Relationship between PGCs Settle and Gonad Development in the Early Chicken Embryo*

B. C. Li, G. H. Chen, X. J. Xiao, J. Qin, S. X. Wu, K. Z. Xie and Olowokeso Olojide**
Animal Science and Veterinary Medicine College, Yangzhou University, Yangzhou city, 225009, China

ABSTRACT : Chick embryos from stage 14 to stage 31 were studied by means of serial section and light microscopy in order to learn the relationship between the settlement sites of the primordial germ cells (PGCs) and the forming genital ridge. The results showed that: when embryo hatched for 53-56 h, the PGCs reached the coelomic epithelial tissue where gonad would be formed, meanwhile the epithelial tissue began thicker before the PGCs reached. Before stage 19, the final region the PGCs arrived was the thickened portion of the coelomic epithelium, the glycogen in the PGCs cytoplasm maintenance remained unchanged. However at the 3.5-5th hatching day, the glycogen in the PGCs cytoplasm reduced gradually. On the 6th hatching day, the gonad of the embryo appeared the feature of ovary, and the glycogen in the PGCs cytoplasm reduced further. On the 7th hatching day, the differentiation of ovary or testis was obvious and the glycogen in the PGCs cytoplasm later disappeared. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 4 : 453-459)***

Key Words : Chicken, Embryo, PGCs, Gonad

INTRODUCTION

The current state of biotechnology stimulates interest in primordial germ cells (PGCs) as a vehicle of new genetic coding for birds. However, areas of technology and biology must be understood better for essential knowledge of PGCs and gonad formation. Ukeshima (1987) researched the relationship between the genital ridge formation and the settle of the PGCs. Prior to 3 hatching day, when the epithelial thickening appears at the future gonadal site, the extravasation PGCs from blood vessel was found penetrate into epithelium of the splanchnopleure (Ando and Fujimoto, 1983; Ukeshima et al., 1987). He pointed out that both of the thickened coelomic epithelium of the splanchnopleure and the PGCs has formed the epithelium of the future gonad. In the present study, the migration characteristic of the PGCs and the change of the extravasation PGCs entered into the gonad formation area was investigated by means of serial sections at the light microscopic level, for further understanding of the theory base for isolation and culture of PGCs.

MATERIALS AND METHODS

Preparation of embryos

Embryos of the recessive white feather fowl were used to observe the PGCs. Fertilized eggs of prior to 19.5 h of incubation and fertilized eggs were incubated at 38.2°C for 3 h to a week and 60-70% relative humidity in a incubator, at the end of incubation each embryo was removed from the eggs. Each group of them was six samples.

Methods

Experimental embryo of chicken was fixed in the Rossman's fluids for 1 day, decolouized in 70% ethyl alcohol for 1 day, then dehydrated in graded series of ethyl alcohol (50, 70, 80, 90, 95, 100%) for 60 min, finally transparent in n-butyl alcohol for 1day at room temperature. Embryos were embedded in paraffin (melting point 55°C) for 30-60 min, and samples were molded and trimmed used blade. The serial sections were made 5-7 μm. They were loosened in 40% ethyl alcohol and hot water(30-40°C), mounted on the silde glass, dried on the hot plate (50-60°C)for 3-6 h. Sections were deparaffined in xylene for 15 min two times, and then hydrated in graded series of ethyl alcohol (100, 90, 80, 70%) for 1-2 min. Sections were stained with H·E or Periodic Acid-Schoff (PAS) reaction. Stained sections were dehydrated and mounted with paramoune mounting solution. Each period of the PGCs and the change of the gonadal formation were observed by light microscope. In order to avoid the interference when identify the PGCs, samples were hydrolyzed in 1% buffer diastase 37°C for 1 h.

The method of making integral section

Fixed the whole blastoderm in Rossman's solution, stained by PAS after dehydration, then mounted with paramoune mounting solution and took photograph.

RESULTS AND ANALYSIS

The PGCs of the chick embryo had abundant cytoplasmic glycogen which can be special stained by PAS reaction and can be easily identified by light microscopy.

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** Corresponding Author: B. C. Li. Tel: +86-514-7979350, Fax: +86-514-7350440, E-mail: ghchen@mail.yzu.edu.cn
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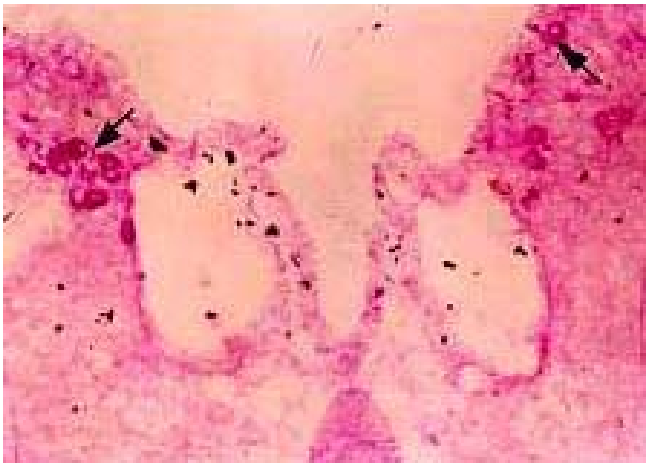

Figure 1. Many PGCs accumulated in the area of thicken coelomic epithelium (stained by PAS, ×400).

Chicken embryos were divided into two periods according to H&H (Hamburger and Hamilton), observed the migration and direction of the PGCs in different development period. At each stage, the number of 6 eggs were used to serial sections.

1) Prior to incubation (n=6). Observed by serial sections, chick embryo developed to 19.5 h, can find 3-4 PGCs in the area pellucida with light stained, but not find in the area opaca, which indicated that the PGCs had appeared when the area pellucida of the chick embryo formed.

2) At stage 1 (n=6), prophase of the primitive streak (incubation time: 3-4 h). Observed the serial sections of the embryo, still can find the classic PGCs distributed in the center of the area pellucida, while none PGCs in the area opaca.

3) At stage 2 (n=6), prometaphase of the primitive streak (incubation time: 6-7 h). Observed two embryonic serial sections, there were 4-5 PGCs in the inner edge of the area opaca, distributed in the border of the tiny area of the area pellucida.

4) At stage 3 (n=6), metaphase of the primitive streak (incubation time: 12-13 h). Observed three integral sections, there were many PGCs distributed at the both side of the extra embryonic area pellucida near the area opaca, 8-10 on the left, 4-6 on the right.

5) At stage 4 (n=6), telophase of the primitive streak (incubation time: 19 h). Observed one integral section, 10-15 PGCs were found in the area pellucida of the anterior region of the primitive streak, a few PGCs also found on the both side of the area pellucida of the primitive embryo. Most were large and round, the diameter was about 12 μm.

6) At stage 5 (n=6), head process period (incubation time: 22 h). Observed three integral sections, many PGCs concentrated on the lateral of the anterior region near the pellucida germinal crescent area of the head, about 18-22 on the left of the embryo, 12-14 on the right, 1-2 in the caudal area pellucida of the embryo.

7) At stage 9 (n=6), the 7 somites period (incubation time: 32 h). There was a blunt pseudopodium in the left area pellucida of the anterior region of the germinal body which not only indicated the PGCs were migrating, also indicated that PGCs are motile. One embryo serial section and one integral section, both of them can find 30-40 PGCs regularly arrayed in the blood vessel of the extra embryonic area pellucida.

8) At stage 10 (n=6), the 10 somites period (incubation time: 36 h). Observed two integral sections, a few PGCs have penetrated into the embryo, 10-12 in the head of the embryo, 1-2 between the inversive seventh and eighth somites from head to tail on the right. A lot of PGCs still distributed in the blood vessel of the extra embryonic area pellucida, about 30-35 on the left, 50-60 on the right.

9) At stage 11 (n=6), the 13 somites (incubation time: 44 h). Three embryo serial sections and integral embryo, which showed PGCs became increasing, about 23-30 in the head of the embryo. On the entrance of the embryo where left mesenteric vein entered, 4-6 in the vessel, 3-5 on the right. Many PGCs still located in the blood vessel of the extra embryonic area pellucida, 52-60 on the left, 40-50 on the right. Either on the right or on the left, most PGCs distributed in the anterior region of the head of the embryo and in the lateral area pellucida, almost no PGCs was found near the middle of the embryo and in the caudal area pellucida, the diameter of the PGCs about 12 μm at this period.

10) At stage 12 (n=6), the 16 somites (incubation time: 48 h). Observed three integral sections, the PGCs distributed in the following way: 10-15 in the head, 1-2 in the heart. More than 40 were in the export way of blood vessel of the heart. 1-2 was in the inversive third somite from tail to head on the left of the embryo. PGCs in the blood vessel of the extra embryonic area pellucida fewer than at stage 11, 21-25 on the left, 20-25 on the right, almost distributed equally on both side of the embryo.

11) At stage 13 (n=6), the 19 somites (incubation time: 52 h). The PGCs increased drasticly at this period. In one of three integral sections, PGCs concentrated on the head and the heart, 50-55 in the small blood vessel near the prosencephalon and the metencephalon of the head, 45-50 in the heart, 18 in the blood vessel of the embryo which at the same horizontal site with the heart, 27-30 in the blood vessel of the left extra embryonic area pellucida of the embryo, 16-20 on the right, same as stage 12. More than 50% PGCs still distributed in the mesenchymal blood vessel of the head, about 40% in the heart.

12) At stage 14 (n=6), the 22 somites (incubation time: 53 h). Observed one integral section, about 18 PGCs in the mesenchymal blood vessel of the head of the embryo, one located on the epiblastic surface of the head, 5 in the

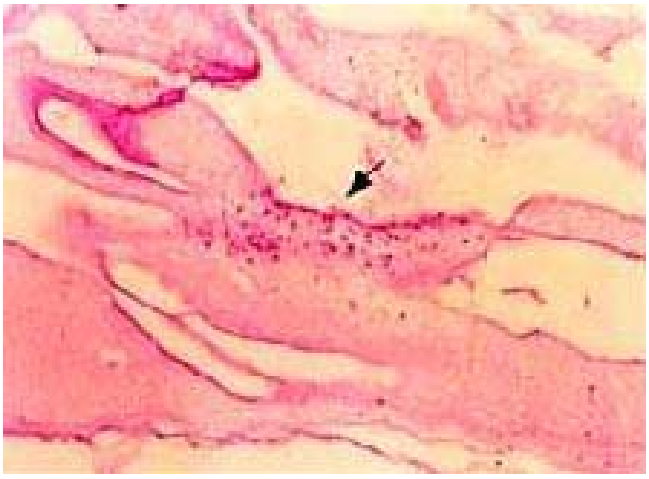

Figure 2. PGCs in the area of mesonephros (stained by PAS, ×40).

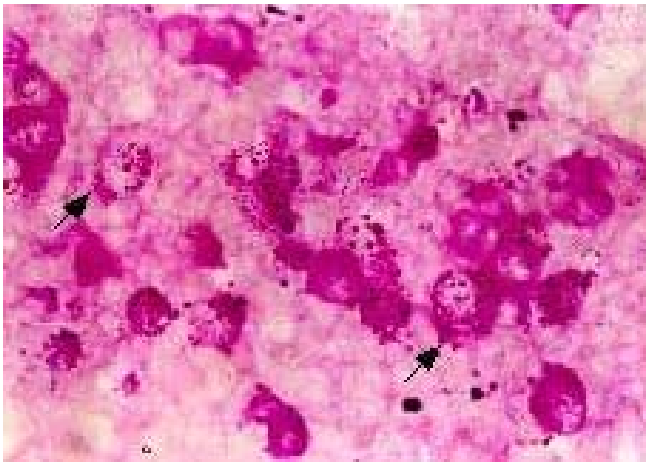

Figure 3. Cytoplasmic glycogen of PGCs in gentital gidge was resolving (stained by PAS, ×100).

ventricle, 20 in the dorsal artery blood vessel, on the right of the embryo level to the forth somite, each in the seventh, tenth, thirteenth somites. 18 somites to 20 somite had four; one in the omphalomesenteric artery, two in the omphalomesenteric vein, while on the left of the embryo, 4 in the tenth somite, 2 in the twelfth somite. PGCs distributed in the mesenchyme of the embryonic area at random. On another section, most PGCs still located in the mesencgyme of the head or in large blood vessel. Three located in the coelomic epithelium of the 18 somite to 20 somite and in the splanchnopleural mesoderm of the dorsal mesentery, one located near the 21 somite.

The result indicated PGCs almost concentrated in blood vessel at stage 12 to 14, especially in the small blood vessel of the mesenchyme of the head.

13) At stage 15 (n=6), the 24-27 somites (incubation time: 54 h). Observed one integral section, hardly can find PGCs except the head of the embryo and its edge (about 20), almost no PGCs in the lumina of the blood vessel. Another sample section distribution of the PGCs changed greatly, about 20 located in the mesenchyme of the head, about 40 located among the heart, the dorsal artery, and the omphalomesenterocartery. The coelomic epithelium covering the coelomic cavity has thickened in almost whole portion of the splanchnopleur and somatopleur. The epithelial cells showed columnar shape and the nuclei were rhomboidal. Not any PGCs were observed in the coelomic epithelium and its neighbour that indicated PGCs still not arrived this area.

14) At stage 16 (n=6), the 26-28 somites (incubation time: 56 h). We can find 3-5 layers of cells of the thickened portion of the coelomic epithelium limited in the 180 μm area from splanchnopleme to coelomic angle, while the region between the coelomic angle and mesonephros which corresponds to the site of gonad in later stage did not thicken, structured by single complanate or columnar cells. At this developmental stage, the number of PGCs increased, but still less than at stage 17. PGCs distributed between the caudal of the embryo and the vitelline artery, ranging in length from 0.8 mm to 1.9 mm in this territory, some PGCs were seen to be incorporated into the thickened coelomic epithelium and others were found in the mesenchyme nearby and in the blood vessel, in some cases PGCs were found between endothelial cells of the vessel. PGCs occasionally found in the splanchnopleure, no PGCs were found in the somatopleure. The PGCs were large size and possessed a conspicuously large and round nucleus, can easily discern from the neighbour cells. It is noted that when the embryo developed to stage 16, PGCs were found in the thickened epithelium and the mesenchyme of the head or blood vessel of the caudal.

15) At stage 17 (n=6), the 29-32 somites (incubation time: 62 h). Observed integral section, PGCs crossed the blood and entered into the left and right dorsal embryo via circulation of blood. Perhaps gonad formation settled in this portion, many PGCs concentrated in this site. Observed the serial section, many PGCs concentrated in the thickened region of the coelomic epithelium. (Figure 1)

16) At stage 19 (n=6), the 37-40 somites (incubation time: 72 h). The thickening of the coelomc epithelium was found in the region from the coelomic epithelium to the future gonadal site, however, some thickened epithelium was still present in the proximal of the splanchnopleure. Most PGCs were found to be involved in these thickened epithelia (Figure 1), some were found in the mesenchyme nearby. As the thickened portion of the epithelium moved toward the mesonephros, the localizing area of the PGCs was alternated, the place was the thickened portion of the epithelium of the splanchnopleure, which indicated that the final region the PGCs arrived was the thickened portion of the coelomic epithelium, this area would participated in forming the gonad.

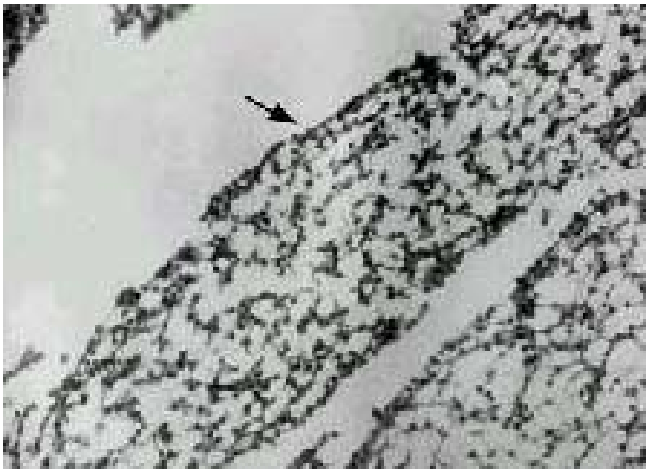

Figure 4. The differentiated gonad showed character of testis (stained by H.E., ×100).

17) At stage 21 (n=6), (incubation time: 82 h). Observed the transverse serial section parallel to the axis of the embryo, found that the embryo began to form mesonephric duct, while in the inner edge of the mesonephric, the genital ridge has formed and began to separate to the mesonephros, especially at the anterior region. Many PGCs concentrated on the genital ridge, about 50-60 in each section, which indicated the PGCs and the thickened portion of the coelomic epithlium form the genital ridge together (Figure 2). Stained by PAS can find the cytoplasm easily reaction with the PAS, but the nucleus couldn't. The glycogen in the cytoplasm began to resolve in most PGCs, germinal ridge located in the conterminous region between the mesonephros and the hind-kindney, the shape and the size of the PGCs in genital ridge changed greatly, some were round oblong, some begin to divide. At the terminal of the genital ridge, there were many PGCs arrived, while less near the mesonephros, the epithelium of the genital ridge has attracted to PGCs. The genital ridge and the inner wall of mesonephric duct did not completely separated, which showed they have a collective parenchyma.

18) At stage 22 (n=6), (incubation time: 4 days). The mesonephros were very bright, the genital ridge and the mesonephros can be identified. They separated each other, while PGCs still arrived in the genital ridge disorderly. Cytoplasmic glycogen of the PGCs was resloving (Figure 3).

19) At stage 26 (n=6), (incubation time: 5 days). The mesonephros development conspiciousness, primitive gonad closed to its inner side, separated obviously to the mesonephros. The primitive gonad was big and long, many PGCs located in it, the cytoplasmic glycogen was resolving, like flower. But still can identify the basic shape of the glycogen in the cytoplasm. About 75 PGCs in every section, the undifferentiated gonad obviously longer than that at stage 21, but still cannot identify the sex, which indicated the final area of the PGCs was gonad.

20) At stage 29 (n=6), (incubation time: 6 days). The genital ridge became more conspicuous, the neuropile closed to the mesonephros and the cortex of the ventral side can be distinguished. Stained by PAS, but had weak staining, some located in the neuropile of the genital gonad, some began to migration toward the cortex, cytoplasmic glycogen resolved and distributed disorder, which indicated the gonad began to differentiation. Stained by H·E, there was one large cell like PGCs in the epithelium of the genital gonad and has obvious gap under it, the genital gonad separated conspicuously into two parts. Both of the embryonic genital gonad showed ovarian characteristic, which indicated the sex began to differentiation at this stage, PGCs took part in incorporating to the gonad.

21) At stage 31 (n=6), (incubation time: 7 days).By PAS-staining was cannot find PGCs, which indicated with the sex differentiation, the PGCs in the gonad changed to the oogonium or the spermatogonium, the glycogen disappeared, both right and left gonad showed inverte eight, gonad on the left like thread, bias located in the inner wall of the mesonephros, gonad on the right lightly short than the left, showed rectangle, can easily identify it was female. Observed the section, the gonad obvious separated into two layers, the exterior contained blood vessel and net like cells, showed ovarian characteristic. Observed another section, the epitheliac cell had two layers, like the early tunica albuginea of the testis development progress of the mammalia, closed to epithelium was one non-classic rhomboidal layer cells, showed testiculate characteristic (Figure 4). There was high stained cell mass cord in the parenchyma, the mass cord separated by some rhomboidal cells. Because the sexual cord separated by the early tunica albuginea that made it separated from the epithelium and forming early shape of the spermiduct (Figure 4, "A" arrowed.)

The major affairs of PGCs migration and embryonic development was showed in Table 1.

DISCUSSION

Origin, migration and concentration of the PGCs

This experiment showed that: a few PGCs entered the germinal body when the embryo developed to stage 10, at stage 11, the PGCs became increased in the germinal body, this result absolutely was not common with the Meyer's (1964) report that the PGCs first appear at stage 12. Meyer (1964) thought that the heart began to shrink when the somite 10 formed. But the heart began formal beat at somite 16. There was blood running in the blood vessel, moreover, the time of the PGCs first appeared in the embryo was in common with the heart formal beat and the blood begin circulation. It was the evidence from the study that: the

Table 1. A timetable for major affairs of PGCs migration and embryonic development

Stage	Incubation time	Major affairs of PGCs migration and embryonic development
	Prior to incubation	PGCs had appeared when the area pellucida formed
Stage 1	3-4 h	Typical PGCs had distributed in the center of area pellucida, embryo in prophase of the primitive streak
Stage 2	6-7 h	PGCs appeared in the inner edge of area opaca, embryo in prometaphase of the primitive streak
Stage 3	12-13 h	PGCs distributed in both side of area pellucida and area opaca, embryo in metaphase of the primitive streak
Stage 4	19 h	PGCs were found in region of primitive streak, embryo in telophase of the primitive streak
Stage 5	22 h	Many PGCs concentrated on the lateral of anterior region, embryo in head process period
Stage 9	32 h	PGCs began to migration, the extra embryonic blood vessel net has formed
Stage 10	36 h	A few of PGCs penetrated into the embryo, formed the vitelline circulation toward to the heart.
Stage 11	44 h	PGCs increased in the head of embryo, heart bent to right
Stage 12	48 h	PGCs distributed eaqually on both side of the embryo
Stage 13	52 h	PGCs increased drastically in head and heart
Stage 14	53 h	PGCs almost concentrated in the medenchymal blood vessel of the head of the embryo
Stage 15	54 h	PGCs only existed in head, the dorsal artery, and the omphalomesenterocartery, the coelomic epithelium thickend in almost portion of splanchnopleur
Stage 16	56 h	PGCs in the thickened epithelium and the mesenchyme of the head or blood vessel of the caudal
Stage 17	62 h	Many PGCs in the thicken region of coelomic epithelium
Stage 19	72 h	Most PGCs were found in thicken epithelia, the thicken portion of the epithelium moved toward the mesonephros
Stage 21	82 h	Many PGCs concentrated on genital ridge, embryo began to form mesonephric
Stage 22	4 d	PGCs arrived in genital ridge disorderly, genital ridge and mesonephors can be identified
Stage 26	5 d	Many PGCs located in primitive gonad, mesonephros development conspiciousness
Stage 29	6 d	PGCs took part in incorporating to the gonad, genital ridge became conspicuous
Stage 31	7 d	PGCs in gonad changed to oogonium or spermatogonium with the sex differentiation

extra embryonic blood vessel net has formed at somite 5 to 7, its differentiation progress first begin at the exterior portion of the extra embryonic area vascculaire, and toward to the direction of the embryo slowly (Li et al., 2003). The omphalomesenter switched on somite 10th, formed the vitelline circulation toward to the heart. The PGCs migrated gradually to the embryo via blood circulation, and some PGCs have reached the embryo.

In avian species, PGCs first arouse from the epiblast and migration to the hypoblast of the area pellucida (the germinal crescent) (Urven et al., 1988). At stage 4, approximately several hours after incubation, with the extra embryonic mesoderm invaded in the germinal crescent and began to form blood island and blood vessel, PGCs entered into the extraembryonic and intra-embryonic blood vessel, until to 65 h of incubation (at stage 17). Following PGCs leaving to the blood vessel, reached the area of the future gonad at stage 20 to 24 (Nakamura et al., 1988). The result of this experiment showed that, PGCs originated from the area pellucida prior to incubation, after 22 h of incubation, many PGCs concentrated in the area pellucida of the anterior lateral region of the germinal body (the germinal crescent area). With the extra embryonic blood vessel development, the PGCs in the germinal crescent first migrated into the extra embryonic blood vessel, PGCs firstly appeared in the germinal body at stage 10 (incubation time: 36 h), major distributed in the head. Stage 11 to 13(incubation time: 44-45 h), the PGCs major distributed in heart and in large blood vessel. At stage 17 (incubation time: 72 h), many PGCs concentrated on the genital ridge of the caudal area of the germinal body, to stage 22 participated in forming the gonad. So the result of this experiment was different from former report, it was possible due to the species difference and environment factor. The result of this experiment showed in the migration progress, the PGCs appeared three peaks of concentration, the first concentrated in the germinal crescent after 22 h of incubation, the second concentrated in the heard and the blood vessel nearby after 44-52 h of incubation, the third concentrated in the germinal ridge after 62 h of incubation. This result supplied theoretical evidence and experiment base for isolation of PGCs and produce transgenic chick.

In this experiment, chick embryo at stage 15-18 of development which correspond to the peak of the PGCs migration to the gonad were observed by means of serial sections. According to the report (Ando and Fulimoto, 1983), the location of the PGCs and the formation of the genital ridge has close relationship. After the PGCs appeared in the near blood vessel of the genital ridge, the PGCs migrated to the thickened region of the coelomic epitehelium via amoeba movement. The result of this experiment showed, with the embryonic development, the thickened portion of the coelomic epithelium changed it site. At stage 16, the coelomic epithelium of the region between the coelomic angle and mesomephros where corresponding

to the site of the gonad did not thicken, the thickened portion of the coelomic epithelium was present only in the splanchnopleure, the original entered PGCs distributed in the thickened area, they formed the coelomic epithelium together. With advance of development, the site of the thickened epithelium, in which the PGCs were involved, moved to the coelomic angle. But the later-arrived PGCs entered the epitheliumconsistent thickening area directly in the prospective gonad. It is reported that the epithelium of the genital ridge in except amniotes originates from splanchnopleure. Observed from this experiment, the thickened epithelium first appeared in the splanchnopleure at stage 16, and had PGCs migrated into it, it was the really epithelium of the gonad, the epithelium of the gonad which is the final site for the PGCs.

The change of the organization when the gonad differentiated

At somite 16 to 23, the mesoderm development gradually into mesonephros (Rodemer and Wartenberg, 1986). At 6-7 days of incubation the mesonephros began to participate in forming the gonad (Hong et al., 1995). There was not a unity view about how the gonad formed. Before the sexual differentiation. It was thought that it origin was from the coelomic epithelium, others think it origin from the blastema of the mesonephros or arise from the diffenerated mesonephros. The result of this experiment showed: the genital gonad origin from the coelomic epithelium, support the first view. It was proved that the gonadal differentiation origin from after the germinal cell arrived the genital ridge, but whether the PGCs were helpful for the gonad differentiation or the gonad in differentiation progress attracted the PGCs is uncertain. Witschi's (1948) work showed: when the genital ridge developed to certain size, after the PGCs arrived, it division quickly, at the same time, the somatic cell and the tissue division too. The later major arise from the inner side of the mesonephros, original neuropil of the genital ridge formed, the epithelium of the genital ridge forming the original cortex. Then the epithelial cell continue developed toward the neuropile forming the cell mass cord, and with the PGCs among the epithelium entered together, which was called original sex cord, sexual difference can not find at this stage, gonad still not differentiated in this research, gonad of the chicken embryo did not take on the testis and the ovarian characteristic at the undifferentiation stage. At 6 days of incubation, the gonad took on the ovarian characteristic, gonad epithelium had one layer cell like PGCs and has lax structure under it, at 7 days of incubation, obvious took on testis characteristic, under the epithelium has formed by 2-3 layers unclassic cells. The ovarian characteristic became more obviously, had differentiated into neuropile and cortex. So we think, at 6 days of incubation, perhaps appeared sexual differentiation, some samples sections showed the gonad began to take on ovarion characteristic. Concluded by the testis cord formed at 7 days of incubation, the testis perhaps began to differentiate at 6 days of incubation, because the testis and the ovary should formed simultaneously. After appearing the sexual differentiation, PGCs began to division, the female appeared at 8-11 days of incubation, the male appeared at 13-15 days of incubation (Han et. al., 1994; Han et al., 1995). The PGCs in males scattered randomly within a developing seminiferous tubule until 13 days of incubation, after this period, they began to differeniate into spermatogonis, in contrast to the embryo of the male, the sexual differentiation of the female embryo occurs in the left ovary at 8 days of incubation, the PGCs began to active meiotic division, forming oogonia at this stage, the right ovary always degradate after birth. But the result of this experiment showed that, at the same incubation condition, sexual differentiation began at 6 days of incubation, that did not in common with Han's (1994) report, perhaps owed to specious, ample time, incubation condition difference, the result of this experiment revealed that, many PGCs are located in the gonad at 6 days of incubation, the classic PGCs cannot be found in the gonad at 7 days of incubation, they had differentiated into female or male germinal cells, which indicated that the structure of the germinal ridge appeared at 6 days of incubation, PGCs began to differentiate to oogonium or spermagonic at 7 days of incubation.

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