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Ultrastructure characteristics of primordial germ cells in stage X of pheasant (*Phasianus colchicus*) embryo

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Abstract

Stage X is one of the formation stages in birds at which the blastoderm area is distinguished by two areas of area pellucida being responsible for formation of embryonic tissues and primordial germ cells, and area opaca forming the extra-embryonic tissues. Primordial germ cells are multi-potent stem cells giving rise to spermatogonia or oogonia. The present study was carried out to describe the characteristics of primordial germ cells in stage X of pheasants' embryo using a transmission electron microscope. The blastoderm was dissected out from embryos which were already incubated for 12 hr. Toluidine blue was used for staining semi-thin sections; lead citrate and uranyl acetate were also used to stain ultra-thin sections. Images of primordial germ cells elucidated that the nucleus was situated eccentrically and had a compact spherical structure. Moreover, the nucleolus appeared elongated and was located eccentrically. The cytoplasm was composed of yolk granules and glycogen particles. Mitochondria were observed as round structures in the cytoplasm. The most important finding was that the primordial germ cells contained yolk granules, mitochondria and small amount of glycogen at this stage.

Introduction

Primordial germ cells (PGCs) are the precursors of gametes found in the very early stages of embryonic development. A significant number of investigations has been done on the morphological aspects of PGCs in different organisms. Although the germ cells exhibit similar cellular characteristics, they show clear differences in their composition and cytochemical features.[1] Several studies have assessed the exact composition and cytochemical properties of germ cells in chickens; but, few have examined these cells in other birds.[2] Using chick-quail chimera experiments, Eyal-Giladi *et al.* have proposed that the avian PGCs were of epiblastic origin.[3] During the early stages of primitive streak formation, germ cells move to the lower layer (i.e., hypoblast).[4] Then, the PGCs translocate to the germinal crescent located farther away from the future gonadal region and localize in the hypoblast layer. From here, they enter the bloodstream in the early stage of fetal blood vessel formation.[4,5]

The PGCs temporarily circulate in the circulatory system and eventually migrate to the gonadal ridge. Germ cells in birds, similar to mammals, reptiles and amphibians, have a large size of 14.00 - 19.00 µm in diameter and a spherical nucleus and contain various cytoplasmic lipid droplets.[6-8] In avian embryos, the PGCs develop through a sequence of cellular procedures as follows: (i) migration of the cell to specific sites, (ii) proliferation and (iii) differentiation. They are initially localized in the central region of the area pellucida (AP), i.e., settling on the expanding hypoblast from the epiblast at stage X, and are then gradually trans-located from this region to another site.[9,10] By scanning electron microscopy (SEM) at stages 4-8, England and Matsumura have examined PGCs in the primitive streak stages in the chick embryo.[11] In stage X of an unincubated chicken, the blastoderm has a single-layered AP and peripheral area opaca (AO), and the AP is subdivided into a central disc and a peripheral marginal zone.[12] The origin of PGCs in the pre-streak of chicken embryos has been investigated utilizing stage-specific embryonic antigen-1 (SSEA-1) and embryonic mouse antigen-1 (EMA1).[13,14]

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The morphological and immunohistochemical traits of PGCs in birds have been also studied using monoclonal and polyclonal antibodies.[15,16] Yoshinaga *et al.* have referred to the properties of PGCs once they appeared in the germinal crescent until migrated to a steady region in gonadal ridges.[17] Furthermore, an electron microscopy study on a chick indicated that the PGCs in the *in vivo* migration phase were associated intimately with neighboring somatic cells through their migration route.[8] By applying the ethylene diamine tetraacetic acid staining procedure, it has been demonstrated that nuclear ribonucleoproteins would be stained preferentially; while, DNA and most of the proteins would remain relatively unaffected.[8,18]

The PGCs' ultrastructure and reactivity to various antibodies such as 2C9 (mouse Immunoglobulin M (IgM), QB2, QCR1 (mouse IgG), and anti-SSEA-1, as well as periodic acid Schiff's (PAS) solution have been tested formerly.[19] Once the time active circulation begins, PGCs are dispersed in the anterior part of the blastoderm and occupy the germinal crescent. During the presomite stages**,** the PGCs are mostly located in the anterior part of the AP. On the other hand, in stages with > 6 somites**,** the PGCs are mainly located laterally on both sides of the embryo's head.[20] Germ cells within the ostrich embryo have been examined, and the morphological characteristics and nuclear organization of cytoplasm in developing germ cells have been studied on embryonic days of 20, 26 and 36 and the hatching day using transmission electron microscope (TEM).[21]

Researches on germ cells are not limited to bird species. For instance, a study on these cells in zebrafish showed that germ cells can be distinguished from the neighboring somatic cells in the early developmental period due to the properties they possess.[22] In support of studies on the origin of germ cells, Yön and Akbulut have explained that the PGCs are specialized in early development in an area located in a different location from gonads.[23] These cells migrate to gonadal precursors by amoeboid movements.

The peripheral cytoplasm of PGCs shows a high alkaline phosphatase activity and is more densely stained compared to the peripheral somatic cells.[24] Since glycogen density in the cytoplasm of PGCs is high, they can be stained with the best carmine and PAS stainings.[23,24] To study the importance of PGCs in producing chimera animals, Hajji *et al.* have produced germline chimeras of birds by transplantation of PGCs taken from the germinal crescent.[25] In another study, a selection of lectin was utilized to assess alterations in the distribution of sugar residues in PGCs during gonadal differentiation and colonization.[26]

Immunohistochemical and PAS staining of the surface antigen of such cells have been used for detection of PGCs; but they are not specific enough to evaluate the development of germline because PAS staining can merely detect PGCs efficiently after stage 4. Also, EMA1 and SSEA-1 are expressed by PGCs as well as undifferentiated cells such as embryonic stem cells.[27] Ginsburg has evaluated the distribution of PGCs or their precursors in stage X blastoderm by counting the number of PGCs and demonstrated that the main population of PGCs is originated from the center of central disc.[28] For identifying PGCs in pre-streak stages of development as early as stage X, immunohistochemical markers such as EMA-1 in chick embryos[29] and quail endothelial cell surface (QH-1) in the quail embryo have been suggested.[15]

Stage X is one of the embryo formation stages in birds at which the blastoderm area is distinguished by an inner single epithelial cell disc, i.e., the AP, and a surrounding multi-layer ring, i.e., the AO. Functionally, AP is responsible for formation of the embryonic tissue and some cells of this region giving rise to the PGCs; while, the extra-embryonic tissues stem from AO.[13,30]

The aims of this study were to identify the characteristics and location of PGCs in early embryonic development. To the best of our knowledge, this is the first electron microscopic study on PGCs in stage X of the pheasants' embryo.

Materials and Methods

Collection and incubation of eggs. Eight fertile pheasants' (*Phasianus colchicus*) eggs were purchased from a commercial farm and incubated at 37.00 ˚C and 60.00% humidity. To prevent the adhesion of the embryo to the eggshell, the eggs were rotated every 60 min.

Embryo separation. Pheasants' embryos were obtained after 12 hr of incubation.

Determination of the chronological age of embryo. Eggs were candled before opening, and it was done in a dark room to ensure the presence of the embryo. Then, eggs were opened at their blunt end with a small scissor (1.00 cm in diameter), as the embryo was in front of the opening side; next, the outer membrane was removed and the embryo was observed under a stereomicroscope (Stemi Sv6; Zeiss, Jena, Germany) and staged according to Hamburger and Hamilton. The blastoderms and the thick albumen were separated from the yolk, dissected out from embryos and incubated for 12 hr.[31]

Preparation of the samples for TEM analysis. To perform a standard TEM analysis, the samples were fixed with glutaraldehyde (2.00%) in cacodylate (0.10 M) at pH of 7.40. To prepare buffer cacodylate, 4.28 g of sodium cacodylate [$(CH_3)_2AsO_2NA$)] (TAAB, Reading, UK) was first dissolved in 200 mL of distilled water to obtain a 1.00 M solution. Then, 8.40 mL of hydrochloric acid (Merck, Darmstadt, Germany) was added to the prepared buffer solution to reach the pH of 7.20. Glutaraldehyde solutions (TAAB) were poured into small vials and kept in a

refrigerator in dark. During sampling, the samples were placed in glutaraldehyde and transferred to the laboratory in a cold condition. After 1 - 2 hr, the samples were taken out and placed in buffer solutions (Sigma, St. Louis, USA) for 1 hr (the buffer was changed every 15 min), to completely remove glutaraldehyde from tissues. Then, the samples were post-fixed in 1.00% osmium tetroxide solution (TAAB) containing 0.10 M phosphate-buffered saline (PBS; Sigma) at the pH of 7.40 for 1 hr at 4.00 ˚C. Upon washing with PBS, samples were dehydrated via a graded series of ethanol and embedded in an epoxy resin 812 (Sigma). In addition, toluidine blue was used for staining semi-thin sections (1.00 µm thickness), and lead citrate along with uranyl acetate were used to stain ultra-thin sections.[32] Semi-thin sections were analyzed through a Leo 912 AB TEM instrument (Zeiss).

Results

After 12 hr of incubation, the pheasant egg often contains a stage X blastoderm. The blastoderm consists of two regions including AO and inner AP (Fig. 1). Given semi-thin sections, PGCs in stage X of pheasant embryo were distinguished easily from the neighboring somatic cells with a large and round shape. They were characterized with a large size and a large spherical nucleus with the nucleolus. Giant blastoderm cells were observed around the PGCs, and PGCs were seen in various sizes; while, the neighboring somatic cells were smaller and normally had a single large nucleus (Figs. 2A and 2B). Moreover, according to the ultra-thin sections, the nucleus of PGCs was compact, dark and eccentrically situated occupying most of the cytoplasm. The PGCs' cytoplasm was stained clearly, and the nucleolus appeared eccentrically elongated. Furthermore, a little amount of chromatin was found in nucleoplasm. The somatic cells were seen smaller than PGC, and giant blastoderm cells were also observed (Figs. 3A and 3B).

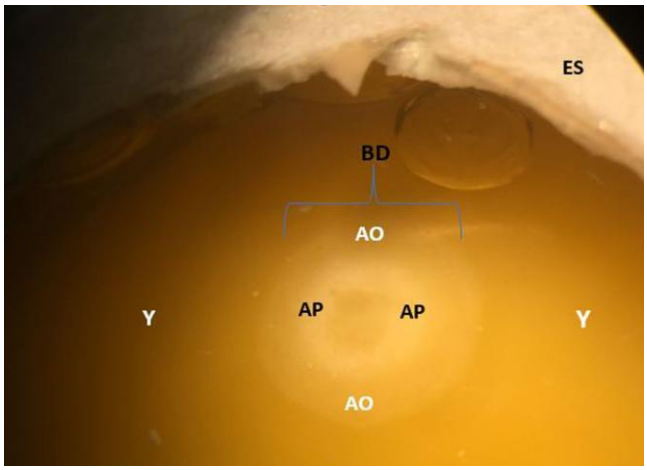

Fig. 1. Pheasant's embryo in stage X. AP: Area pellucida; AO: Area opaca; ES: Egg shell; Y: Yolk; BD: Blastodisk (100×).

The PGCs were found in contact with somatic cells and the neighboring PGCs through cell junctions (Fig. 3B). The PGCs in the early stages contained a large amount of yolk and a small amount of glycogen (Figs. 3B and 3C). Also, mitochondria were round and they were distributed in the cytoplasm (Fig. 3C).

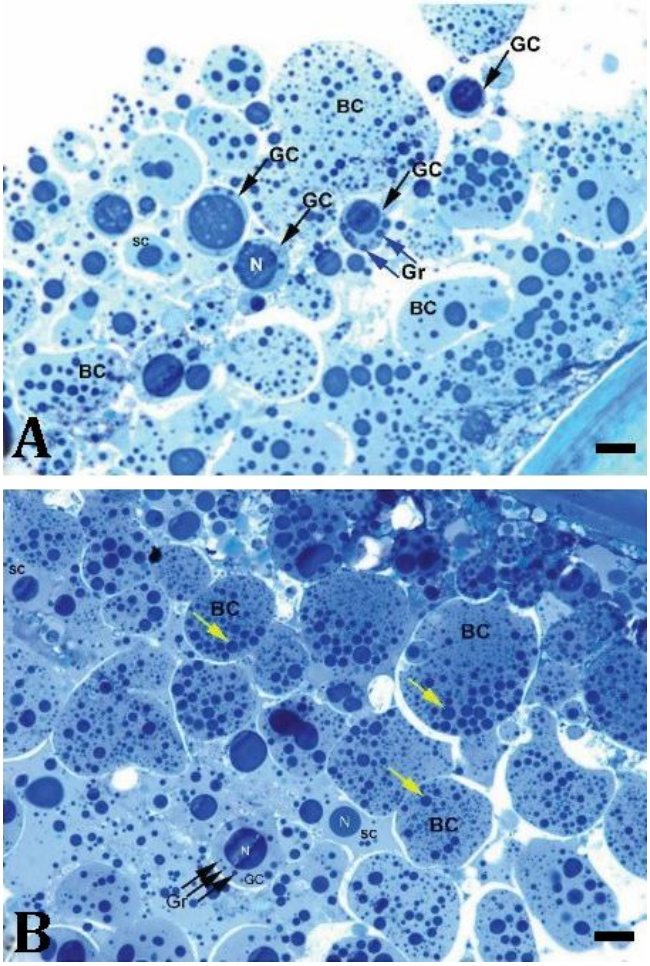

Fig. 2. Semi-thin sections of the stage X of pheasant embryo by using toluidine blue staining. **A)** Showing primordial germ cells (GC) in different sizes: N: Nucleus; BC: Giant blastoderm cells; SC: Somatic cell; Gr: Granules of primordial germ cell. **B)** GC: Primordial germ cells; N: Nucleus; SC: Somatic cells; Gr: Granules of primordial germ cell (Black arrows); BC: Giant blastoderm cells; Yellow arrows: Yolk granules. (bars = 10.00 µm).

Discussion

In birds, the PGCs originate from the central disc of AP and migrate to the germinal crescent region. These cells circulate mostly through blood vessels and settle at the primitive gonad.[8,12] The PGCs carry genetic information to the next generation.[21,26] Thus, these cells have been used to produce germline chimera using the chicken-pheasant system.[19] Therefore, it can be useful to study the characteristics of PGCs in stage X of pheasant embryo using TEM.

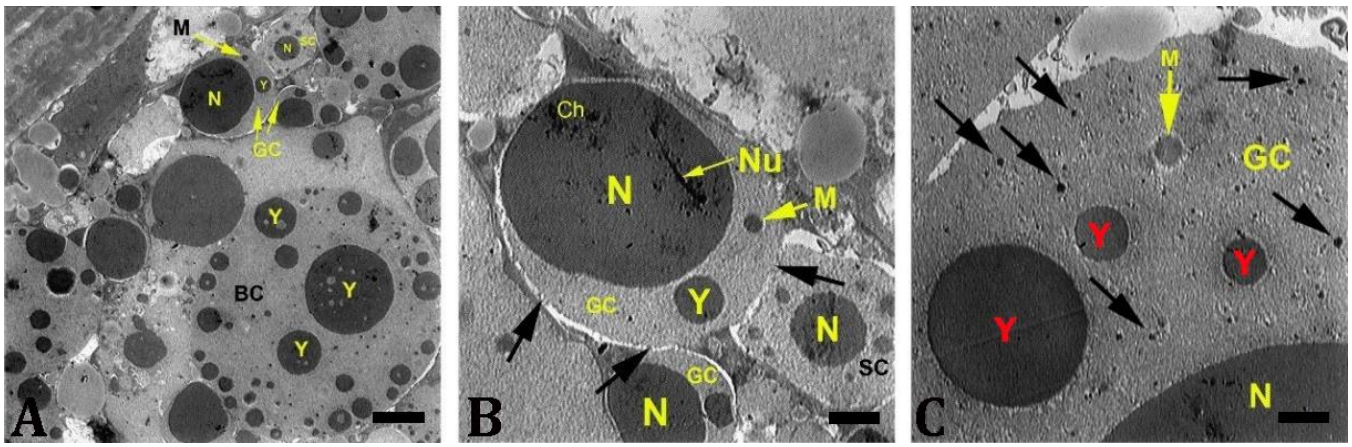

Fig. 3. A) Electron micrograph of the stage X of pheasant embryo. GC: Primordial germ cells; SC: Somatic cell; N: Nucleus; BC: Giant blastoderm cell; M: Mitochondria; Y: Yolk granules. (bar = 3.20 µm). **B)** Electron micrograph of primordial germ cells (GC) in contact with somatic cells (SC) through cell junctions (black arrows). N: Nucleus; Nu: Nucleolus; Ch: Chromatin; Y: Yolk granule; M: Mitochondria. (bar = 1.00 µm). **C)** Electron micrograph of cytoplasmic granules and mitochondria in a primordial germ cell (GC). Y: Yolk granule; Black arrows: Glycogen granules; M: Mitochondria; N: Nucleus. (bar = 1.00 µm).

To the best of our knowledge, this is the first report describing the ultra-structural characteristics of the pheasant embryo PGCs in stage X. The present study indicated several ultra-structure similarities to those previously reported in various vertebrate species including chick and quail.[17,33]

To study germ cells, it is of crucial importance to find a suitable marker for the identification of these cells, such as PAS or immunohistochemical stainings. The PAS staining is not specific enough because it can detect PGCs only after stage 4 Also, immunohistochemical staining is expressed by PGCs as well as undifferentiated cells including embryonic stem cells.[27] In this research, the features of PGCs in stage X of pheasant embryo were studied by correlative semi-thin and TEM sections. At stage X in chick, the blastoderm is composed of two regions including a single layer, i.e., AP, being surrounded peripherally by a thicker ring of cells, i.e., the AO.[12] The obtained results in the current study were in agreement with this description.

The TEM observation of stage X of pheasant embryo demonstrated that PGCs had the typical morphology of avian PGCs similar to that of chick PGCs.[19] The semi-thin sections revealed that the PGCs were characterized by a large size, in comparison with somatic cells, and a round shape located near the somatic cells with a dark staining of toluidine blue. In contrast, the somatic cells were found smaller and lighter. These results were in agreement with those reported by a study on PGCs through the germinal crescent area of quail embryo[17] and those demonstrated in two separate researches on PGCs in chick[33] and in the ovary of ostrich.[21]

According to research by Kim *et al.* ultra-thin section of PGCs had a large eccentric nucleus and a high nucleus to cytoplasm ratio.[19] The findings of the present study were in line with those of this research, as the morphology of the cells in stage X was different from that of cells in the blood, and the PGCs in blood were involved in a pseudo-pod-like cytoplasmic processes through the endothelial gaps of the capillary walls; so, the PGCs, as a whole, changed shape.[34] This morphological difference in PGCs at different stages might result from a decrease or increase in metabolic activity during embryonic development.

England and Matsumura have examined the PGCs of a chick embryo in the hypoblast layer by SEM and found that the PGCs are spherical and easily recognizable by their shape and large size. They also have realized that the surface of PGCs is covered with numerous microvilli, except towards the region where it contacts with the nurse cell processes; so, the surface becomes smooth.[11] Similar observations about PGCs were reported for other avian species.[17,35,36] In the current study, the cytoplasm of PGCs contained a round nucleus with nucleolus, and some organelles such as mitochondria were also observed. This finding was in disagreement with that reported by Kim *et al.* showing that the nucleus of gonadal PGCs in a pheasant is flattened.[19] Also, a few small accumulations of chromatin were observed being distributed in the nucleus; so, it was concluded that chromatin prevalence in the cells could signify an active transcription of many of their genes.

According to a study by Didier and Fargeix, since PGCs in stage X are inactive, the possible changes in nuclear activities of the PGCs during different stages of embryonic development might closely depend on their entrance into a highly proliferating stage.[37] In our study, some granular vesicles with various sizes such as yolk granules and glycogen particles were observed in the cytoplasm of PGCs in stage X.

The eggs of birds contain different granules representing storage sites for proteins. Among them, yolk granules provide essential nutrients for the development of the embryo; while, the other granules contain proteins such as enzymes and extra-cellular matrix components

being necessary for the early development of the fetus.[38] The lipid droplets are a form of fat storage in cells providing the required energy for growth and development of cells and are also involved in regulatory of maturation.[39] Glycogen, the essential stockpiling type of glucose, is a fast and available type of energy that can be provided to tissues upon request.[40]

Yolk granules, lipids droplets and glycogen particles are found in the cytoplasm of PGCs.[27,34] Also, another study in the quail confirmed that the PGCs cytoplasm contained lipid granules.[26] However, in the present research, lipids droplets were not observed. This could also be related to the particular characteristics of pheasant PGCs.

As detected in PGCs of pheasant, the germ cells were frequently inter-connected with somatic cells via the desmosome junction which was in accordance with the results reported by Kheirabadi *et al.* [21] A study by Ukeshima revealed that the nucleus of oogonia included fine particles and lacked the heterochromatin aggregation as seen in somatic cells.[41]

In the present study, mitochondria were found in the cytoplasm of PGCs and the characteristic pattern of the nucleolus in our study differed from that of the chick, i.e., fragmented nucleolus.[8] The size of PGCs remained unchanged during their migration from the germinal crescent area to the posterior parts of embryo; while, in the vessels, they appeared more round and smaller.[42] In turtle's mesothelium of a genital ridge or of the coelomic angle, PGCs are spherical in profile; whereas, those in the mesenchyme are usually irregular in shape with pseudopods or cytoplasmic processes, suggesting that PGCs migrate by an active amoeboid movement.[43] The presence of glycogen in PGCs is a characteristic of these cells with some exceptions, where in quail PGCs, there is no glycogen granule in the cytoplasm; however, based on the results of this study, a small amount of glycogen was observed in the cytoplasm of PGCs.[12,17,44]

The glycogen granules differ in their shapes inside the embryo, as they may be found as spherical granules, scattered particles in the cytoplasm or dense star-shaped clusters in different areas of the bird embryo.[8,45,46] In the current study, glycogen particles were scattered in the cytoplasm of PGCs. Also, Kim *et al.* in agreement with our results, have reported that pheasant PGCs contain less glycogen in their cytoplasm.[19] A similar finding has been reported in the mouse embryo.[47] It was concluded that the PGCs in stage X of pheasant embryo demonstrated the characteristics of PGCs observed in other birds including a large size, a nucleus situated eccentrically with a compact spherical structure and an eccentrically elongated nucleolus. Besides, PGCs had chromatin masses in the nucleus and glycogen particles and yolk granules in different sizes as well as round mitochondria in the cytoplasm.

In this study, Cytoplasmic granules in the PGCs were not stained specifically with the colors used in the electron microscopy technique. Therefore, histochemical and immunostaining methods are suggested to identify and characterize PGCs and cytoplasmic granules at stage X in the pheasant embryo.

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Conflict of interest

The authors declare that they have no conflict of interest.

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