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Short Paper

Ostrich (*Struthio camelus*) primordial germ cells in embryonic blood and presumptive gonad: characterization by PAS and immunohistochemistry

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Abstract

Background: Among the birds, chicken was the model used in the majority of studies on germ cells and other birds like quail, turkey and pheasant had some interest but ostrich has been lacking from these researches. **Aims:** The present study was conducted to confirm the presence of ostrich primordial germ cells (PGCs) in the embryonic blood and to determine the appropriate time for having them in the blood. **Methods:** Embryos were extracted in embryonic day (E) 6-12, their blood was obtained and the rest of the embryos were processed for histological examinations. Staining by periodic acid-Schiff (PAS) method and immunohistochemistry (IHC) using stage-specific embryonic antigen 1 (SSEA1) and stage-specific embryonic antigen 4 (SSEA4) antibodies (Abs) were used for identification of the PGCs. **Results:** While the blood circulating ostrich PGCs were SSEA1-negative and SSEA4-positive, in the presumptive gonads PGCs showed negative immunoreactivity for both Abs. Although the ostrich PGCs were PAS-positive in both blood and presumptive gonad, their PAS-positive contents reduced during development from E 10 to E 12. **Conclusion:** E 9, when the hind limb buds appear, is the best time for detecting PGCs in the ostrich embryonic blood.

Key words: Immunohistochemistry, Ostrich embryo, PGC, Presumptive gonad, SSEA

Introduction

Development of primordial germ cells (PGCs) in the vertebrate embryos usually occurs through a complex procedure and studies concerning this procedure have been widely carried out in all vertebrate species but mainly were pivoted on mammals. Unlike mammals, avian PGCs select a different way to develop by starting a journey from the epiblast (Karagenç *et al*., 1996), settling in the germinal crescent (Swift, 1914; Ginsburg and Eyal-Giladi, 1986) then floating in blood (Kuwana *et al*., 1986) to reach their rendezvous in gonads (Ando and Fujimoto, 1983). Our understanding of avian germ cell development is based on studies on chick embryos and somewhat on other studies using quail, pheasant and turkey (D'costa and Petitte, 1999; Kim *et al*., 2005; Armengol *et al*., 2007). Anyway, to generalize this known pattern to other species the first step is PGCs identification in their embryonic blood.

Regarding the egg availability and relative ease of ostrich embryo manipulation, it is a suitable case for investigations on avian PGCs. The first step of the present study was confirmation of PGCs presence in the embryonic blood and the second step was the determination of an appropriate time for having them in the blood. We also considered a comparison of the results with other avian PGCs.

Materials and Methods

Embryos

From the total number of 21 ostrich eggs at the first stage, 12 eggs were incubated at 36-37°C and 25 ± 2% relative humidity with tilting to 90 degree at 4 h intervals for 6, 8, 10, and 12 days. At the second step 9 eggs were incubated in the same conditions for 8, 9, and 10 days. The ethic-scientific committee of the Ferdowsi University of Mashhad approved all procedures for experiments on animals.

Blood samples

Blood samples were taken from the extraembryonic blood vessels and in some cases from the dorsal aorta. After drying, the blood smears were fixed by 4% formaldehyde in 95% ethanol for 1 min at room temperature (RT) and used for immunohistochemistry (IHC) and periodic acid-Schiff (PAS) staining.

Tissue processing

After bleeding, embryos were fixed in 4% formaldehyde overnight at 4°C. Then embryos processed by routine tissue processing method and were blocked in paraffin. Five-µm-thick paraffin sections on polylysine-coated slides were used for IHC and PAS staining.

Whole mount embryo staining by DAPI (4′,6-diamidino-2-phenylindole)

A 9-day-old embryo was fixed as explained above, washed in phosphate buffered solution (PBS) and placed on a glass slide and mounted with mounting medium containing an antifade reagent and 10 µg/ml DAPI (invitrogen) in PBS. After completely sealing the coverslip it was kept in darkness until microscopic exploration. This staining was done to show the morphological traits of the 9-day-old embryo.

PAS staining and IHC

Periodic acid-Schiff staining was carried out following the appropriate method (D'costa and Petitte, 1999). After fixation, blood smears and paraffin sections were used for IHC. Firstly, they were incubated 15 min in 0.3% H_2O_2 in darkness then heat-induced antigen retrieval was applied to paraffin sections as previously described (Man and Tavassoli, 1996). Following 1 h treatment with normal goat serum at RT, slides were incubated overnight at 4°C in 1:100 dilutions of mouse anti-stage-specific embryonic antigen 1 (SSEA1) or anti-stage-specific embryonic antigen 4 (SSEA4) antibodies (Abs) (Santa Cruz Biotechnology, Inc., US). Subsequently, the slides which were treated with anti-SSEA1 Ab were incubated with donkey anti-mouse immunoglobulin M (IgM) conjugated to horseradish peroxidase (HRP) (1:300; Jackson ImmunoResearch Laboratories, Inc., US). Other slides, treated with anti-SSEA4 Ab, were incubated in anti-mouse immunoglobulin G (IgG) conjugated with HRP (1:300; dianova; Ord., Germany) for 1 h at RT. After incubation with DAB-substrate (Sigma-Aldrich, St. Louis, MO, US) for 5 min at RT, the slides were washed again by PBS. Some sections were used as negative controls using PBS instead of the primary Ab. Undifferentiated P19 cells were used as control-positive for SSEA1 Ab (Nye *et al*., 1994).

Imaging

Images were collected on a light microscope (model BX60; Olympus) and a fluorescent microscope (model BX51; Olympus) equipped with Olympus (model: DP12 and DP25) digital cameras and processed using Image (NIH, US) and Snagit Editor (TechSmith, US) software.

Results

Stage of embryos

The 6-day-old embryo was very translucent and its extraembryonic blood vessels had just formed without obvious blood circulation. In the 8-day-old embryo, the heartbeat had started and blood vessels containing the circulating blood were completely visible but the embryo was not completely opaque yet. The 9-day-old embryo had the same morphology except for the emersion of rudimentary hind limb buds (Fig. 1). In the 10-day-old embryo, the body mass was increased enough to make the embryo completely opaque, hind limb buds completely appeared, blood vessels grew thicker and the heartbeat went more visible. Pigmentation of the retina occurred and the color of optic cups turned darker, making them visible. In the 12-day-old embryos, these traits became more distinct, besides the beak appeared and wing and foot buds became more distinct.

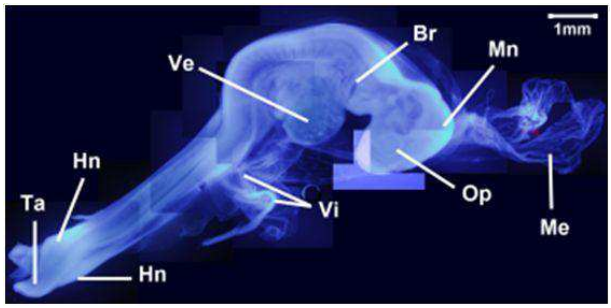

Fig. 1: Fluorescent micrograph of whole mount 9-day-old ostrich embryo stained with DAPI showing the appearance of hind limb buds (Hn). Images were captured in ×40 magnification and assembled by Snagit software. Br: Branchial arches, Me: Embryonic membranes, Mn: Mesencephalon, Op: Optic cup, Ta: Tail bud, Ve: Ventricle, and Vi: Vitelline vessels

Primary detection of PGCs by PAS staining

In light microscopy blood smears belonging to the 8- and 9-day-old embryos contained large spherical cells with numerous distinct PAS-positive vesicles (Figs. 2A-C). Because of their higher number, finding a PGC in the smears from a 9-day-old embryo was much easier than 8-day-old embryos. In the blood smears from other embryos no distinct cell with mentioned morphology was found. In paraffin sections from 10-day-old embryos, there were some PGCs with large volume and distinct PAS-positive contents in the construction of the primitive gonad (Figs. 3A and B). The PAS-positive contents of PGCs were decreased in E 12 relative to E 10 (Figs. 3C and D).

Confirmation by IHC

The samples whose blood smears and paraffin sections contained PGCs were subsequently used for IHC by SSEA1 and SSEA4 Abs. Primordial germ cells on blood smears reacted with SSEA4 Ab (Figs. 4A and B) but did not react with SSEA1 Ab (Fig. 5A). On the paraffin sections of 9-day-old embryos, PGCs remaining in the blood vessels also reacted with SSEA4 Ab (Figs. 4C and D) but not with SSEA1 Ab (Fig. 5B). There was no sign of reaction to both Abs on the primitive gonads of 10- and 12-day-old embryos. The P19 cells which were treated with SSEA1 Ab the same as the blood smears showed the efficiency of this Ab (Figs. 5C and D).

Discussion

Since the timing of embryonic development in ostrich is not similar to the fowl, we could not use the Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1951). Accordingly, we were compelled to use

the phrase of "embryonic day (E)". The results of the first stage of study showed that we can take blood from E 8 upward. Nonetheless, we managed to detect PGCs by PAS staining only in the blood of the 8-day-old embryos, therefore, we focused on E 8, 9, and 10 at the second stage of study. It is known that carbohydrates staining methods like PAS are effective for identifying chicken PGCs, because of their numerous cytoplasmic glycogen

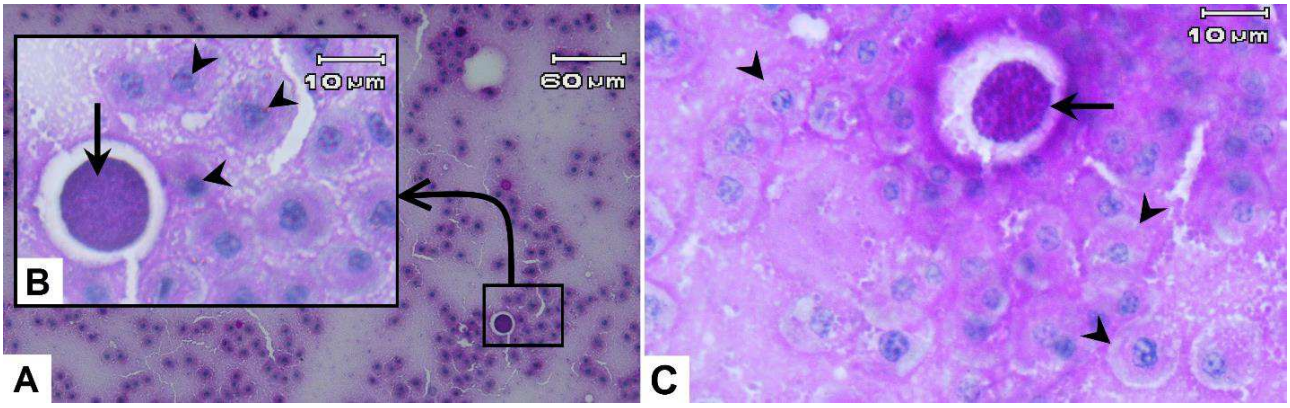

Fig. 2: Photomicrographs of blood smear from a 9-day-old ostrich embryo, stained with PAS (**A**&**B**), and PAS-positive vesicles of PGC with more visibility in (**C**). Blood globules (arrowheads), PGCs (arrows)

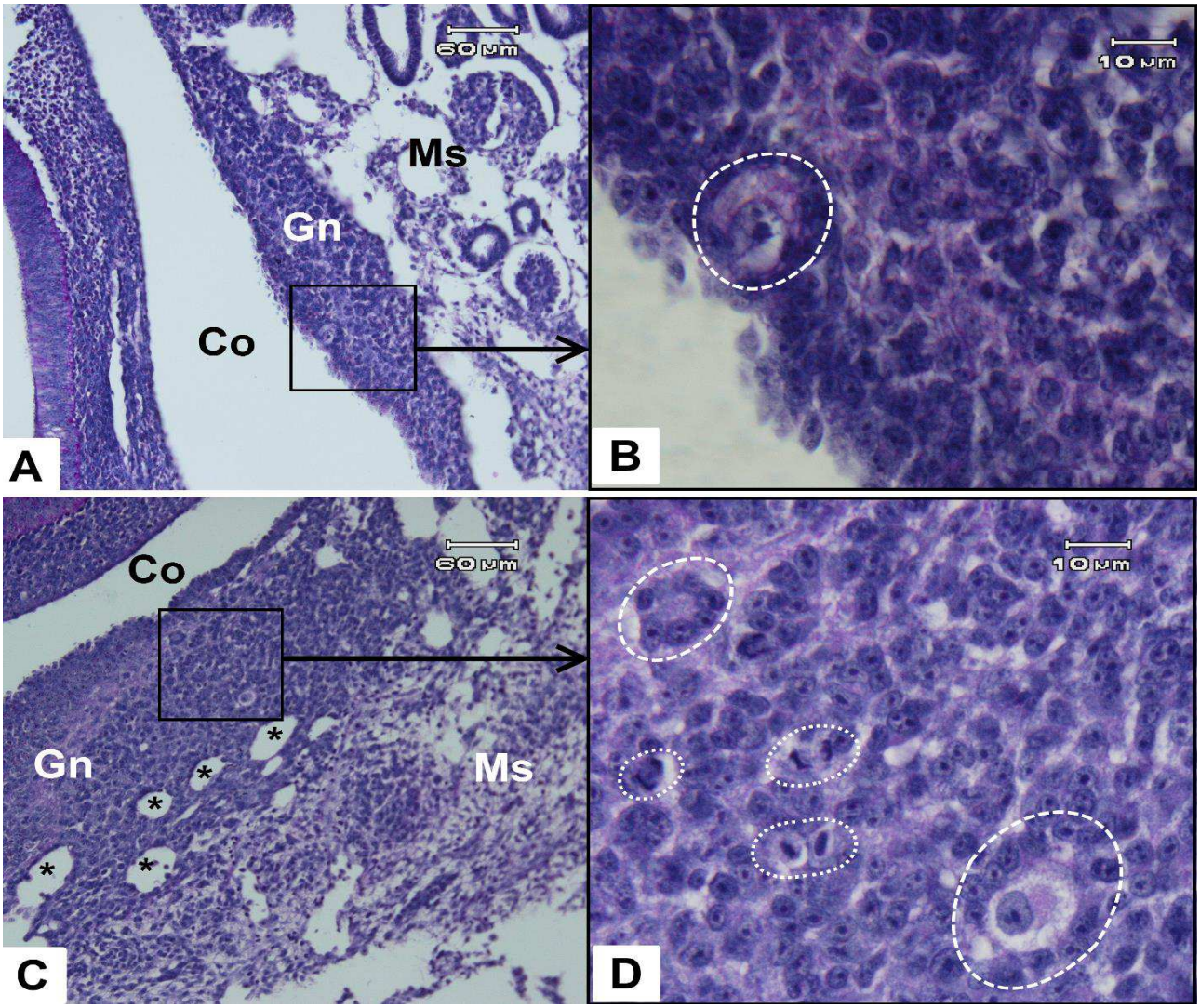

Fig. 3: Photomicrographs of presumptive gonads of a 10-day-old (**A**, **B**), and a 12-day-old (**C**, **D**) ostrich embryos demonstrating PGCs (circled by broken lines) among the mesenchymal cells (some of them are in mitosis circled by dotted line). Note the low PAS-positive content of the PGC in (**D**) relative to (**B**). Co: Coelom, Gn: Presumptive gonad, Ms: Mesonephros, and asterisks: Blood vessels

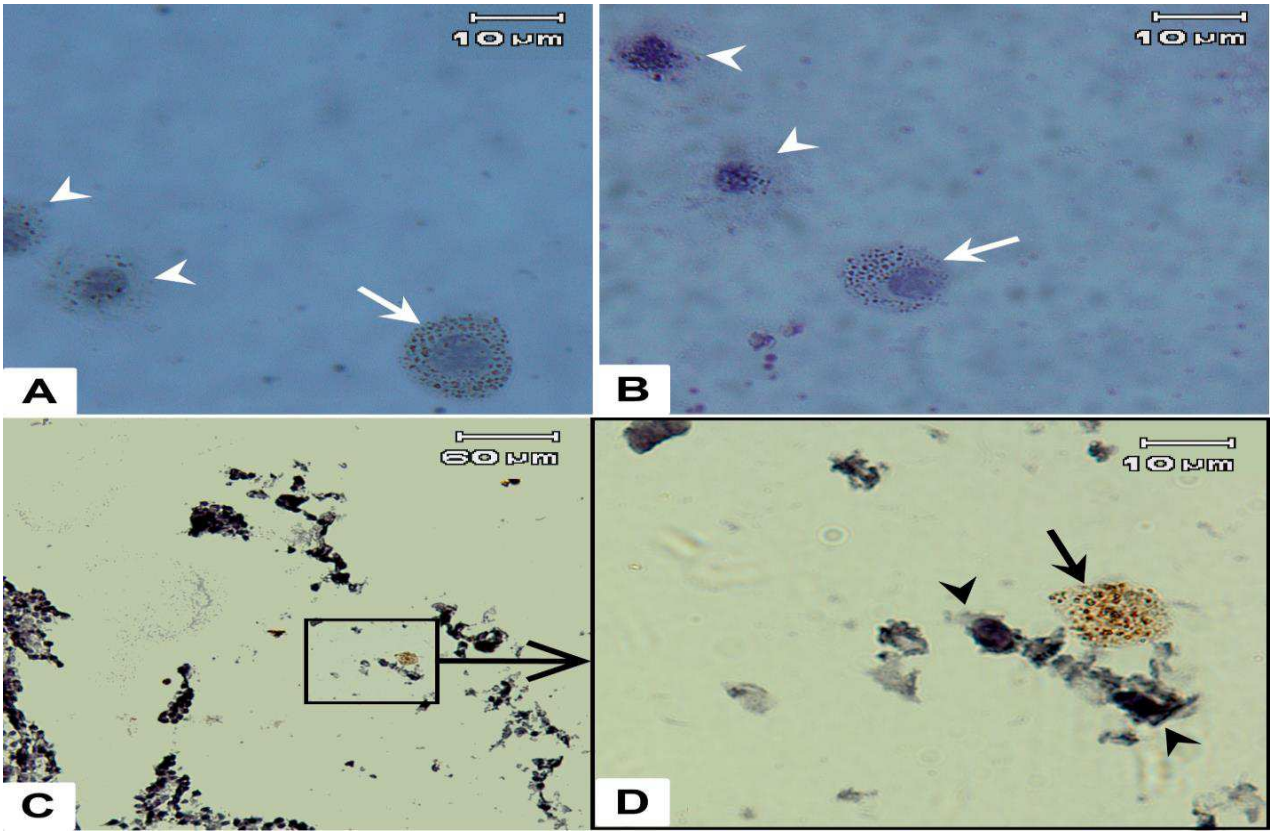

Fig. 4: Photomicrographs of blood smears (**A**, **B**), and paraffin sections (**C**, **D**) of 9-day-old ostrich embryos treated by SSEA4 and visualized by HRP-DAB system, showing the positive immunoreactivity of PGCs (arrows). Note numerous SSEA4-positive vesicles in the cytoplasm of PGCs. Blood globules (arrowheads)

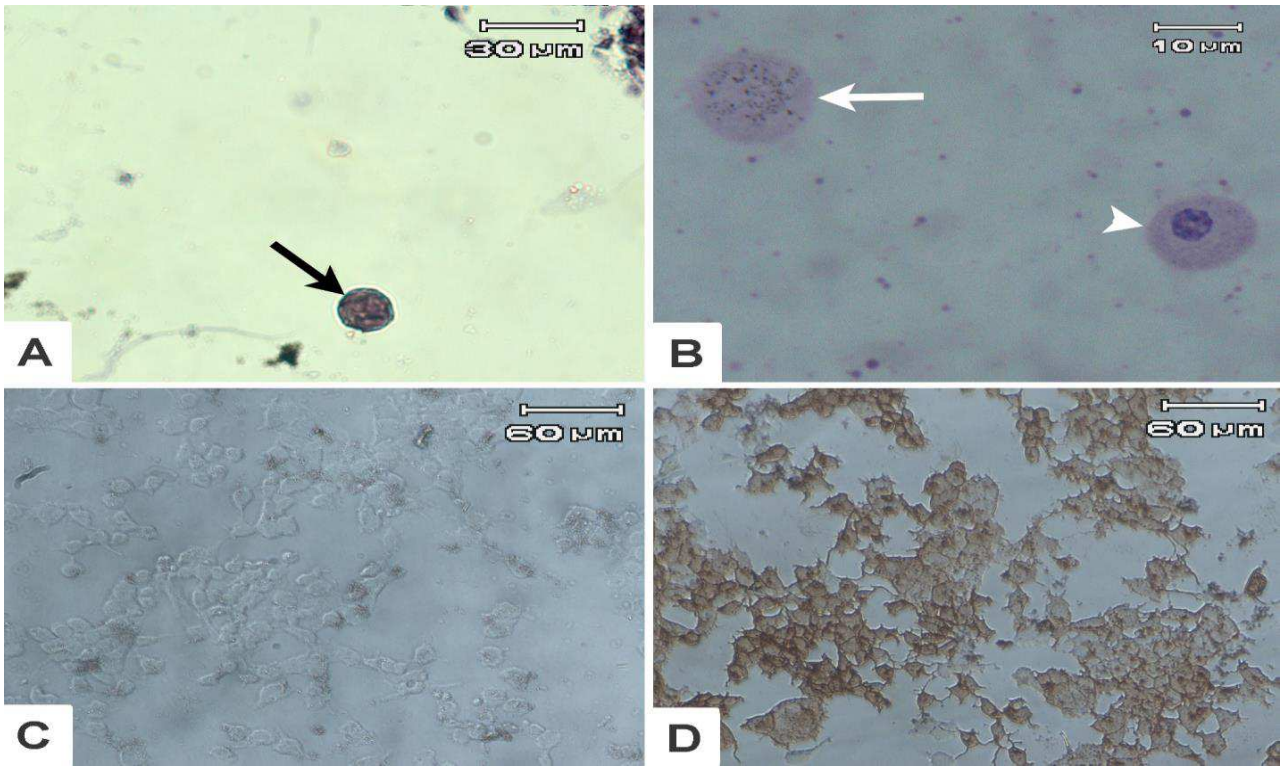

Fig. 5: Photomicrographs of blood PGCs in a paraffin section (**A**) and a blood smear (**B**) of 9-day-old embryo treated with SSEA1 Ab and visualized by HRP-DAB and haematoxylin counterstaining showing the negative immunoreactivity of PGCs (arrows) and blood globule (arrowheads). Photomicrographs of undifferentiated P19 cells treated only with secondary Ab (colorless cells in **C**) and treated with both SSEA1 Ab and then secondary Ab (colored cells in **D**) visualized by HRP-DAB system

vesicles (Meyer, 1960), consequently, we decided to identify the ostrich PGCs by PAS staining and confirm it with specific Abs. Our results confirmed that PAS staining is a suitable method for identifying the PGCs in blood smears and paraffin sections of the primitive gonad. Moreover, we perceived that the PAS-positive contents of PGCs undergo a downward trend from E 9 to E 12.

Furthermore, the results offer a relative prediction for the presence of PGCs in the embryonic blood in another member of Struthioniformes order, emu. That, as regards the appearance of hind limb buds in the emu embryo was reported at E 10 (Nagai *et al*., 2011), which is the equivalent of stage 17/18 HH, and considering the published images of 10-day-old emu embryo we could predict that the E 10 is the best time to find the emu PGCs in the blood.

Antibody markers are usually used for characterization of PGCs epitopes especially the SSEA family which is one of the best-analyzed antigens on the PGCs in mammalian and avian embryos. Among them SSEA1, stage-specific embryonic antigen 3 (SSEA3) and SSEA4 are frequently used and efficiency of SSEA1 and SSEA4 has been well established in birds (Karagenç *et al*., 1996; D'costa and Petitte, 1999; Kim *et al*., 2005; Han, 2009; Chan, 2010). While SSEA1 immunoreactivity of PGCs is previously reported in fowl and turkey (D'costa and Petitte, 1999; Kim *et al*., 2005), our results revealed that the ostrich PGCs like those of rat are SSEA1-negative (Encinas *et al*., 2012). Since the efficiency of SSEA1 Ab is confirmed by control-positive cells (P19 cells), we were confident about Ab efficiency and indeed we took these results as SSEA1-negative characteristics of ostrich PGCs.

We observed that once the ostrich PGCs settle in the gonads they lose the immunoreactivity to SSEA4 Ab. To ensure our method efficiency we used both Abs on paraffin sections and we observed that the PGCs remaining in the blood vessels showed the same immunoreactivity similar to the blood smears and again no reaction was found in the presumptive gonad. Putting together these results leads us to the conclusion that blood circulating ostrich PGCs are PAS and SSEA4-positive and SSEA1 negative, while in the presumptive gonads they become PAS-positive, SSEA4 negative and SSEA1 negative. As previously reported this difference might result from an alteration in the cell characteristics, such as a decrease or increase in metabolic activity before or after migration (Kim *et al*., 2005).

In conclusion, our findings propose that the best time for obtaining ostrich PGCs circulating in the embryonic blood is E 9, when the hind limb buds appear, and both PAS staining and IHC with SSEA4 Ab could be used efficiently to identification of these cells. However, in the case of gonadal PGCs, only PAS staining is proposed as an efficient confirmation method.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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