PRIMORDIAL GERM CELLS IN AVES
— Review —

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Summary

Primordial germ cells (PGCs) in aves are the progenitor cells for the gametes. These cells first appear in the epiblast (Eyal-Giladi et al., 1981), then translocate and concentrate to endoderm of germinal crescent area in the junction of the area opaca and area pellucida lateral to the primitive streak in stage 4 through 7. They separate from the endoderm, temporarily circulate via the blood vascular system, leave the blood vessels, and finally settle down in the gonadal anlagen at stage 20-24 where they rapidly proliferate to form germ cells. Recently, several attempts have been made to introduce foreign gene into the avian genome to form a transgenic chicken. The stem cells most readily available as vehicles for genetic manipulation of germline in avian species are the PGCs. PGCs have recently been manipulated genetically and used successfully as a vector for gene transfer.
(Key Words : Primordial Germ Cell (PGC), Germinal Crescent, Germline Transgenic Chicken)

Introduction

In aves, PGCs arise from the epiblast and migrate to the hypoblast. During gastrulation, the PGCs migrate anteriorly via the hypoblast and reside in the extraembryonic area, which is a so-called germinal crescents located in the anterior region of the blastodisc at very developmental stage (Eyal-Giladi et al., 1981; Urven et al., 1988). And then these cells migrate into gonadal ridge via vascular network (Fujimoto et al., 1976). PGCs settle down in the gonadal primordium at stage 20-24 where they rapidly proliferate to form germ cells (Nakamura et al., 1988). The isolated PGCs are potentially suitable vehicles for manipulation of chicken genome.

Shuman (1981) reported that the isolated PGCs can be successfully transferred to the host embryo by utilizing the microinjection technique. Donor PGCs colonized in the host gonad were identified by chromosomal markers. In addition, it was demonstrated that PGCs did not influence the sexual differentiation of the gonad. Simkiss et al. (1990) reported positive results by amplification of DNA with polymerase chain reaction (PCR) following Southern blot analysis. This was verified by examination of 5 day embryos, 10 day urogenital regions, and 18 day gonads. PGCs were transfected *in vitro* and expressed the exogenous plasmid DNA, indicating that PGCs are a possible vector for direct gene transfer into the germline (Han et al., 1993, 1994). More recently, PGCs were obtained from germinal crescent or blood vessel (dorsal aorta) and transfected with defective retroviruses carrying the *neo* and *lacZ* genes. These transfected PGCs were microinjected into recipient embryos to form chimeras. The produced chimeras were grew to sexual maturity and produced offspring. Analysis of Southern blot of the DNA from the chimeric bird sperm and the offspring blood was carried. As a results, both the chimera sperm and transgenic offspring blood contain the foreign DNA (Vick et al., 1993).

This review is focusing on characteristics, migratory route, manipulation and culture of PGCs for production of transgenic chicken.

Characteristics of PGC in Aves

PGCs are the embryonic cells antecedent to mature sex cells in aves. Ginsburg and Eyal-Giladi

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Received April 20, 1994
Accepted June 27, 1994

(1987) stated that PGCs are present in the central portion of the area pellucida in preoviposital (stage X) embryos (Eyal-Giladi and Kochav's nomenclature, 1976) and that further differentiation of PGCs is independent of other processes forming in the embryos. Meyer (1960, 1964) found that embryos histochemically stained with the Periodic Acid-Schiff (PAS) show the localization of PGCs which stain a deep purplish red due to abundant glycogen content in the cytoplasm. PAS staining makes it possible to selectively identify PGCs. However, this procedure cannot be used for isolating viable PGCs because it is lethal to cells. Some carbohydrates on avian PGCs have been shown to react with several antibodies raised against cell surface antigens on other embryonic cells (Pardanaud et al., 1987a, 1987b; Urven et al., 1988; Didier et al., 1990). The carbohydrates are known to be remarkably altered in the process of development and differentiation, and some are thought to play an important role in many developmental events during embryogenesis (Muramatsu, 1988). Lectins are especially helpful for studying structure and changes of cell surface during embryonic development, and they are candidates for lineage markers of embryonic cells, including PGC (Fazel et al., 1987, 1990). Yoshinaga et al. (1992) examined the distribution of binding sites for several biotinylated lectins in chick and quail developing embryos. Lectin from *Sojanum tuberosum* reacted with PGCs in chick and quail embryo, whereas lectin from *Wistaria floribunda* and *Griffonia simplicifolia* II reacted in the quail and chick PGC, respectively. In chick embryo, like previous studies using the PAS procedure (Meyer, 1964; Fujimoto et al., 1976), these biotinylated lectins easily identified PGCs during stages from the germinal crescent through the migration.

Yoshinaga et al. (1993) recently reported that several ultrastructural characteristics of quail PGC not described previously in chick PGC. They observed that no glycogen particles in quail PGC were detected in the cytoplasm at any stage examined, that electron dense and membrane bounded granules were found in the PGC cytoplasm during the sexually indifferent gonadal stages, and that quail PGC were characterized by a prominent nucleolus associated with condensed chromatin (heterochromatin). The size range of PGCs in the germinal crescent are between 12 μm to 18 μm in diameter. PGCs are large in comparison to other somatic cells. In the gonad analogue region, the size of the PGCs varies between 10 and 25 μm in diameter (Meyer, 1964). The nucleus is large, 8 μm in diameter, and the PGCs are round or oval in shape distinguishing the PGCs from other somatic cells. PGCs are derived from the germ wall endoderm of the germinal crescent along the border between the area pellucida and area opaca (Swift, 1914; Clawson and Domm, 1969). As embryological development continues, the PGCs separate from the endoderm, enter the forming vascular system, and migrate into the germinal ridges (Swift, 1914; Fujimoto et al., 1976).

Clawson and Domm (1969) believed that the PGCs originated only from the cells of a particular area located in the endodermal layer of the blastodisc. They characterized PGCs as bearing PAS-positive granules in the germinal crescent area of the junction of the area opaca and area pellucida lateral to the primitive streak in stages 4 (primitive streak) through 7 (1-2 somites) (Hamburger and Hamilton's nomenclature, 1951). The PGCs originate prior to the definitive streak stage, indicating that it must begin at stage 3 or earlier. They also reported that germ cells may arise from inside the endodermal loop, located in the space between the endoderm and ectoderm. The loops have been observed in stages 4 through 8 (4 somites).

The PGCs separate from the endoderm during stages 4 to 8 (primitive streak stage to 4 somites) (Clawson and Domm, 1969; Fujimoto et al., 1976). This process of separation generally begins at the junction of the superior surface of the endoderm contacting the PGCs. There are 100-250 PGCs in each blastodisc at these stages. Many PGCs are present as free cells between the endoderm and ectoderm and in areas with mesodermal elements. At this separation state, the PGCs showed a high content of glycogen, lipids, and yolk in the cytoplasm (Clawson and Domm, 1969; Fujimoto et al., 1976). Once the PGCs are freed into the space between the endoderm and ectoderm in the area pellucida, PGCs migrate via the embryonic vasculature system to the developing gonads.

Urven et al. (1988) found that chick PGCs can be labeled by a monoclonal antibody (EMA-1) raised against mouse embryo carcinoma (Nulli SCC1) cells. This antibody was used to identify PGCs throughout germ cell development and migration in the unincubated embryos (stage X-XI). Some embryos were found to have EMA-1-positive cells in a scattered fashion in the epiblast. During

hypoblast formation (stage XII, 6-12 hours of incubation), individual EMA-1-labeled cells separate from the basal surface of the epiblast and enter the blastocoel. These results correspond to the reports from Eyal-Giladi et al. (1981), which states that avian PGCs are of epiblastic origin. This is in distinct contrast to earlier studies (Clawson and Domm, 1969), when PGCs were thought to have originated from cells in the endodermal layer of the blastodisc. It is now thought that PGCs are derived from the epiblast at stages prior to those at which they are identifiable by either morphological or histochemical ciriteria (Urven et al., 1988; Ginsburg and Eyal-Giladi, 1986, 1987). Because all cells of the embryo stain deeply, PAS is not capable of PGCs identification in stages X-XI (unincubated) and stage XII (hypoblast formation) embryos (Urven et al., 1988).

Monoclonal antibodies (QH1) which recognize quail endothelial and haemopoietic cells was cross reacted with quail PGCs (Pardanaud et al., 1987a, 1987b). They found that some cells (2-3) were already positive to QH1, in unincubated blastulas (stage X-XI, at laying), and the number of PGCs increased to more than 100 by the primitive streak stage (18 hours of incubation). Approximately 20 PGCs were noted at stage XII-XIII (12 hours of incubation) and 90 PGCs at stage 2-3 (15-17 hours of incubation). Following the primitive streak stage (stage 4, 18 hours of incubation), the number of PGCs did not increase significantly until the 7 somite stage (stage 9, 27 hours of incubation). A conspicuous increase of PGC in stage 9 was detected by Clawson and Domm (1969). Pardanaud et al. (1987b) concluded that the low number of PGCs in the unincubated and 12 hour blastula corresponds to the segregation of PGCs from the somatic cells of the blastula at this period of development. According to Ginsburg and Eyal-Giladi (1987), most of the PGCs originated from the central disc of the area pellucida, and not from the periphery of the area pellucida. They found that PGCs may already be determined at stage X and further differentiation of PGCs is independent of the embryo forming process. Ginsburg and Eyal Giladi (1986, 1987) indicated that PGCs begin migration from the epiblast towards the hypoblast from stage XII. Migrating cells are carried by both the hypoblast and mesoderm into the germinal crescent. The germinal crescent and endoderm hypoblast are secondary locations in the PGC development.

PCGs Migratory Route

Ginsburg and Eyal-Giladi (1986) suggested that the earliest PGCs land on the expanding hypoblast from the epiblast, and centrifugal expansion of the hypoblast during stage XII-XIII to stage 6 is a passive method of PGC migration into the germinal crescent. The chick embryo PGCs adhere to, and migrate along a fibrous band, present on basement membranes of the epiblast at the anterior margins of the area pellucida that forms the germinal crescent (Wakely and England, 1979). Critchley et al. (1979) have suggested that the fibronectin pattern is laid down by the ectoderm either alone or in association with endoderm and that fibronectin contributes to a contact guidance system laid down by the ectoderm and utilized by PGCs and mesoderm cells. England et al. (1986) transplanted PGCs from *Xenopus laevis* embryos into stage 4 chicken embryos and found that the fibrous band of extracellular materials, which contains fibronectin, collagen type I, and sulphate glycosaminoglycans, was responsible for the preferential migratory pathway for chick embryo PGCs. The fibrous bands serve as a physical as well as a chemical cue for guiding moving cells to a predetermined destination.

Fujimoto et al. (1976) demonstrated that some of the migratory PGCs possess pseudopodia, suggesting that their amoeboid movement as a possible mechanism for migration. Lee et al. (1978) observed that because of the alterations in cell surface properties of the PGCs due to concanavalin A, migration of the PGCs from the germinal crescent area to other parts of the stage 6 12 embryo was inhibited.

The net result is that PGCs originating from the epiblast migrate to the deeper layers of the hypoblast and mesoblast and become concentrated between these two layers within the germinal crescent (Ginsburg and Eyal-Giladi, 1987; England et al., 1986). As development proceeds, PGCs penetrate into or are enclosed by the blood vessels by stages 9-10 (30-38 hours of incubation) without changing morphology. Most PGCs were found in the blood stream at stage 12 (16 somites) (Lee et al., 1978; Fujimoto et al., 1976). The intravascular PGCs are generally round in shape, possibly due to passive migration through the blood stream

(Fujimoto et al., 1976). Changes in enzyme activity of the PGCs during migration into the developing gonads of the early chick embryo was described by Swartz (1982). He demonstrated a change in alkaline and acid phosphatase activity in the PGCs, suggesting alterations in metabolic activities. The detached prevascular PGCs contain a high glycogen, glucose, galactose, and mannose content with abundant yolk granules (Didier et al., 1981). Clawson and Domm (1962) found that PGCs in circulation contain a high glycogen content, but in the developing gonads the amount of glycogen and yolk decrease suggesting that PGCs use their glycogen and yolk as a source of energy during migration. The intravascular PGCs were reported to be smaller in size (9-16 μm in diameter) than PGCs in the germinal crescent (12-20 μm in diameter) (Lee et al., 1978).

The circulatory PGCs in blood vessels successfully reach the sites destined to form the gonad and invade the thickened coelomic epithelium after 2-3 days of incubation (Ukeshima et al., 1987). Dubois et al. (1976) reported that the thickened coelomic epithelium releases the attraction factor for the circulating PGCs. The PGCs, after extravasation, penetrate into the coelomic epithelium of the splanchnopleure where PGCs escape from the vessels (Ando and Fujimoto, 1983). Ukeshima et al. (1987) found: 1) stages 15-18 (24-36 somites) to be the most frequent extravasation periods; 2) the coelomic epithelium covers the coelomic cavity whole portions of the splanchnopleure and somatopleure at stage 15 and 3) the PGC are located in the thickened coelomic epithelium from stage 16.

Ukeshima et al. (1991) observed the chick PGCs with special reference to the extravasation in their migration course using scanning electron microscope and transmission electron microscope. They reported that PGCs in the vessels showed a round profile, possessing many microvilli, while extravasating PGCs were rather oligovillous except the one site of the cell, where long filopodia extended toward the wall of the vessels. These filopodia seemed to adhere to the wall of the vessels prior to emerging out. After extravasation, PGCs moved toward adjacent prospective gonadal epithelium and invaded it by amoeboidism. Following the settling down in the epithelium, PGCs showed rugged surface with few microvilli.

Nakamura et al. (1988) reported that PGCs start to concentrate in the future gonad from stage 16 and are in the settlement stage in the gonadal primordium at stage 20-24. Approximately 20% of the total number of PGCs were observed in extragonadal regions such as the head and mesenchyme surrounding the neural tube. Nakamura et al. (1991) examined the trend of ectopic distribution of PGCs in the chick embryo when its future gonadal region (caudal third region) had been removed at an early stage (stage 10). They reported that, when the chick embryo lacked gonads, the PGCs could be concentrated in the head region and migrated from the capillaries into the mesenchyme.

At stages 16-23 the number of PGCs was estimated to be approximately 150 (stage 16) -1000 (stage 23). The number of PGCs at stage 24-29 (3-5 days of incubation) was estimated to range from 329 (stage 24) to 1359 (stage 29) by Dider et al. (1981).

PGCs migrate from germinal crescent to the germinal ridge to form the definitive gonad. The migration of PGCs occurs after about 2.5 days of incubation (stage 16) and most of these cells reach the germinal ridge by 3 days (stage 18-19) at 38℃. When the incubation temperature is reduced to 36℃, the pulse of PGCs occurs at a significantly earlier stage of development (stage 15) than for embryos at 38℃ (stage 16) (Al-Thani and Simkiss, 1991). The incubation temperature affected the migration of PGCs during the embryonic development. They proposed from this result that (1) the different organ system have slightly different temperature sensitivities, (2) there is some absolute time effect in PGCs formation, (3) the phenomenon may be a physical response.

The attraction of PGCs to the gonadal anlagen (germinal ridge) was tested by Kuwana et al. (1986). They placed PGCs isolated from the circulatory blood at stage 13 embryos between the germinal ridge and other embryonic tissues such as the neural tube, heart, allantois, and liver *in vitro*. The PGCs showed directional movement toward the germinal ridge, indicating that they are attracted by some factor emitted from the germinal ridge. One of the PGCs migration possibility is that the developing gonad produces a chemotactic substance that attracts PGCs and retains them in the capillaries bordering the gonad (Rogulska, 1969). Another possibility is that the endothelial cells of the gonadal capillaries have a cell surface

compound that causes the PGCs to adhere there specifically. Thus, the chicken gonad attracts circulating PGCs from the turkey and even the mouse (Reynaud, 1969; Rogulsak et al., 1971).

Gonadal Soma Formation

After extravasation of PGCs, the cells move actively into the coelomic epithelium of the coelomic angle at day 2-3 of incubation. Initially, the gonadal anlagen is located in the dorsal splanchnic mesoderm on both sides of the dosal mesentery. The epithelial thickening appears at this site prior to 3 days of incubation (Ukeshima et al., 1987). The thickened splanchnic epithelium of the coelom, which initially incorporates the PGCs, becomes the superficial epithelium of the genital ridge in later stages. This thickened portion at stage 16 is thought to be the definitive gonadal anlagen (Ukeshima et al., 1987).

Rodemer et al. (1986) suggested that the intermediate mesoderm from somites 16 to 23 develops into the mesonephros. The mesonephros begins its participation in gonadal soma formation between day 6 and 7, the time of sexual differentiation (Rodemer-Lenz, 1989). The information about the origin of the gonadal soma in sexually indifferent gonads is variable. Contradicting reports consider the gonadal soma to originate from areas such as the coelomic epithelium, the mesonephric blastema, and differentiated mesonephros (Cited from Rodemer-Lenz, 1989). The PGCs appear to arrive at both the right and left gonad primordia in equal numbers prior to the 4th day of incubation, but following this period the distribution of the PGCs becomes asymmetrical. An active migration from the right to left gonad occurs in both sexes which results in a greater concentration of PGCs on the left side (Swift, 1914).

Sexual differentiation of the gonad occurs following the arrival of the germ cells. It is unclear whether the arriving PGCs contribute to the differentiation or products of the gonadal differentiation process attracts PGCs. Germ cell multiplication occurs at the next stage. The proliferation stage of the germ cell in the female embryos takes place between the 8th and 11th day of incubation whereas in males, between the 13th and 15th day of incubation (Romanoff, 1960). PGCs in males remain scattered randomly within a developing seminiferous tubule until day 13 of incubation (stage 39). After this period, they begin to differentiate into spermatogenia. In contrast to male embryos, the sexual differentiation of female embryos occurs in the left ovary at the 8th day of incubation (stage 34). The PGCs begin active meiotic division, forming oogonia at this stage. The right ovary generally regresses to a rudimentary vestige after hatching (Van Krey, 1990).

Manipulation of PGCs

Shuman (1981) reported that the isolated PGCs can be successfully transferred to the host embryo by utilizing the microinjection technique. Donor PGCs colonized in the host gonad were identified by chromosomal markers. In addition, it was demonstrated that PGCs did not influence the sexual differentiation of the gonad. Wentworth et al. (1989) produced the autogenic quail by injection of PGCs into the extraembryonic vein of stage 20 quail embryos. Also, gonadal chimeras in chicken were reported by Gonzales (1989). This was accomplished by transferring PGCs into the intravasculature of developing embryos. These chimeras were identified by chromosomal markers in the progeny of male germ cell recipients. Two out of six males tested positive.

PGCs with retroviral DNAs have been transferred to host embryo which is free from the retrovirus, by Simkiss et al. (1989). By infecting the PGCs with a defective retrovirus carrying the *E. coli lacZ* gene, and injecting these cells into the circulatory system of a host embryo, Simkiss et al. (1990) reported positive results by amplification of DNA with polymerase chain reaction (PCR) following Southern blot analysis. This was verified by examination of day 5 embryos, day 10 urogenital regions, and day 18 gonads.

Petitte et al. (1991) examined the fate of PGCs transferred from Dwarf White Leghorn to Barred Plymouth Rock (BPR) embryo to form functional gametes. Hatched chicks were raised until sexual maturity and testcross were carried with BPR. The chimeras were assessed with feather color as a phenotypic marker. But they did not obtain the chimera chicks. Tajima et al. (1993) reported that the germline of PGC recipient chickens was consisted of 2 distinct populations of germ cells, suggesting the success of a germline chimera production in chickens by transferring PGCs.

PGCs were transfected *in vitro* and expressed

the exogenous plasmid DNA, indicating that PGCs are a possible vector for direct gene transfer into the germ line (Han et al., 1993, 1994). More recently, PGCs were obtained from germinal crescent or blood vessel (dorsal aorta) and transfected with defective retroviruses carrying the *neo* and *lacZ* genes. These transfected PGCs were microinjected into recipient embryos to form chimeras. The produced chimeras were grew to sexual maturity and produced offspring. Analysis of Southern blot of the DNA from the chimeric bird sperm and the offspring blood was carried out. As a result, both the chimeric sperm and transgenic offspring blood contain the foreign DNA (Vick, 1993). This is the first example of the direct use of PGCs to produce transgenic offspring by retroviral vector.

Culture of PGCs

During the past decade a number of studies have increased our knowledge of the origin, migration and differentiation of vertebrate and avian PGCs (Meyer, 1960, 1964; Pardanaud et al., 1987a, 1987b; Urven et al., 1988; Didier et al., 1990). However, we know little of the biochemical characteristics of PGCs and the changes that may occur in energy metabolism, protein synthesis or nucleic acid synthesis during the critical period of their early development. For such studies and genetic manipulation of chicken germline, *in vitro* culture of PGCs would clearly be very useful.

Recently, Cherny et al. (1994) reported that bovine PGCs was cultured for 7 days onto a feeder layer of bovine embryonic fibroblasts obtained from a conceptus of equivalent gestational age, mitotically inactivated using mitomycin C and cultured in conventional ES medium. The PGC-derived cells were labelled with FITC and injected into early bovine IVF/IVM blastocysts (Stokes et al., 1994). Incorporation of the labelled cells (5-7 cells per embryo) into the inner cell mass of the developing blastocysts was observed using a fluorescence microscope and proliferation was observed microscopically. They are presently in the process of transferring bovine embryos injected with PGC derived cells into recipient cows and testing resulting fetuses for evidence of chimerism.

Tsai et al. (1992) examined long-term culture of chick PGCs isolated from the blastodisc and germinal crescent. They demonstrated that PGCs obtained from the blastodisc were able to proliferate or differentiate *in vitro*, but the PGCs from the germinal crescent did not replicate, suggesting that chicken PGC could be cultured *in vitro*.

Discussion

A number of studies have increased our knowledge of the origin, migration and differentiation of vertebrate PGCs. Although there has been considerable study of PGC cycles from early embryos to inclusion in the gonad, it is not altogether clear when there is an advantageous stage for coincidental presence of vector and PGC replication. The germinal crescent, blood and embryonic gonads are optimal sources of PGCs, but the low number of available PGC makes isolation difficult. A possible way to overcome this condition is to develop an efficient technique which can separate PGCs from other embryonic cells. Use of delayed plating down of PGCs (Shuman, 1981), Percoll density gradient, Fluorescent activated cell sorter (Wentworth et al., 1989), antibody directed flow sorting (McCarrey et al., 1987) and Ficoll density centrifugation collection (Yasuda et al., 1992) are various methods that can be used to separate PGCs. Although development of these methods has improved the separation process, the amount of pure PGCs produced for gene transfer is still low.

Another possibility for separation of PGCs is to develop a culture system to stimulate replication of PGCs *in vitro*. The genetically manipulated PGCs could be transplanted into the germinal crescent and blood vessel. Embryonic stem cells can also provide the source for gene transfer by making germline chimeras. The establishment of PGC line or embryonic stem cell line will greatly accelerate gene transfer in chicken. Embryonic stem cell lines and PGC lines in culture could be manipulated genetically according to a designed plan and produce animals of a desired genotype.

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