e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 67 (2019), No 2
https://doi.org/10.3409/fb_67-2.08

The Developmental Changes in the Extra-Embryonic Vascular System in the Circulating Phase of Primordial Germ Cells in Aves

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Accepted June 17, 2019 *Published online June 24, 2019* *Issue online July 18, 2019*

Original article

SZCZERBA A., KUWANA T., BEDNARCZYK M. 2019. The developmental changes in the extra-embryonic vascular system in the circulating phase of primordial germ cells in Aves. Folia Biologica (Kraków) **67**: 79-83.

There are many reports regarding the molecular control of the asymmetric developments that occur during the early embryonic period in many experimental animals, including avian species. Generally, only the left ovary develops and becomes a mature reproductive organ in matured avian females. In mature avian males, the left testis tends to be dominant in respect to size. However, these reports do not to provide an answer to why avian primordial germ cells (PGCs) mainly colonize in the left gonadal rudiment just after their circulation period. In the present study, dynamic changes in the vascular networks were observed throughout the circulating phase of PGCs in the early embryonic stages, mainly in chickens. India ink was injected into the bloodstream of chicken and quail embryos at stages 13 to 17 in order to visualize the changes in the vascular networks in the extra and intra embryonic regions. The blood flow pathway was also observed via the movement of the injected India ink particles. Throughout stages 13 to 17, the branching level of the vitelline veins from the dorsal aortas was at the 19th and 20th somites in the quail and chicken embryos respectively. Furthermore, the posterior vitelline vein did not develop by stage 14; it began to develop at stage 15 or later. The blood flow in the gonadal region became asymmetrical in the left and right sides in parallel with the development of the posterior vitelline vein. The asymmetry we observed in the blood flow might be the cause of the asymmetric proportions of the PGCs just after their circulation in the left and right gonadal regions.

Key words: vascular system, avian embryo, primordial germ cells, circulation, posterior vitelline vein, blood flow, asymmetry.

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Avian primordial germ cells (PGCs) are the reproductive stem cells of future ova or sperm. The precursors of PGCs are of epiblastic origin in chickens, as a model of the avian species (EYAL-GILADI *et al.* 1981; CHOJNACKA-PUCHTA *et al*. 2012). PGCs originate from the central disc and not from the periphery of the area pellucida in early chicken blastodermal stages (GINSBURG & EYAL-GILADI 1987). They are then transported passively into the anterior region of the extra-embryonic area, in a so-called germinal crescent (HAMBURGER & HAMILTON 1951) through the formation and movement of developed mesoderm (gastrulation) until stage 10 (SWIFT 1914). PGCs next begin to enter the extra-embryonic vascular system at the germinal crescent region (FUJIMOTO *et al*. 1976) and circulate temporarily via the developing vascular system after separation from the endoderm of germinal crescent region at stages 4 to 8. After their circulation through the embryonic bloodstream, they are gradually trapped in the fine capillary networks in the right and left gonadal regions and migrate interstitially toward the future gonads.

The avian ovary develops as a functional gonad from the left embryonic gonadal rudiments; in adult females, the right gonad is merely rudimentary. In males, the number of PGCs in the left developing gonad is significantly higher than that in the right gonad, just before hatching (ONO *et al.*

1996; NAITO *et al*. 2009; NAKAJIMA *et al*. 2011; INTARAPAT & STERN 2013). After hatching, both gonads develop into functional testes and have the same size in sex-matured adult individuals, although the left testis tends to be slightly larger than the right one in most bird species (KEMPENAERS *et al*. 2002).

An increase in the number of germ cells was observed (SCHWARTZ & DOMM 1972) during the period of migration from the age of 18 hours to five days of incubation (stages 3-27). This was primarily considered to be due to the proliferation of intra embryonic cells, since no significant increase in the number of extra-embryonic germ cells was seen during this period. The number of germ cells during this period ranged from 43 (at 18 hours) to 2,211 (at 120 hours). Two periods of intense proliferation were observed, the first between 48 and 72 hours and the second between 96 and 120 hours. Dividing germ cells were found in the chick embryos throughout the entire migratory period.

There are many reports regarding the molecular control of asymmetric development during the early embryonic period in many experimental animals, including avian species (LEVIN 2005). However, these reports did not provide a direct and clear answer as to why avian PGCs mainly colonize in the left gonadal rudiment after their circulation period.

According to INTARAPAT & STERN (2013), differences in germ cell distribution could be observed between the left and right gonads of Cvh-positive cells in 9-day-old chicken embryos (stage 35) . This confirms the results that were obtained studying 5 to 12-day-old embryos (GUIOLI & LOVELL-BADGE 2007) (stages 26 to 38). However, these results only show the differences in the number of PGCs between the left and right developing gonads. There was less information regarding the difference in the number of PGCs between the future gonadal left and right regions at the end of their circulation.

NAKAMURA *et al*. (2007) found that the number of PGCs in the left future gonadal region was always higher than that in the right one at the end of PGC circulation (in stages 15, 16, and 17). In a study, PKH26 fluorescent dye-labeled exogenous PGCs were injected into a recipient embryo; the number of cells was seen to be higher in the left gonad than in the right gonad (NAKAJIMA *et al*. 2014). However, it is still unclear why the PGCs, just after their circulation via the embryonic bloodstream, colonize mainly in the left gonadal region and not the right.

In the present study, the developmental changes in the extra-embryonic vascular system throughout the periods of PGCs circulation (stages 13 to 17) were investigated among different avian species (chicken, Japanese quail). The aim of this study was to clarify the character of the developmental changes in the extra-embryonic vascular system regarding the circulation of PGCs and to determine the cause for the colonization of PGCs mainly in the left gonadal rudiment.

Materials and Methods

Preparation of chicken and quail embryos

120 Fertilized eggs of chicken breed Ross 308 were supplied by Drobex-Agro Sp. z o.o. (Solec Kujawski, Poland) and 108 Japanese quail eggs were supplied by Krzysztof Drążek (Czudec, Poland). The eggs were divided into 5 groups, to obtain embryos from stages 13 to 17. The eggs were incubated in automatic incubators with electronically controlled conditions of incubation (temperature 37.8°C and relative humidity 60%). The minimum number of typical and properly developed embryos used for comparisons with each developmental stage was 10.

Injection of India ink into the embryonic bloodstream

India ink (Rotring black drawing ink, ROTRING, Germany) was diluted with four times the volume of PBS just before injection. Five microliters of prepared India ink solution were slowly injected into the embryonic blood circulation from a point in the terminal vein with a glass micropipette to clarify the distribution of the embryonic vascular system, especially at the extra-embryonic region. After injection, the embryos were incubated for 15 minutes on a heating plate (temperature 37.8°C).

The injected embryos were isolated from the yolk by using a filter paper ring, which was cut out from filter paper (# 1 filter, Toyo Filter, Japan) using a compass cutter (CMP-1, OLFA Corp., Osaka, Japan). The filter paper ring was placed on the embryonic region and the surrounding yolk membrane was cut to remove the embryonic disk from the yolk. After removing the disk, another filter paper ring was attached to the lower side of the embryonic disk. The remaining yolk was rinsed off within a petri dish that was filled with PBS. The cleaned embryonic disks were observed under a stereo microscope (X-type, Olympus, Japan) to count the number of developed somites and to record the development of their vascular system.

Results

In the present study, it was seen that the development of the omphalomesenteric artery (vitelline arteries) and the posterior vitelline vein are closely related to embryonic development. First, the level of the right and left roots of the omphalomesenteric arteries were at the 20th somite in chickens (Fig. 1a), and at the 19th somite in quails (Fig.1b). However, the precise determination of the number of somites was possible only under a stereoscope.

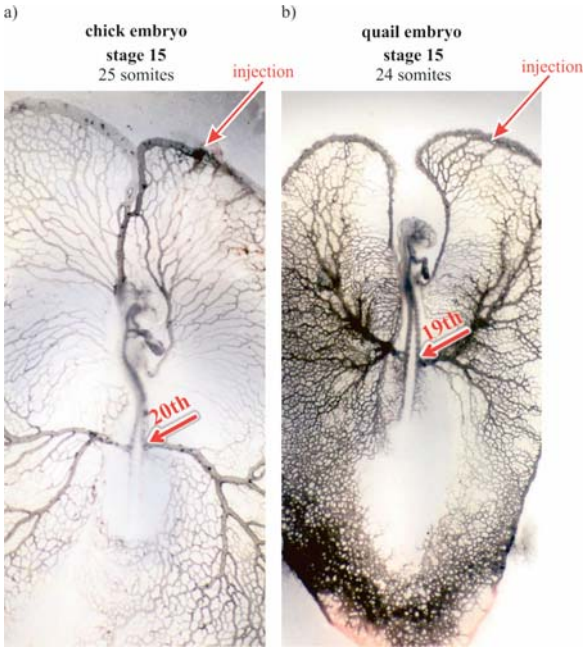

Fig. 1. The level of the right and left roots of the omphalomesenteric arteries a – the 20th somite in chickens, b – the 19th somite in quails.

Second, the posterior vitelline vein appeared at stage 15 with 25 somites (sometimes with 24 somites) in the chicken embryos (Fig. 2a-b). In the quail embryos, it appeared at the end of stage 14, with 24 somites. In both chicken and quail embryos, the vitelline vein was completely established as a large and visible vein after stage 16.

Before the formation of the posterior vitelline vein, the blood circulation in the right and left gonadal regions takes place in blood vessels of a similar volume. However, after the formation of the posterior vitelline vein, the area of the blood vessels in the left gonadal region increases in comparison to the right one. This is because the posterior vitelline vein is formed only on the left side of the embryo; therefore, the bloodstream in the capillary network on the left side can flow directly into the vein, while the bloodstream in the right-sided

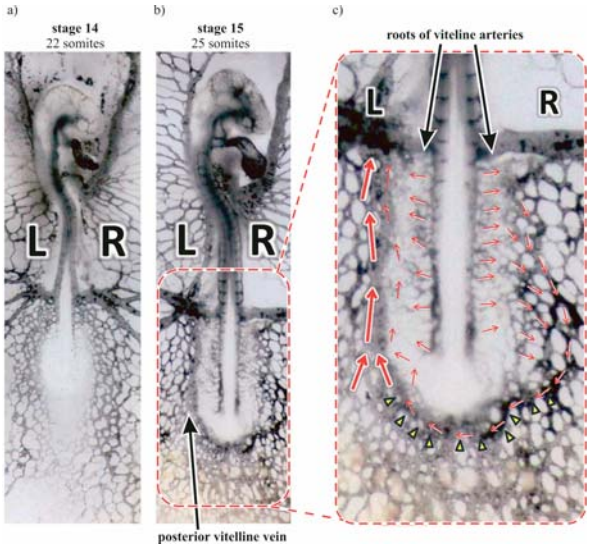

Fig. 2. Difference of blood flow in the gonadal regions between stages 14 and 15 in chickens. a – the posterior vitelline vein appears just after the end of stage 14 (22 somites); the blood flow in the left and right gonadal regions. There is no development of the posterior vitelline vein; b – it appears clearly in the embryo at stage 15. c – the small red arrows indicate the direction of blood flow in the micro-capillary networks and the big red arrows indicate the blood flow in the posterior vitelline vein. The yellow arrows indicate the path of a small vein from the right gonadal region to the posterior vitelline vein at stage 15.

network cannot flow into any other veins, as shown in Figure 2c.

In the present study, we were able to determine one of the important causes of these phenomena. The capillary networks start to develop equally after stage 13 in both the left and right sides of the future gonadal regions, which is just posterior to the root of the omphalomesenteric arteries at the level of the 20th somite. However, the blood flow in the future gonadal regions does not develop until the end of stage 14 with 22 somites, because there is no functional outflow vein. In the early half of stage 15 with 23 or 24 somites, the posterior vitelline vein rapidly develops on the left side of the gonadal region and the blood flow at the gonadal regions obtains its outflow vein. At the same time, the presence of the posterior vitelline vein, is only located on the left side and is the cause of unequal blood flow between the left and right gonadal regions, as shown in Figure 3.

Regarding the timing of fusion between the right and left anterior vitelline veins, no meaningful relationship with embryonic development was found.

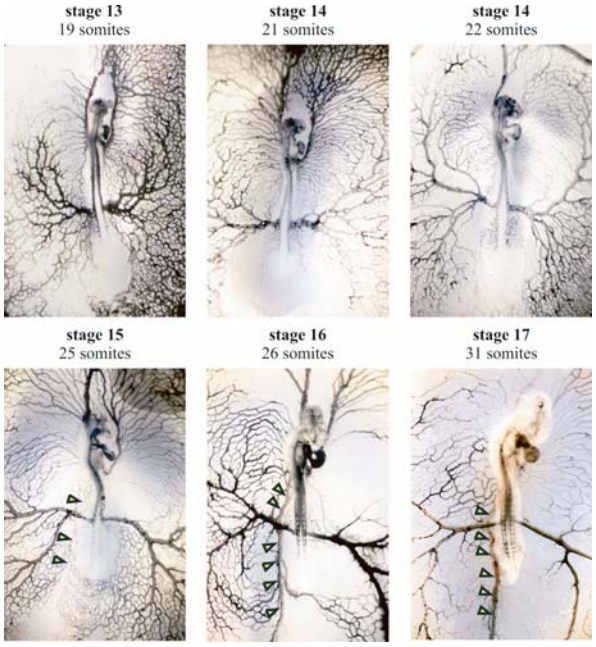

Fig. 3. Development of vascular systems in the extra-embryonic region. Transmitted stereo-microscopic photographs of chick embryos in stages 13 to 17 after injection of India ink into their blood circulation through the right marginal vein. The yellow arrow heads indicate position of the posterior vitelline vein.

Discussion

The number of developed somites is one of the most important criteria used to determine the embryonic stages based on the stage of embryonic developments (HAMBURGER & HAMILTON 1951); however, it might not be easy to count the somites after cervical flexure and the rotation of the back from stage 14 onward. In the present study, we show that the right and left omphalomesenteric arteries branch at the level of the 20th somite in chickens and the 19th in quails.

NAKAMURA *et al*. (2007) reported the detailed distribution changes of PGCs from stage X (EYAL-GILADI & KOCHAV 1976) to stage 17 using immunohistochemical CVH staining on PGCs. They report that the circulating PGCs start to accumulate within the intermediate mesoderm at the future gonadal region only from stage 15 onward, and that the PGCs are distributed more in the left side of the gonadal region than in the right side after stage 15 in both males and females. This report shows that PGCs circulate via the embryonic vascular system from stage 11 and suddenly start to accumulate in the future gonadal regions. Moreover, the cells accumulate more on the left side than on the right side, although both capillary networks seem to develop equally throughout the period of PGC circulation.

According to HAMBURGER and HAMILTON (1951), beyond stage 14, it is increasingly difficult to accurately determine the number of somites. This is partly due to the dispersal of the anteriormost somites into the mesoderm and in later stages, into the curvature of the tail. The total somite counts given for the following stages are typical, but sufficiently variable so as not to be diagnostic. In the present study, we showed that the position of the omphalomesenteric arteries is at the level of 20th somite in chicken embryos and 19th in quail embryos. This new criterion should be useful to determine the number of somites throughout stages 13 to 17.

Acknowledgments

The work reported herein was supported by the Polish Ministry of Higher Education – statutory activity of the Department of Animal Biotechnology and Genetics; and partially by the National Centre for Research and Development, grant no. PBS3/A8/30/2015.

Author Contributions

Research concept and design: T.K., A.S.; Collection and/or assembly of data: A.S., T.K.; Data analysis and interpretation: A.S., T.K., M.B.; Writing the article: A.S., T.K., M.B.; Critical revision of the article: M.B.; Final approval of article: M.B.

Conflict of Interest

The authors declare no conflict of interest.

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