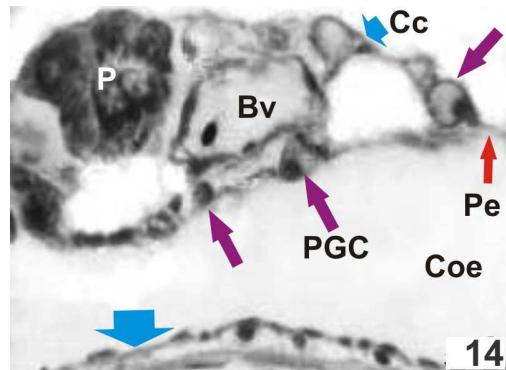


Fig.15. Magnified view of 40 mm stage showing dividing PGCs (arrow) with prominent nuclei. X400.

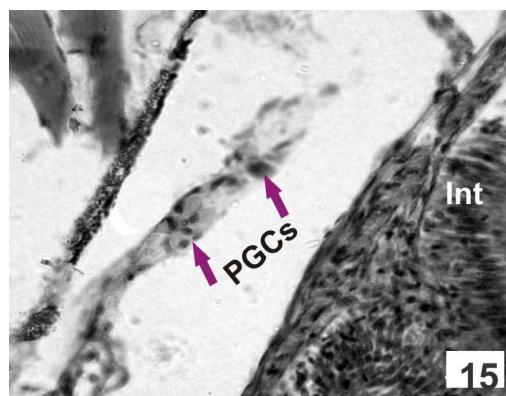
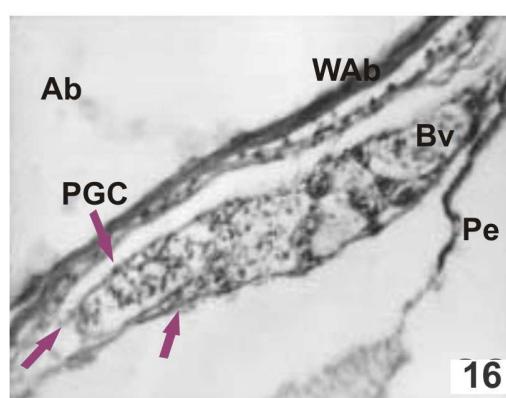


Fig.16. T.S. of 50 mm stage (a.h.) showing PGCs, peritoneum (Pe), blood vessel (Bv) and air bladder (Ab). X160.



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