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# Suitable Stages for Isolation and Culture PGCs from Chicken Embryos

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**Abstract:** The aim of this study was to evaluate the efficiency of isolation and culture of PGCs from various tissues of chicken embryos at specific developmental stages including: the circulating blood of stage 14 embryos (hatched for 48-52hrs), the genital ridge of stage 19 embryos (hatched for 68-72hrs) and the gonad of stage 28 embryos (hatched for 128-132hrs). Ficoll density-gradient centrifugation is a standard method for the purification of PGCs from fetal blood. The genital ridge and gonadal tissue contain more PGCs in total but must first be digested with trypsin-EDTA to give a single cell suspension containing a mixture of PGCs and other contaminating cell types. In these experiments, we cultured PGCs from the genital ridge and from gonadal tissue before and after Ficoll density-gradient purification. In all cases, PGCs were subsequently cultured in TCM-199 medium supplemented with 10% fetal calf serum. The results demonstrated that trypsin-EDTA alone of the genital ridge of stage 19 embryos yielded a total of 2.7×104 per embryo of which 89.5% were viable. After Ficoll density-gradient purification of these cells the yield was 1.8×104 of which 87.5% were viable. Processing of the gonadal tissue of stage 28 embryos yielded a total of 3.1×104 PGCs per embryo of which 90.0% were viable. It was clear that the PGC yield with trypsin-EDTA alone was higher ($P<0.01$) than the yield of the full procedure which included the Ficoll density-gradient purification step. The results of PGC culture from the three developmental stages indicated that the survival time was longest (80-88 hours) for PGCs obtained from stage 19 embryos. At this stage, a large number of PGCs had accumulated in the genital ridge which facilitated the isolation procedure. These results suggest that the highest yield of PGCs per embryo can be achieved by trypsin-EDTA treatment of genital ridge tissue from stage 19 chicken embryos.

**Key words:** Chicken embryo, primordial germ cells (PGCs), isolation, culture

## Introduction

The current state of development of the technology for production of transgenic chickens has stimulated interest in primordial germ cells (PGCs) as a vehicle for the introduction of new genetic material. PGCs are the first identifiable progenitors of gametes and they display a unique migration pathway during early development of chickens (Eyal-Giladi *et al*., 1981). The authors hypothesized that chicken PGCs could be isolated, cultured and introduced into the gonadal environment where they produced functional gametes which could participate in normal fertilization. The transfer of exogenous genes via such PGCs into germ cells would be an efficient method for the production of transgenic chickens (Wentworth, 1989). Most research on PGCs has been based on their isolation from fetal blood by Ficoll density-gradient centrifugation. However, this protocol has not yet been tested for use in the isolation of PGCs which have migrated to the gonad or the genital ridge at stages 19 and 28 of embryonic development. The present study was aimed at finding suitable methods for the isolation and culture of PGCs from all three stages of chicken embryos as a first step towards production of transgenic chickens.

## Materials and Methods

**Experimental stocks:** White Leghorn chickens were obtained from the Jiangsu Institute of Poultry Science, China.

**Preparation of fertilized eggs:** Fertilized eggs were collected 2 to 7 days after artificial insemination, then they were incubated at 38°C and 70% relative humidity for 48-52 hrs (to produce stage 14 embryos), 68-72 hrs (to produce stage 19 embryos), or 128-132 hrs (to produce stage 28 embryos). Developmental stages of the chicken embryos were determined according to the system of Hamburger and Hamilton (1951) and Li (2003).

**Isolation and primary culture of PGCs from three developmental stages:** The blood from stage 14, the genital ridges from stage 19 and the gonads from stage 28 were collected by suspension in TCM-199 medium containing 10% fetal calf serum. PGC collection was performed using the following methods:

For isolation of PGCs at stage 14 (incubated for 48-52hrs), blood was collected as described by Li and Chen (2001). An aliquot of 100 μl of this blood was

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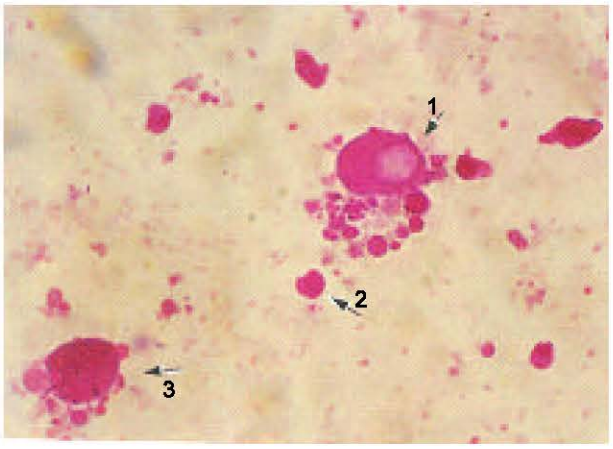


Fig. 1: PGCs obtained from getal blood and stained by PAS (X1000)

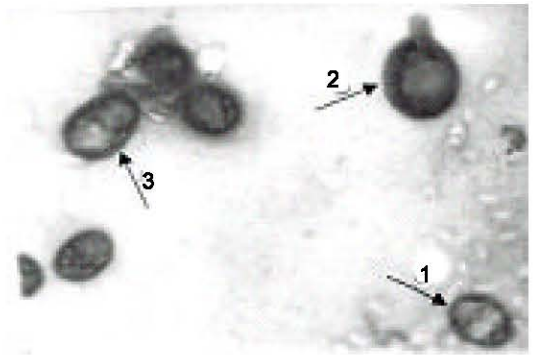


Fig. 2: PGCs derived form stage 28 chicken embryos and stained with PAS (indicated with arrows). Some PGCs were oval and some had pseudopodia

dispersed in 0.9 ml of TCM-199 medium at room temperature. The TCM-199 medium was supplemented with 10% FCS to prevent the PGCs from adhering to and aggregating with blood cells. Blood samples were spun at 200g for 5 min at room temperature. The cell pellet was then resuspended in 0.1 ml of TCM-199 mixed with 0.9 ml of 16% Ficoll in a microfuge tube (1.5ml capacity). This preparation was then overlaid with 0.2 ml of 6.3% Ficoll. After centrifugation at 600g for 30 min, 0.3-0.6 ml of the PGC-rich fraction, located at the interface between the 16% and 6.3% Ficoll solutions, was drawn into a micropipette. This was washed in TCM-199 containing 10% FCS and centrifuged at 200g for 5 min. The PGCs were resuspended in a small volume of TCM-199 medium and residual Ficoll was removed by two additional washing steps with centrifugation at 200 for 5 min, and finally placed in the center of a tissue culture dish at 38°C with saturated humidity and atmosphere of 5% $CO_2$ in air.

**Preparation of the chicken genital ridge:** embryos collected from stage 19 embryos (incubated for 70-72 hrs) were rinsed with $Ca^{2+}$- and $Mg^{2+}$- free phosphate-buffered saline (PBS) to remove the yolk. The abdomen of the embryo was carefully dissected under a stereoscope and the genital ridge was removed with sharp forceps and transferred to a culture dish.
Preparation of the chicken gonad: chicken embryos were collected from stage 28 embryos (incubated for 130-132 hrs). The abdomen of the embryo was carefully dissected under a stereoscope and the gonad, located between the mesonephros and the dorsal cellular kidney, was carefully removed.

**Trypsin-EDTA isolation procedure:** For trypsin-EDTA isolation of PGCs from stage 19 and 28 embryos, genital ridges or gonads were placed in PBS containing 0.25% (w/v) trypsin and 0.53 mM EDTA at room temperature for 5 min. Then, the cells were dissociated by gentle pipetting. After centrifugation at 200g for 8 min, approximately 1×104 isolated cells were cultured at 38°C with saturated humidity and 5% $CO_2$ in air, with daily medium changes.
Ficoll density-gradient purification: Ficoll density-gradient centrifugation to isolate PGCs from chicken embryos at stage 19 and 28: the procedure of collecting PGCs from genital ridges and gonads was similar to the method described above for embryonic blood PGCs at stage 14.

**Calculation of PGC content:** PGCs% = (The viable PGCs / The total number of cells) × 100%

**Trypan blue staining:** The viability of isolated PGCs was evaluated using 0.4% Trypan blue staining for 2 min, unstained cells were viable cells. The viable PGCs were counted on a counting plate.

**PAS (Periodic Acid-Schiff) staining:** The PGCs were fixed to the tissue culture dish in 95% alcohol for 10 min and rinsed with 1× PBS twice. For PAS–staining, the PGCs were immersed in periodic acid solution for 10 min at room temperature. After washing with 1×PBS, the fixed PGCs were immersed in Schiff's solution for 30 min at room temperature. Then they were washed with 1× PBS twice. The PAS stained PGCs (Mayer, 1964) were observed though inverted microscope.

**Statistical analysis:** Data were analyzed with Student's T-test and the LSD test using the statistical program, SPSS 11.0.

## Results and Discussion

**The morphology of PGCs:** Under the phase contrast microscope, the morphological characterization of freshly isolated PGCs, either from blood or from genital ridge and gonad, showed a large round cell with

Table 1: PGC yield after trypsin-EDTA digestion and after Ficoll density gradient purification.
The number of cells per embryo is the combined mean for both stage 19 and stage 28 embryos.

| stages | Protocol | Number of cells per embryo | Viable somatic cells (%) | No. of PGCs released per embryo | Viable PGCs (%) | PGCs/ml in cell suspension |
|---|---|---|---|---|---|---|
| 19 stage | Trypsin-EDTA treatment | $2.7x10^4±0.12x10^{4*}$ [150] | 92.4±3.1 | 72.0±5.1* [150] | 89.5±3.6 | 3.2±0.5 |
| | Ficoll density centrifugation | $1.8x10^4±0.14x10^4$ [150] | 90.9±3.8 | 40.0±4.6 [150] | 87.5±3.2 | 7.2±0.4* |
| 28 stage | Trypsin-EDTA treatment | $3.1x10^4±0.30x10^{4*}$ [150] | 90.0±3.2 | 78.0±5.1* [150] | 90.0±3.2 | 2.6±0.5* |
| | Ficoll density centrifugation | $1.9x10^4±0.22x10^4$ [150] | 90.0±3.7 | 48.0±6.0* [150] | 85.0±3.3 | 6.7±0.3* |

Notes: Numbers in bracket indicate the total number of embryos on which the data are based.
The number of viable cells was determined by trypan blue staining. PGCs were identified by morphological criteria and by the PAS reaction. * Means the difference is significant at the 0.01 level ($p<0.01$).

Table 2: The number of PGCs isolated at different stages of chicken embryos development

| Stage | Repeats | The number of viable embryos | PGC content before isolation (%) | After isolation | |
|---|---|---|---|---|---|
| | | | | The number of PGCs | PGCs (%) |
| 14 | 20 | 25 | 0.011±0.0011** | 526±53.0 | 3.3±0.3* |
| 19 | 25 | 20 | 0.0017±0.0005* | 1120±130.0** | 3.1±0.2* |
| 28 | 20 | 15 | 0.0013±0.0003 | 943±105.0* | 2.4±0.2 |

*Indicates that the difference is significant at the 0.05 level ($p<0.05$); ** Indicates that the difference is very significant at the 0.01 level ($p<0.01$).

diameter from 12 to 18 µm. The cytoplasm contained abundant glycogen deposits which are specifically stained a brilliant magenta by Periodic Acid-Schiff (PAS) reaction. The nucleus was usually acentric and about 9µm in diameter and not stained by the PAS reaction (Fig. 1).

During embryonic development, the glycogen deposits in the cytoplasm of the PGC changed from an even distribution to a polar distribution, and the glycogen content of the yolk granule and the fat droplets decreased. Glycogen was seen in the cytoplasm of embryos up to stage 30.

PGCs obtained by the trypsin-EDTA treatment were contaminated with other somatic cell types which could be removed by Ficoll density-gradient centrifugation. PGCs derived from the chicken gonad differed in morphology: some were oval and others exhibited pseudopodia (Fig. 2).

Fig. 2 PGCs derived from stage 28 chicken embryos and stained with PAS (indicated with arrows). Some PGCs were oval and some had pseudopodia.

**Comparison of PGC yield:** The mean PGC yield after Trypsin-EDTA treatment and Ficoll density-gradient purification for embryos at stages 19 and 28 is shown in Table 1.

Table 1 shows the total number of cells obtained per embryo, the total number of PGCs and the number of PGCs obtained per embryo. It is clear that there is a significant amount of loss of PGCs caused by the Ficoll density-gradient centrifugation ($p<0.01$) however there was no significant difference in the percentage of viable PGCs ($P>0.05$).

In order to collect a large number of viable PGCs at different developmental stages from chicken embryos, we investigated a protocol which consisted of trypsin-EDTA treatment followed by Ficoll density-gradient purification. trypsin-EDTA treatment is necessary for isolation of PGCs at stages 19 and 28 because of the necessity to break up these solid tissues. It is unavoidable that during Ficoll purification, repeated centrifugation and rinsing steps resulted in some loss of cells. Furthermore, in the Ficoll protocol, only about 60% of PGCs are accumulated in the extraction layer of the Ficoll gradient. Cell viability (as defined by Trypan Blue staining) and survival time was reduced by Ficoll purification. Following cultivation for 72 hours, the percentage of viable PGCs after Ficoll purification was only 10.35% (711/6900), compared to 29.7% (2762/9300) after trypsin-EDTA digestion alone. After cultivation for 84 hours, the percentage of viable PGCs was 20.5% (1907/9300) with cells which had only experienced the trypsin-EDTA protocol while the number of PGCs which had subsequently been purified on a Ficoll gradient was reduced to 6.2% (428/6900). The decrease in the number of viable PGCs might be due to

Table 3: Comparison of survival time of PGCs at different stages

| Stages | Number of Repeats | % Viable PGCs after increasing time in culture | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Freshy isolated | 24hr later | 48hr later | 60hr later | 72hr later | 80hr later | 88hr later |
| 14 | 20 | 85.6±4.9[a] | 40.7±2.3 | 23.6±2.8 | 9.4±3.4[a] | / | / | / |
| 19 | 25 | 85.7±5.6[ab] | 50.4±2.7 | 33.5±2.1 | 21.3±1.7[b] | 10.3±0.7 | 3.9±0.3 | / |
| 28 | 20 | 80.5±4.4[c] | 45.5±3.1 | 31.7±1.3 | 19.3±1.2[c] | 7.9±0.6 | / | / |

*Indicates the difference is significant at the 0.05 level ($p<0.05$).

the lack of growth factors. For example, Coucouvanis *et al*. (1993) suggested that lack of growth factors leads to cell death. IGF and EGF are known to be important for PGC survival and differentiation(Il-Kuk Chang, 1992, Jiang *et al*., 1997) . We observed that when the number of somatic cells was limited, there was an increased level of PGC death. During culture, somatic cells tended to age and die gradually, perhaps resulting in an increasing deficiency in secreted growth factors. Our results revealed that PGCs derived from all three stages only survived for 3-4 days in medium containing 10% FCS but not supplemented with additional growth factors.

**Comparison of the number of PGCs obtained from three different development stages:** PGCs from stage 19 and 28 embryos were prepared by trypsin-EDTA treatment followed by Ficoll density-gradient purification. PGCs from stage 14 embryos were prepared by standard Ficoll density-gradient purification of fetal blood. The results are shown in Table 2.

In Table 2, the number of PGCs counted under a microscope following PAS staining showed significant differences among stage 14, 19 and 28 embryos ($p<0.01$). Stage 14 embryos had more PGCs as a percentage of total cells than the other two stages. The number of PGCs before isolation from stage 19 was significantly higher than that of the stage 28 ($p<0.05$). The number of PGCs after isolation at stage 19 was significantly higher than that of the stages 14 and 28 ($p<0.01$).

**Results of primary PGC culture:** PGC suspensions obtained from stage 19 and 28 embryos were transferred to tissue culture flasks and cultured in TCM-199 containing 10% fetal calf serum with the pH adjusted with $NaHCO_3$ to 7.2. After one hour in culture, somatic cells attached to the surface of the culture flask and formed a fibroblast like layer, while PGCs remained suspended in the medium. After culturing for 24hrs the PGCs tended to aggregate into small clumps of 10 cells which were attached to the fibroblast feeder layer. After 72 hours in culture, the rate of viable PGCs among the surviving cells was 29.7% after trypsin-EDTA isolation and 10.3% after Ficoll gradient purification. After 84 hours, the percentages of viable PGCs were 20.5% and 6.2%, respectively. In the following 72 hours, the PGCs began gradually to shrink, the color of cytoplasm became darker and the characteristic profile was lost.

Il-KUK Chang (1992) and Han and Zhou (1996) reported purification efficiency of 86% and 50% after Ficoll density-gradient purification from fetal blood. These values are higher than what we observed. The main reason could be that we adopted a different method for calculation of the number of PGCs. In our study, the total number of PGCs plus other somatic cells were used as a denominator, while the other authors used the total number of PGCs and the number of yolk granules as a denominator. This would explain the relatively larger percentage of PGCs that they reported. Another explanation could be the effect of using different chicken embryos and methods of obtaining blood. In our study, the proportion of PGCs in blood at stage 14 was 3.3% and our protocol resulted in 21 PGCs per embryo. However, there have been reports of 30-60 PGCs per embryo from blood at stage 14 (Il-Kuk Chang, 1992; Han and Zhou, 1996; Li and Chen, 2001). The process of producing a chimaeric chicken requires approximately 200 PGCs (Natio *et al*., 1994) and the observed yield of only 21 PGCs per embryo obtained by direct purification from blood is not yet sufficient to be practical for this application.

Our results demonstrated that collection of PGCs from stage 19 embryos gave a dramatic improvement in yield. There are more viable PGCs at this stage and the purification procedure is easier. Although PGCs can be purified from stage 28 embryos, we observed that survival time of these cells was shorter, about 70-80 hours. Since PGCs begin to differentiate at stage 30 (Leichthammer *et al*., 1990), one would expect that the totipotency of PGCs would already have decreased by stage 28. Based on this assumption, we suggest that collection of PGCs from the genital ridge at stage 19 is preferable.

**The results of PGC culture at different stages:** Under the phase contrast microscope, the PGCs isolated from all three stages tended to aggregate into small clumps of cells after 24 hours in culture as described above. There were 4-5 aggregates per dish (3-5 embryos pooled for each dish) following isolation from the embryonic blood at stage 14. When PGCs were derived

from the genital ridge and gonad, there were typically 8-9 aggregates per dish (3-5 embryos pooled for each dish). The number of cells making up a colony and the number of colonies derived from embryonic blood at stage 14 were both less than what was obtained from genital ridge or gonads of stage 19 and 28 embryos. Our results revealed that most PGC colonies appeared within the first 24 hours of cultivation, in following days the number of colonies diminished until none could be found. The survival time of PGCs cultured at different stages is shown in Table 3.

Table 3 shows that the percentage of viable PGCs derived from chicken embryos at these three stages decreased dramatically after about 24 hours of culture. After a cultivation period of about 60 hours, the percentage of viable PGCs at stage 19 and 28 was significantly higher than that of stage 14 ($p<0.05$). Table 3 also shows that the survival time of PGCs at stage 14 was shortest at 60-70 hours, while the survival time of PGCs at stage 19 was the longest, 80-88 hours. The survival time of stage 28 PGCs was intermediate, 72-80hrs.

Table 3 shows that the percentage of viable PGCs derived from chicken embryos at these three stages decreased dramatically after about 24 hours of culture. This may be due to the fact that the PGCs were separated from embryo and had entered a new environment and that the less viable PGCs died off rather quickly. The fact that the medium used contained only 10% FCS without growth factor supplementation and lacked supplementation with critical nutrients might explain why the cells began to die. After a cultivation period of about 60 hours, the percentage of viable PGCs at stage 19 and 28 was significantly higher than that of stage 14 ($p<0.05$). The reason may be that many stromal cells at stage 19 and 28 were obtained with the PGCs during purification, these stromal cells may have secreted growth factors and prolonged the survival time of PGCs from the more advanced stages. Table 3 also shows that the survival time of PGCs at stage 14 was shortest at 60-70 hours, while the survival time of PGCs at stage 19 was the longest, 80-88 hours. The survival time of stage 28 PGCs was intermediate, 72-80hrs.

The number of cells making up a colony and the number of colonies derived from embryonic blood at stage 14 were both less than what was obtained from genital ridge or gonads of stage 19 and 28 embryos. This may be due to the fact that the absolute number of PGCs was higher in gonadal tissue than in blood. The destination of migrating PGCs in these embryos is not blood but the gonads where PGCs began to differentiate and proliferate. The increased size of the aggregates which form from later stage embryos may reflect the fact that during the phase in which PGCs migrate through the embryo as single cells they probably do not express factors which would cause them to adhere to each other or to other cells but once they populate the gonadal tissue this situation may change and adhesion could be expected to increase. Our results revealed that most PGC colonies appeared within the first 24 hours of cultivation, in following days the number of colonies diminished until none could be found. The reason for this may be that in culture, the PGCs cease to produce aggregation factors and revert to the phenotype of blood derived PGCs.

The percentage of viable PGCs was generally higher when they were freshly isolated, whereas after culturing for 24 hours, the percentage of viable PGCs decreased dramatically. The reason for this might be the damage to the PGCs during the process of isolation and culture, resulting in cell death. It has been reported that PGCs growing in vitro on a preconditioned feeder layer matrix could survive more than 5 days (Il-KUK Chang, 1992). In the present study, Ficoll density-gradient centrifugation increased the proportions of PGCs to other cell types in preparations from embryos of stages 19 and 28 from 0.0013% to 2.4% and from 0.0017% to 3.1%, respectively. After purification from these two stages, a large number of stromal cells remained mingled with PGCs and these may have secreted growth factors which contributed to survival of the PGCs. The lack of such cells in the blood at stage 14 might explain the shorter survival time of PGCs isolated from blood as compared to the survival time of PGCs derived from the genital ridge or gonad. The difference in survival time between stage 19 and stage 28 may be due to the fact that stage 19 PGCs had only recently arrived in the gonad and had no close connections with gonad matrix cells, while the stage 28 PGCs had resided for a period in the gonad and had formed close connections with gonad matrix cells (Li *et al*., 2004). During PGC isolation, damage to the PGCs was unavoidable and causing shortened survival time for the PGCs from stage 28 as opposed to stage 19 embryos under the same culture conditions. This also suggests that isolation and culture of PGCs at stage 19 from chicken embryo will be the basis for the most suitable protocol.

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