Substantial Evidence to Localize the Developmental Origin of Primordial Germ Cells in the Chicken

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Abstract The developmental origin of the primordial germ cells (pgcs) in avian embryos was substantially localized at the center of area pellucida. A cell cluster consisting of about 700 cells was isolated from : 1) the center of area pellucida, 2) the outer of area pellucida and 3) the area opaca of the stage X⁵ blastoderm. The isolated cell cluster was dissociated and about 500 cells were micro-injected into the recipient stage X blastoderm, from which a cell cluster of about 700 cells at the center of area pellucida were removed. These chimeric embryos were cultured until stage 15⁸. When the donor cells from area 2) or 3) were microinjected into the recipient, the mean number of pgcs per 1 μ l of blood decreased significantly to 7 or 11 ($P < 0.05$), respectively, as compared to intact embryos with 62. When the cells were replenished with the donor cells from area 1), no reduction in the pgcs number was observed. The replenishment of the recipients' cells by the cells from area 2) or 3) could not restore the original number of pgc. Therefore, it was concluded that the pgcs originated in the central part of the area pellucida and that there were few pgcs at the outer of area pellucida or area opaca.

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Determination of the origin of primordial germ cells (pgcs) and its use as the vector of foreign genes can lead to the development of transgenic poultry^{4,9,11,15-17,21}. Extensive efforts have been made to determine the origin of the avian pgcs. The pgcs were localized in the epiblast of stage X⁵ blastoderm by immuno-staining using epitopes such as EMA-1, SSEA-1 and QH-1^{7,13,18,25}. Despite extensive efforts in these studies, the localization of pgcs within the blastoderm has not been determined precisely. There is little evidence to support that putative pgcs which are detected by the immuno-staining could be differentiated into the gametes, *i.e.*, spermatozoa in males and ova in females^{3,6}.

To determine the origin of pgcs more directly, deletion and chimeric studies were performed^{10,22}. When a cell cluster, of about 700 cells, was removed from the center of area pellucida at the stage X blastoderm and cultured *ex vivo*, the mean number of pgcs in the circulating blood significantly decreased in the embryo at stage 15⁸, as compared to intact embryos. The decrease of pgc numbers in the embryos at stage 15 recovered in chimeras produced by an injection of the donor blastoderm into a recipient blastoderm from which cells at the center of area pellucida were removed. It was concluded that the pgcs originated at the central part of area pellucida of stage X⁵ blastoderms. However, the region of

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blastoderm from which donor cells were obtained was not clearly defined in the last studies.

In the present study, the donor cells from a clearly defined region of the blastoderm : 1) the center of area pellucida, 2) the outer of area pellucida and 3) the area opaca, were microinjected into the recipient blastoderm from which the cells at the center of area pellucida was removed. The number of pgcs in the circulating blood in the stage 15⁸⁾ embryo was counted and compared to intact embryos. This approach would substantially clarify the developmental origin of the pgcs in the blastoderm.

Materials and Methods

Chimeric embryo

The donor and recipient embryos were from White Leghorn chickens. The donor embryos, stage X⁵⁾ blastoderm in freshly laid and unincubated eggs, were separated from the egg yolk and washed with phosphate-buffered saline (PBS). A cell cluster, consisting of about 700 cells, was isolated from a clearly defined region of the blastoderm : 1) the center of area pellucida, 2) the outer of area pellucida and 3) the area opaca. The cluster was dissociated with trypsin and dispersed in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% chicken serum²⁾. The recipients were fertilized eggs containing a stage X blastoderm. According to the method of Kagami *et al.*¹⁰⁾, a cell cluster containing approximately 700 cells was aspirated from the center of the area pellucida. Initially prepared donor cells of about 500 were microinjected into the treated recipients. The manipulated embryos were ex vivo cultured^{14,19)} until stage 15⁸⁾.

pgc number in circulating blood

Blood samples of about 5 μ l were collected from the stage 15⁸⁾ embryos. Each blood sample was diluted to 200 μ l with PBS and centrifuged for 5 minutes at 1,000 g to wash the cells. The supernatant was removed and the cell pellets were resuspended in 200 μ l of PBS. The cells were fixed with 3.7% formaldehyde in PBS. The fixed samples of 7 μ l were placed on a glass cell culture slide and covered with 0.05% celloidin dissolved in ethanol. The fixed cells were then stained with periodic acid-Shiff (PAS)¹²⁾ to iden-

tify the pgcs and the number of pgcs (PAS-positive cells) were counted under a light microscope²⁴⁾. The *t*-test was used to identify statistically significant differences in the mean number of pgc between the derivation of donor.

Results

When the blood samples from stage 15⁸⁾ embryos were stained by the PAS staining, only the pgcs were stained dark red, whilst the other blood cells were not stained at all. In the intact stage 15 embryos (Table 1), the number of pgcs per 1 μ l of embryonic blood was 62. When the 500 donor cells, derived from the area 2) or 3), were microinjected into the treated recipient, the mean number of circulating pgcs per 1 μ l of blood significantly decreased to 7 or 11 ($P < 0.05$) in the embryo at stage 15 as compared to the intact embryos with 62. When the cells were replenished with the 500 donor cells derived from area 1), the number of pgcs per 1 μ l of embryonic blood was 60 which was not significantly different from the intact embryos. The replenishment of recipient cells by use of the donor cells from area 1) could restore the number of pgc at the stage 15, but could not restore the number with donors from area 2) or 3).

Discussion

Recent studies on the production of germline chimeras indicate that the blastoderm, as well as isolated

Table 1. Number of PGCs in chimeric embryos at stage 15 (Hamburger and Hamilton, 1951)

Derivation of donor	No. of embryos counted	No of PGCs in the embryos ¹⁾
Control	15	62.3 $\pm$ 9.5 ^a
CAP ²⁾	25	59.7 $\pm$ 7.5 ^a
OAP ³⁾	23	7.4 $\pm$ 6.2 ^b
AO ⁴⁾	27	11.1 $\pm$ 7.1 ^b

¹⁾ The number of PGCs $\pm$ standard deviation per μ l of embryonic blood at stage 15.

²⁾ CAP, Center of Area Pellucida ;

³⁾ OAP, Outer of Area Pellucida ;

⁴⁾ AO, Area Opaca.

^{a, b} Values without common superscripts are significantly different ($P < 0.05$).

pgcs, could be used to transfer foreign DNA into the avian germline^{1, 15, 20, 23, 26}. Determination of the origin of pgcs in the developing embryo can lead to the development of transgenic poultry^{17, 22}. By means of immuno-staining using epitopes such as EMA-1, SSEA-1 and QH-1, extensive efforts have been made in determining the origin of the avian pgcs^{7, 13, 18, 25}. The pgcs were localized in the epiblast of stage X blastoderms. However, the localization of pgcs within the blastoderm has not been determined precisely, with little evidence to support that putative pgcs which are detected by immuno-staining could be differentiated into the gametes, *i.e.*, spermatozoa in males and ova in females^{3, 6}.

Kagami *et al.*¹⁰ first determined the origin of avian pgcs directly, both *in vivo* and *in vitro*, with deletion and chimeric studies. It was found that the pgcs originated at the central part of the area pellucida of stage X blastoderms. However, the region of blastoderm from which donor cells were obtained was not clearly defined in the last study. In the present study, the donor cells from a clearly defined region of the blastoderm : 1) the center of area pellucida, 2) the outer of area pellucida and 3) the area opaca, was microinjected into the recipient blastoderms from which cells at the center of the area pellucida were removed. When the donor cells from area 2) or 3) were microinjected into the treated recipient, the mean number of circulating pgcs per 1 μ l of blood significantly decreased in the embryo at stage 15, as compared to intact embryos. When the cells were replenished with donor cells derived from area 1), no reduction in the pgcs number was observed. The replenishment of the recipient cells by use of the cells from area 2) or 3) could not restore the number of pgc.

These results clarify conclusively that the pgcs originate at the central part of the area pellucida and that there are few pgcs at the outer of area pellucida or of area opaca.

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