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RESEARCH ARTICLE

The Development of Primordial Germ Cells (PGCs) and Testis in the Quail Embryo

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ABSTRACT

Histological methods were conducted to detect the development of quail primordial germ cells (PGCs), the pattern of gonads differentiation and different characteristics of testis structure at various developmental stages. We found that on day 4 of post-incubation, all PGCs migrated to gonad ridge and formed undifferentiated gonads; on day 5 the gonads differentiated into testis or ovary. On day 7 to 9 of post-incubation, the morphological characteristics of testis were distinguished, subsequently testicular cord appeared and the PGCs differentiated into spermatogonium on day 10. On day 12 of post-incubation, spermatogonia seemed like clusters of grape or chain in the middle of convoluted seminiferous tubule; simultaneously, a few of sustentacular cells and vascular were developed for filling into the testis lumen. Then on day 15, the seminiferous tubule become thicker and the amount of spermatogonia increased by mitosis. Finally, on hatch day (day 17), the testes were almost mature with numerous mesenchymal cells, connective tissue, vascular and clear and intensive seminiferous tubules. These results can lay data for future studies on sex determination mechanism and the more utilization of PGCs in poultry.

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INTRODUCTION

Quail, as an avian species, has become a desirable animal model in experimental embryology and reproductive biology (Zacchei, 1961). Chimeras produced from quail and chicken are also widely used in the study of developmental biology (Ono *et al.*, 1998; Park *et al.*, 2008). Although the cytochemical characteristics of PGCs have been described in chicken, little is known about such characteristics in quail of interest to experimental biology (Li *et al.*, 2001; Qin *et al.*, 2006). By date, studies on quail PGCs presence, migration patterns and gonadal differentiation are rare because of its unique characteristics. Yoshinaga *et al.* (1993) observed Japanese quail (*Coturnix coturnix japonica*) PGCs by means of electron microscope, and Clara *et al.* (2007) developed QH1, LEA, LEA, WGA, RCA-I and WFA as markers to identify the PGCs, focusing on its migration patterns from the appearance in germinal crescent area to the colonization in the genital ridge. They found no glycogen granules were in the PGCs cytoplasm at any stage of embryonic period and dense membrane-coated particles were found in cytoplasm at undifferentiated gonads; besides, nucleus had a prominent condensed nucleolus which was compact in germinal crescent period but became dispersed in genital ridge. These above characteristics are thought to make the identification of quail PGCs difficult. Though some researchers have taken the isolation and in vitro-culture quail PGCs into account (Kimmell *et al.*, 2007; Zhang *et al.*, 2011), it is important to fill a vacancy on the gonadal differentiation and development after PGCs settled down. Feng *et al.* (2007) had analyzed the expression of some genes involved in chicken gonadal development. And we had discussed previously the migration and accumulation of PGCs in the quail embryo and the development of the ovary (Chang *et al.*, 2010; Chen *et al.*, 2011). In this study, serial slices of quail embryo gonads from day 4 of post-incubation to hatch day were taken as materials to determine its gonadal differentiation time and to observe testicular morphological characteristics during the whole embryonic period. The aim is to lay the foundation for further understanding of sex determination mechanism and the more utilization of PGCs such as inducing PGCs into other tissues and transferring interesting genes via PGCs in poultry.

MATERIALS AND METHODS

Materials: Fertilized eggs in this experiment were obtained from Taizhou quail breed center, Jiangsu

Province, China. Every 3~5 quail eggs were used from day 4 of post-incubation to hatch day. Here, fertilized eggs were incubated at 38°C and a relative humidity of 60%.The quail embryos from day 4 to day 6 were wholly fixed in Rossman's solution. The chemical composition of Rossman's solution was as follows: picric acid in absolute alcohol, 90 ml; formaldehyde, 10 ml. The quail embryo gonads from day 7 of post-incubation to hatch day were fixed in 4% formaldehyde.

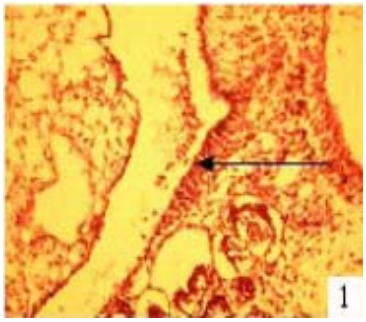
Embryo collection: To collect embryos from day 4 to 6 of post-incubation, the blunt end of the egg (environmental control: temperature, 38°C; humidity, 60%) was gently knocked out with ophthalmic forceps and the shell and shell membrane were removed; after the embryo was transferred into Petri dish filled with warm (38.5°C) 0.75% saline, embryo sac and the outer membrane surrounding the embryo were cut off, and then the embryo was rinsed with clean warm 0.75% saline gently for 3 times. Finally, the embryo was soaked in Rossman's solution overnight.

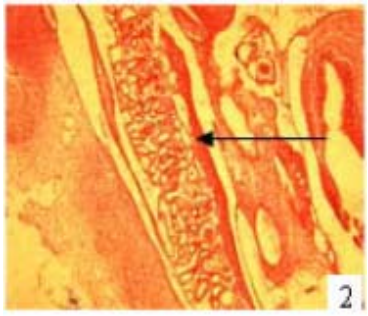
Gonads collection: To collect gonads from day 7 to hatch day, the belly of the embryo was anatomized with ophthalmic forceps and the parts except gonad and kidney were removed; then the samples were transferred into 4% formaldehyde and fixed overnight.

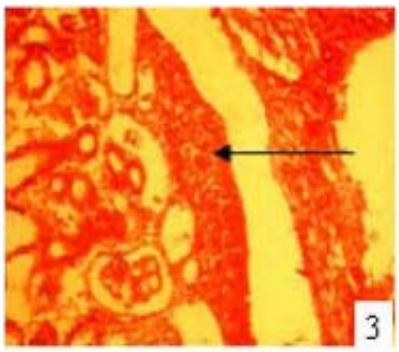
Tissue slicing and staining: The above samples were embedded with paraffin. Sections (5~7μm) were cut with a microtome and mounted on pretreated glass slides, then the slices were stained with Hematoxylin-Eosin (HE). Finally, they were observed under the optical microscope and photographed.

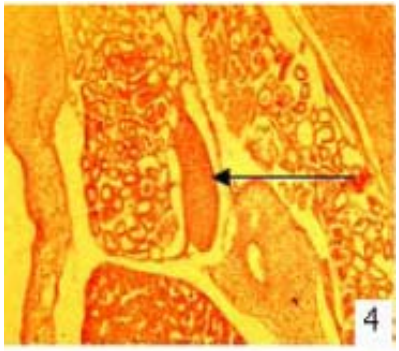
RESULTS

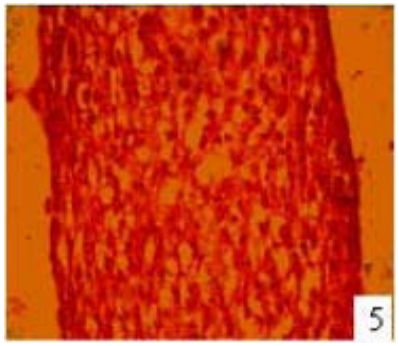
The histological results observed by the microscope were shown in Fig. 1 and 2.











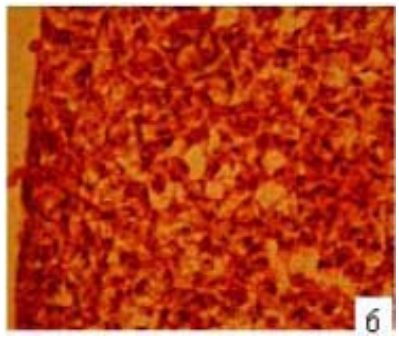


Fig. 1: Results of the whole quail embryo and its gonad slices from day 4 to 7 of post-incubation.

Day 4: The whole embryo slices, in which PGCs gathered in the epithelial thickening zone of body cavity; here genital ridge and kidney were not completely separated. The arrow showed genital ridge area, ×400.
Day 5: Gonads differentiated into ovary, and it seemed like locusts lying on the kidney. The arrow showed the differentiated ovary, ×100.
Day 5: The arrow indicated ovary containing medulla and cortex, ×400.
Day 5: The arrow pointed to the differentiated testis, ×100.
Day 5: Observation of testis not containing medulla and cortex, evenly distributed, ×1000.
Day 7: Testicular cord in early stage, ×1000.

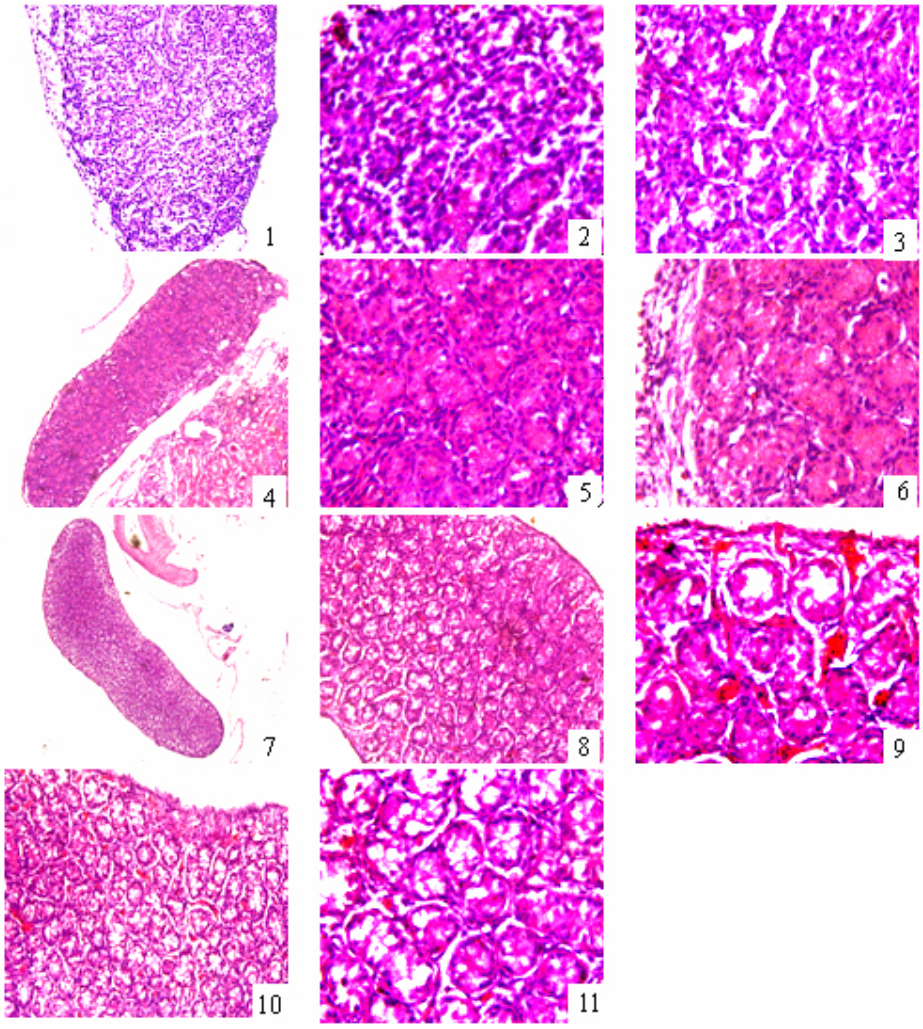


Fig. 2: Results of testis slice of quail from day 10 to hatch day, stained by HE

Day 10: The local observation of testis×100.
Day 12: The testis cord×1000.
Day 14: The typical seminiferous tubules cord×1000.
Day 15: The whole slice of testis and mesonephron×40.
Day 15: The seminiferous cords were closely intertwined×1000.
Day 16: The outer layer was myoid cells, the seminiferous cords became longer×1000.
Day 17: The testis showed like shoes×40.
Day 17: The local observation of testis×100.
Day 17: The seminiferous cords were more mature, containing elastic fiber, vascular and lymphatic between them×1000.
Hatch day: The local observation of testis×100
Hatch day: The testis cord×1000

Genital region on day 4: Successive quail embryo and gonad slices showed that the kidney had generated at this stage. In the inner side of kidney tube, primitive genital ridge had already appeared and began to depart from kidney, but had no obvious boundaries & shared the same parenchyma with kidney tube, which suggested that some kidney cells had involved in the formation of genital ridge. Here, PGCs had already migrated to the primitive genital ridge and were neatly arranged in epithelial thickening zone of body cavity which seemed like a slight belt.

Gonads on day 5: Kidney under the microscope was obvious and clear to see at this stage, and the gonad was close to the inner side edge of kidney. Gonad elongated vertically along the inner side edge of kidney and was larger than on day 4. A layer cells like PGCs could be found in gonadal epithelium, and the gonad was divided into two parts which seemed like ovary cortex and medulla. On the contrary, some slices of gonads showed evenly distributed cells without distinction of ovary-like cortex and medulla, which indicated the developmental trend towards testis. In general, day 5 of post-incubation was just the period of gonadal differentiation.

Gonads on day 7: At this stage, testicular features had appeared and testis was a long strip-like tissue with a thin layer of cells outside but many germ cells inside. The significant difference from ovary was no cortex in testis.

Testis on day 10: Testis showed the shape of two slender ends with a wide middle, and some layers of pinacocytes were on the edge. Under the epithelium, there were some prismatic mesenchymal cells. Funicular structure appeared inside of testis. Germ cells aligned as round or oval which formed early phase sex cords or so-called testicular cord. In the testicular cord, the spermatogonial cells and Sertoli cells were developed. Spermatogonial cells were large and round cells with clear cytoplasm and boundary, which dispersed in the center or around the testicular cord. Sertoli cells were nondirectional, small and long round cells with unclear boundary and deep staining nuclear. Sertoli cells were more than spermatogonial cells and distributed along the basement membrane.

Testis on day 12: After HE staining, the external morphology of testis was similar to that of day 11, but convoluted seminiferous tubules were more and larger, some of which showed a circle-shaped, and some elongated oval-shaped. There were a layer of big round germ cells and some undifferentiated cells dispersed. The undifferentiated cells were oval-shaped and deep staining because of a little invisible cytoplasm. Germ cells termed Spermatogonia were arranged into a neat circle along the basement membrane and dyed bright. Some red blood vessels began to appear between convoluted seminiferous tubules. Generally, throughout the whole embryonic period, testicular cord were all filled with a number of mesenchymal cells which were dyed red because of eosinophilic cytoplasm. But on day 12 of post-incubation, some mature mesenchymal cells seemed like spermatogonia with a large size and a round or flat round shape.

Testis on day 13: The morphology of testis has little difference from that of day 12, but the amount of seminiferous tubules increased with radially distributed spermatogonia inside. Inside of basement membrane, there were a little irregular cone-shaped or round small Sertoli's cells. Simultaneously, lots of fibrous connective tissue, a few mesenchymal cells as well as some capillaries were filled in the testicular interstitial substance.

Testis on day 14: Typical seminiferous tubules like grape bunch could be obviously observed. Spermatogonia were more loosely distributed on the edge and in the center of seminiferous tubules, but there were no cytoplasmic bridges among them. The amount of grape-like or chain-like spermatogonial cells mass increased gradually. At this stage, spermatogonia were large with light staining cytoplasm and meiosis had not happened yet. Sertoli's cells were oval with deep staining and almost invisible because of little cytoplasm involved. Besides many fiber-like connective tissues, there were lots of interstitial cells and capillaries in testis interstitial tissue. The amount of mesenchymal cells was much more than that of day 13, and the cells were distributed in crowds.

Testis on day 15 and 16: Testis was shoes-like and shown as two wide sides and a slight middle. Seminiferous tubules became bigger and closer to each other, some of which were round or irregular round because of crushing. Inside of the testis, it was solid-like, and a single-layer or two–layers of spermatogonia were arranged and attached to basement membrane. Some spermatogonia began to meiosis, so the amount was gradually increased with sparse mesenchymal cells. On day 16, the outside of testis were full of multilayer muscle-like cells. Seminiferous tubules became longer and bigger, while spermatogonia became smaller and seemed to be at a fast-proliferated stage.

Testis on day 17 and hatch day: Testis was also shoes-like but became longer and bigger than on day 16. Seminiferous tubules were clear and intensive, and the lumen became not solid-like. Big elastic fibers, blood vessels, lymphatic tubes, etc., were filled among the seminiferous tubules, and different forms of mesenchymal cells outside tubules became sparse. Besides, linear extracellular matrix also appeared. On hatch day, the morphological characteristics were very similar to that of the day 17.

DISCUSSION

Selective glycosides on PGCs surface play an important role in migration, colonization and early differentiation to oocytes or spermatogenic cells. Clara *et al.* (2007) confirmed that the development time of quail's gonad was different from that of chicken through the research on the specific binding antibody and lectins of PGCs surface glycoside. In chick embryo, Swartz (1982) reported that acid phosphatase activity did not appear within PGCs until 3 days of incubation, and then in only a few PGCs in the active phase of their migration in the dorsal mesentery, suggesting that there is no large wave of degeneration of these cells during migration. Li *et al.* (2005) reported that chicken gonads began to differentiate at 29HH (according to Hamburger and Hamilton, 1951), namely, on day 6 of post-incubation, and on day 7 male gonads began to show testis characteristics. In this study, quail PGCs started to migrate into genital ridge and separated from kidney on day 4 (stage 18 of Zacchei, 1961). On day 5 (stage 20 of Zacchei), the gonad differentiated into testis or ovary and morphology of testis was obviously observed on day 7 to 9 (stage 23~26 of Zacchei). Many reports also confirmed the exact time of gonad differentiation (on day 6 of post-incubation) by PCR detection of embryonic sex chromosome ZZ and ZW (Clara *et al.*, 2007), which was one day later than our results. In addition, Kingston and Bumstead (1995) reported that the time of female PGCs differentiation into ovary was earlier than that of male PGCs differentiation into testis in chicken, and PGCs in ovary began to meiosis and differentiated into oogonia on day 8 (34HH), while male PGCs began to differentiate into spermatogonia on day 13 (39HH). By contrast, in quail, PGCs differentiated into oogonia on day 7 (stage 23 of Zacchei) and differentiated into spermatogonia on day 10 (stage 27 of Zacchei) which showed significant difference from chicken.

Gonadal development depends on the development of cortical and medulla parts which differentiated from mesoderm at early embryonic stage (Ottinger and Brinkley, 1979). The unique tissues have a two-way differentiation potentiality, which involved in a series of complex regulation mechanisms during the process of differentiation into testis. In quail's testis development, up to date we know the expression of DMRT-1, anti-

Mullerian hormone (AMH), Sox9, as well as P450 17α-hydroxylase takes a key role. All are involved together in regulating the gonad formation process and sex determination in embryo (Clara *et al*., 2007). By contrast, in mammals, at least the orderly expression of six genes including SRY guides the developmental direction of gonad towards testis. Besides these differences in development mechanisms, the differentiation time is also different. Mammalian testis differentiation occurs earlier than ovary with the essentials of MIS and androgenic hormones. But with regard to non-mammalian vertebrates, ovary occurrence appears before testis occurrence and estrogen plays an important role in female sex determination and differentiation (Morohashi, 2002). In our research, quail's testicular and ovarian occurrence was synchronous without difference.

In general, testicular cells form two functional areas: testicular cords and mesenchymal cells. The key point of testis development is the formation of testicular cords. At early stages of testis development, Sertoli's cells polarized off and began to gather around the tufted original spermatogenic cells, subsequently triggered the development of testicular cords. Then Sertoli's cells in testicular cords revolve around the spermatogonia and form the basement membrane among pertubular myoid cells. In this experiment, we found testicular cords formed on day 10, and there were a few mature mesenchymal cells and vessels in lumen on day 12. These results revealed some key time features in testis development, which can provide foundation for further researches on sex determination mechanism and other tissues differentiation in quail. It can also lay data for the more utilization of PGCs such as inducing PGCs into other tissues and transferring interesting genes via PGCs in poultry.

Conclusions

In this study, we found that on day 4 post-incubation, all PGCs migrated to gonad ridge and formed undifferentiated gonads; on day 5 the gonad differentiated into testis or ovary. On day 7 to 9 post-incubation, the morphological characteristics of testis were distinguished, subsequently testicular cord appeared and the PGCs differentiated into spermatogonium on day 10. On day 12 post-incubation, spermatogonia seemed like clusters of grape or chain in the middle of convoluted seminiferous tubule; simultaneously, a few of sustentacular cells and vascular were developed to fill into the testis lumen. Then on day 15, the seminiferous tubule become thicker and the amount of spermatogonia increased by mitosis. Finally, on hatch day (day 17), the testis almost be mature with numerous mesenchymal cells, connective tissue, vascular and clear and intensive seminiferous tubules. These results can lay data for future studies on sex determination mechanism and the more utilization of PGCs in poultry.

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