Soluble Factors and the Emergence of Chick Primordial Germ Cells In Vitro

L. Karagenç and J. N. Petitte

Department of Poultry Science, North Carolina State University, Raleigh, North Carolina 27695-7608

ABSTRACT Previous observations obtained from a culture of blastodermal cells on a mouse fibroblast feeder layer (STO) suggested that STO cells provide a factor or factors that facilitate development of avian primordial germ cells (PGC) from dispersed embryo cells. The purpose of the current study was to test the hypothesis that soluble factors produced by STO cells are responsible, at least in part, in supporting the development of PGC in culture. To this end, blastodermal cells cultured on feeder layers of CV-1 cells yielded a small number of PGC. When blastodermal cells were cultured on STO cells, a marked increase in PGC was observed. The addition of STO cell-conditioned medium (STO-CM) to blastodermal cells cultured on gelatin-coated plastic and on feeder layers of CV-1 cells resulted in a significant increase in the number of PGC, indicating that soluble factors produced by STO cells can enhance the development of chicken PGC in culture. Supplementation of blastodermal cells with SCF (100 ng/mL) or CNTF (2 ng/mL) or with CNTF and SCF together resulted in a significant increase in the number of PGC after 48 h of culture on feeder layers of CV-1 cells. However, addition of bFGF (100 ng/mL) did not increase PGC. We concluded from these observations that the culture of blastodermal cells on feeder layers of STO and CV-1 cells can be used as a useful biological system in examining the regulatory factors that govern the ontogeny of the germ cell lineage in the avian embryo.

(Key words: chick, primordial germ cells, ciliary neurotrophic factor, stem cell factor, basic fibroblast growth factor)

INTRODUCTION

Morphological aspects of the biology of chicken primordial germ cells (PGC) from their appearance at preprimitive streak stages of development until their settlement in the gonadal ridge is relatively well documented (Nieuwkoop and Sutasurya, 1979; Ginsburg, 1997; Petitte and Karagenç, 1996). Yet, the cellular and molecular mechanisms governing the segregation of avian PGC are currently unknown. However, experimental evidence obtained to date suggests that segregation and further development of PGC is an epigenetic event and requires local cell-cell interactions (Ginsburg, 1997; Karagenç et al., 1996; Karagenç, 1998). For example, when individual stage IX to XIV embryos are dispersed and cultured on glass cover slips, 25 to 45 PGC per embryo are detected only with stages X to XIV but not with stage IX blastoderms (Karagenç et al., 1996). However, when the culture of blastodermal cells obtained from stages IX to XIII is carried out on feeder layers of the mouse fibroblast cell line STO, PGC can be identified in cultures initiated not only with stage X to XIII embryos as expected but also with stage IX embryos. Furthermore, PGC per embryo obtained on STO feeder layers were much higher than those observed in cultures without feeder layers and was comparable to that found in the germinal crescent region (Karagenç et al., 1996).

These observations suggested that STO cells facilitate development of avian PGC from dispersed embryo cells that do not have the capacity to spontaneously yield PGC under in vitro conditions. It is most likely that STO cells provide a factor or factors necessary to implement developmental events leading to the emergence of PGC in culture.

The purpose of the current study was twofold: 1) to test the hypothesis that soluble factors that are produced by STO cells are responsible, at least in part, in the execution of such a developmental program, and 2) to examine the effect of basic fibroblast growth factor (bFGF), stem cell factor (SCF) and ciliary neurotrophic factor (CNTF) in the development of PGC in culture. To this end, blastodermal cells obtained from dispersed stage X embryos were cul-

Received for publication December 14, 1998.
Accepted for publication September 30, 1999.
*Salaries and research support provided by state and federal funds appropriated to the North Carolina Agricultural Research Service, North Carolina State University. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service, nor criticism of similar products not mentioned.
*To whom correspondence should be addressed: J_PETITTE@NCSU.EDU.

Abbreviation Key: bFGF = basic fibroblast growth factor; CNTF = ciliary neurotrophic factor; DMEM = Dulbecco's Modified Essential Medium; FBS = fetal bovine serum; PGC = primordial germ cells; SCF = stem cell factor; SSEA-1 = stage-specific embryonic antigen-1; STO-CM = STO-conditioned media; LIF = leukemia inhibitory factor; LIFR = leukemia inhibitory factor receptor; OSM = oncostatin M.
tured on feeder layers of CV-1 cells, a mammalian cell line that does not support development of mouse PGC in culture (Dolci et al., 1991).

**MATERIALS AND METHODS**

**Preparation of Feeder Layers**

Feeder layers of STO and CV-1 cells were prepared as described by Cooke et al. (1993). Briefly, STO and CV-1 cells were cultured in Dulbecco’s Modified Essential Medium (DMEM), supplemented with antibiotics, 2 mM L-Glutamine, and 10% fetal bovine serum (DMEM-FBS). After reaching approximately 90% confluence, cells were incubated in 0.05% trypsin and 0.53 mM EDTA in PBS at 37 C for 3 min. Trypsin activity was neutralized by adding 8 mL DMEM-FBS to each flask, and a single-cell suspension was obtained by trituration. Cells were exposed to 5,000 Rads of gamma radiation from 60Co, centrifuged at 311 x g for 5 min, and resuspended in DMEM-FBS containing 10% DMSO. The same lot of irradiated frozen feeder cells was used in all experiments. Feeder layers of STO and CV-1 cells were prepared in 96-well-microtiter plates using 62,500 cells per well.

**Preparation and Culture of Blastodermal Cells**

Embryos were obtained from White Leghorn hens. The *area pellucidae* from stage X (Eyal-Giladi and Kochav, 1976) embryos were pooled in DMEM-FBS and were dissociated enzymatically using 0.0125% trypsin and 0.005% EDTA in PBS at 37 C for 10 min. The trypsin-EDTA solution was gently removed, and the cells were resuspended in 500 µL DMEM-FBS. The fragmented *area pellucidae* were dispersed further to obtain a single-cell suspension by gentle trituration using a 200-µL pipette tip. The cells were centrifuged at 311 x g for 5 min and were resuspended in fresh culture medium. The concentration of cells and the blastodermal cells obtained per area *pellucida* were determined using a hemocytometer. Five thousand cells were seeded into each well of 96-well microtiter plates.

The culture of blastodermal cells was carried out at 37 C and 5% CO2 for 48 h. At the end of the culture period, the cultures were fixed in 4% (wt/vol) paraformaldehyde (pH 7.2). The staining procedure was carried out using an avidin/biotin-conjugated-alkaline phosphatase system (Vectastatin ABC-AP kit), using anti-stage-specific embryonic antigen-1 (SSEA-1; MC-480) ascites fluid diluted 1:1,000 as the primary antibody. Cells expressing the antigen were detected using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (BCIP) as the substrate in the presence of 3.75 mM levamisol. The PGC were identified on the basis of morphology and SSEA-1 expression. The number of PGC obtained per well was used to calculate the number of PGC per area *pellucida*, which was referred to as the number of PGC per embryo equivalent.

**Preparation of STO Cell-Conditioned Media**

The STO cells were cultured in 75-mL tissue culture flasks in 20 mL DMEM-FBS. When cells reached 95% confluence, the spent medium was aspirated, and cells were rinsed in PBS twice. Cells were incubated in 0.05% trypsin and 0.53 mM EDTA in PBS at 37 C for 3 min. Trypsin activity was neutralized by adding 8 mL DMEM-FBS to each flask, and a single cell suspension was obtained by trituration. The STO cell suspension (2.5 mL) was seeded into a new 75-mL flask containing 20 mL fresh DMEM-FBS. The STO cell-conditioned media (STO-CM) was prepared by growing STO cells at 37 C and 5% CO2 for 65 h. The medium was harvested once and was used after filtering through a 0.2-µm filter. The STO-CM prepared in this manner was kept at −70 C, and the same batch of the conditioned media was used in all experiments.

**Effect of Basic Fibroblast Growth Factor, Stem Cell Factor, and Ciliary Neurotrophic Factor on the Development of Primordial Germ Cells in Culture**

The isolation and culture of blastodermal cells in DMEM-FBS on feeder layers of CV-1 cells were performed as described above. Bovine bFGF and recombinant rat CNTF were obtained from R & D Systems. The CNTF and bFGF were reconstituted in sterile PBS (pH 7.4) containing 0.5% bovine serum albumen and were stored at −70 C in small aliquots to avoid repeated freeze-thaw cycles. Soluble chicken recombinant SCF was expressed in Escherichia coli and was purified as described by Bartunek et al. (1996). All factors were diluted in DMEM-FBS immediately before use and were added to cultures immediately after seeding blastodermal cells onto feeder layers of CV-1 cells.

**Experimental Design and Statistical Analysis**

The first series of experiments, consisting of three independent experiments performed at different times, was designed to test the hypothesis that soluble factors produced by STO cells enhance the development of chicken PGC in culture. Each experiment was performed using a single 96-micotiter plate with 4 to 20 wells for each treatment. Treatment groups for this series of experiments were as follows: culture of blastodermal cells on feeder layers of CV-1 cells (n = 27); culture of blastodermal cells on feeder layers of CV-1 cells in the presence of STO-CM (n = 43); culture of blastodermal cells on gelatin-coated plas-
tic \((n = 19)\); and culture of blastodermal cells on gelatin-coated plastic in the presence of STO-CM \((n = 14)\). The culture of blastodermal cells on STO cells \((n = 39)\) served as a positive control.

The second series of experiments, consisting of three independent experiments performed at different times, was designed to examine the effect of bFGF on the emergence of PGC in culture. Treatment groups were as follows: culture of blastodermal cells on feeder layers of CV-1 cells \((n = 15)\) and culture of blastodermal cells on feeder layers of CV-1 cells in the presence of bFGF \((100 \text{ ng/mL}; n = 12)\). The culture of blastodermal cells on STO cells \((n = 12)\) served as a positive control.

The third series of experiments consisted of three independent experiments performed at different times. Treatment groups for this series of experiments were as follows: culture of blastodermal cells on feeder layers of CV-1 cells \((n = 18)\); culture of blastodermal cells on feeder layers of CV-1 cells in the presence of SCF \((100 \text{ ng/mL}; n = 24)\), CNTF \((2 \text{ ng/mL}; n = 18)\), or SCF \((100 \text{ ng/mL})\) and CNTF \((2 \text{ ng/mL})\) \((n = 29)\). The culture of blastodermal cells on STO cells \((n = 27)\) served as a positive control.

The data were analyzed for significance at the 5% level. In analyzing the data, each well in a 96-microtiter plate was considered a replicate. In all experiments, the number of PGC per embryo equivalent was subjected to ANOVA with the general linear model procedure of SAS® software (SAS Institute, 1990). The variation originating from the interaction between date and treatment was used as the error term. The treatment means were compared with LSMEANS procedure of SAS® software (SAS Institute, 1990).

**RESULTS**

The number of PGC obtained on feeder layers of CV-1 cells was significantly \((P \leq 0.05)\) less than that obtained on STO feeder layers (Figure 1). In contrast, the number of PGC obtained on CV-1 feeder layers did not differ significantly \((P \geq 0.05)\) from that obtained on gelatin-coated plastic (Figure 1). Furthermore, addition of STO-CM to blastodermal cells cultured on feeder layers of CV-1 cells or on gelatin-coated plastic resulted in a significant increase \((P \leq 0.05)\) in the number of PGC after 48 h of culture (Figure 1). However, the number of PGC obtained in the presence of STO-CM did not differ significantly \((P \geq 0.05)\) between cultures carried out on CV-1 feeder layers and gelatin-coated plastic.

The response of blastodermal cells cultured on gelatin-coated plastic and feeder layers of CV-1 cells was qualitatively different than that observed with STO cells. A typical response of blastodermal cells cultured on STO cells was to form patches of SSEA-1 positive cells after 48 h of culture (Figure 2A). In contrast, blastodermal cells cultured either on gelatin-coated tissue culture plastic or on feeder layers of CV-1 cells did not give rise to SSEA-1 positive patches (Figure 2B, C) and formed isolated, small clumps consisting of SSEA-1 negative embryonic cells (Figure 2C). In some cases, blastodermal cells cultured on CV-1 cells could give rise to distinguished colonies of PGC in the presence of STO-CM (Figure 2D).

In an attempt to mimic the effect of STO-CM on the emergence of PGC in cultures carried out on feeder layers of CV-1 cells, blastodermal cells were cultured on CV-1 cells in the presence of bFGF, SCF, or CNTF. The culture of blastodermal cells on feeder layers of CV-1 cells in the presence of bFGF \((100 \text{ ng/mL})\) did not result in any increase in the number of PGC after 48 h of culture (Figure 3). However, supplementation of cultures with SCF \((100 \text{ ng/mL})\) or CNTF \((2 \text{ ng/mL})\) or with SCF \((100 \text{ ng/mL})\) and CNTF \((2 \text{ ng/mL})\) resulted in a significant increase \((P \leq 0.05)\) in PGC (Figure 4). The PGC obtained in the presence of SCF \((100 \text{ ng/mL})\) in combination with CNTF \((2 \text{ ng/mL})\) was significantly \((P \leq 0.05)\) higher than that obtained with SCF or CNTF only (Figure 4).

**DISCUSSION**

In all vertebrates, the segregation of the germ cell lineage is characterized by the appearance of PGC at an early stage of development. In the chick embryo, PGC can be detected first at stage X when formation of the *area pellucida* is completed (Ginsburg and Eyal-Giladi, 1987; 1989; Karagenç et al., 1996). Developmental mechanisms governing the emergence of PGC in relation to the formation of the *area pellucida* remain to be elucidated. However, the maintenance of cellular organization within this region appears to be essential for the emergence of PGC. For example,
when individual stage IX to XIV embryos are dispersed and cultured on glass cover slips, 25 to 45 PGC per embryo are detected only with stage X to XIV blastoderms but not with stage IX blastoderms (Karagenc et al., 1996). In the present study, similar numbers of PGC were obtained when blastodermal cells obtained from dispersed stage X embryos were cultured either on gelatin-coated tissue culture plastic or on feeder layers of CV-1 cells (Figure 1). However, when blastodermal cells from the same pool of embryos were cultured on feeder layers of STO cells, a marked increase in the number of PGC was observed (Figure 1). A similar response with STO cells was previously described by Karagenc et al. (1996) and Petite and Karagenc (1996). Furthermore, the addition of STO-CM to blastodermal cells cultured either on gelatin-coated plastic or on feeder layers of CV-1 cells led to a significant increase in the number of PGC (Figure 1).

It is evident from these observations that STO cells provide a soluble factor or factors that enhance the development of chicken PGC in culture. In principle, these unidentified factors could support either the survival or the proliferation of PGC or the differentiation of early embryonic cells towards the germ cell lineage. The data presented in this study, however, do not allow us to distinguish between these possibilities.

The cytokines and growth factors that are involved in the ontogeny of the germ cell lineage are currently unknown in the avian embryo. However, a substantial body of evidence obtained from studies carried out with mouse PGC indicates that during normal development the survival, proliferation and migration of germ cells could be regulated by a complex set of growth factors or cytokines (Donovan, 1994). Some of these growth factors include bFGF, SCF, and leukemia inhibitory factor (LIF) (Dolci et al., 1991, 1993; Godin et al., 1991; Matsui et al., 1991, 1992; Resnick et al., 1992, 1998).

It was previously demonstrated that bFGF can stimulate the proliferation of mouse PGC in culture (Matsui et al., 1992; Resnick et al., 1992). Nevertheless, the addition of bFGF (100 ng/mL) to blastodermal cells cultured on feeder layers of CV-1 cells did not increase the number of PGC obtained after 48 h of culture compared with that observed in control cultures (Figure 3). A similar effect of bFGF was also observed in cultures carried out on feeder layers of STO cells (Karagenc, 1998). These observations do not support a functional role for bFGF in the emergence of PGC at
The cultures were identically significantly on STO feeder layers of STO cells (Petitte and Karagenç, 1996; Godin et al., 1994). The evidence reported in the current study indicates that, like SCF, CNTF can stimulate an increase in the number of PGC after 48 h of culture (Figure 4). Furthermore, when blastodermal cells are supplemented with both SCF and CNTF, a more dramatic increase in the number of PGC is observed (Figure 4). Two lines of evidence are in support of these observations. First, gp130-dependent cytokines are active in blastodermal cell cultures (Pain et al., 1996). Second, specific transcripts for both isoforms of SCF and its receptor c-kit are present in whole embryos at prestreak stages of development. Nevertheless, the present data do not rule out a role for bFGF at later stages of germ-line development.

In contrast to the effect of bFGF observed in the present culture conditions, SCF can lead to a significant increase in the number of PGC when blastodermal cells are cultured on feeder layers of CV-1 cells, which do not support the emergence of PGC (Figure 4). It is important to note that CV-1 cells also do not support the development of mouse PGC (Dolci et al., 1991). Furthermore, some reports indicate that the inhibitory effect of CV-1 cells on the development of mouse PGC relates directly to a failure in the activation of SCF/c-kit signaling pathway (Dolci et al., 1991). The present study suggests that SCF is one of the regulatory factors involved in the development of avian PGC. This hypothesis is substantiated by the observation that specific transcripts for both isoforms of SCF and its receptor c-kit are present in whole embryos at prestreak stages of development and in blastodermal cell cultures performed on feeder layers of STO cells (Petitte and Karagenç, 1996; Karagenç, 1998). Specific details regarding the function of SCF in the development of avian PGC remain to be determined. Evidence obtained from studies carried out with mouse PGC indicate that SCF alone acts mainly as a survival factor (Dolci et al., 1991; Godin et al., 1991). However, there is compelling evidence suggesting that SCF might also act as a mitogen in the presence of other factors, such as LIF (Matsui et al., 1988).

The LIF is a pleiotropic cytokine that is known for its differentiation-inhibiting activity on mouse embryonic stem cells (Smith et al., 1988). The function of LIF is mediated by LIF receptor (LIFR), which forms a heterodimeric high-affinity LIF receptor with gp130, capable of transducing signals in response to LIF (Gearing et al., 1992). Other cytokines such as IL-6, oncostatin M (OSM), and CNTF also utilize LIFR and gp130 for signal transduction (Gearing et al., 1992). For example, LIFR/gp130 functions as a high-affinity receptor not only for LIF but also for OSM (Gearing et al., 1992). The heterodimerization of a low-affinity receptor for CNTF (CNTFα) with LIFR/gp130 results in the formation of a tripartite, high-affinity receptor for CNTF (Davis et al., 1993). It is because of this redundancy at the receptor level that IL-6, OSM, and CNTF can substitute for LIF in inhibiting the differentiation of mouse embryonic stem cells (Nichols et al., 1994; Wolf et al., 1994; Yoshida et al., 1994).

![FIGURE 3. The effect of basic fibroblast growth factor (bFGF) on the number of primordial germ cells (PGC) obtained in cultures carried out on feeder layers of CV-1 cells. Each experiment was performed with 4 to 7 wells, and numbers are the means ±SEM of three independent experiments. Five thousand cells were seeded into each well of 96-well microtiter plates. In each experiment, blastodermal cells obtained from the same pool of embryos were cultured on CV-1 feeder cells in the absence (CV-1) and presence of bFGF (CV-1-bFGF; 100 ng/mL). The culture of blastodermal cells obtained from the same pool of embryos on feeder layers of mitotically arrested STO cells (STO) served as a positive control. The cells were cultured for 48 h at 37 C and 5% CO2. The cultures were fixed in 4% (wt/vol) paraformaldehyde (pH 7.2), and PGC were identified on the basis of morphology and stage-specific embryonic antigen-1 expression. Bars with different letters are statistically significant (P ≤ 0.05) from one another.](Image)

![FIGURE 4. The effect of recombinant rat ciliary neurotrophic factor (CNTF) and avian stem cell factor (SCF) on the number of primordial germ cells (PGC) obtained in cultures carried out on feeder layers of CV-1 cells. Each experiment was performed with 4 to 10 wells, and numbers are the means ±SEM of three independent experiments. Five thousand cells were seeded into each well of 96-well microtiter plates. In each experiment, blastodermal cells obtained from the same pool of embryos were cultured on CV-1 feeder cells in the absence (CV-1) and presence of CNTF (CV-1+CNTF; 2 ng/mL) or SCF (100 ng/mL; CV-1+SCF) or SCF (100 ng/mL) in combination with CNTF (2 ng/mL) (CV-1+SCF+CNTF). The culture of blastodermal cells obtained from the same pool of embryos on feeder layers of mitotically arrested STO cells served as a positive control. The cells were cultured for 48 h at 37 C and 5% CO2. The cultures were fixed in 4% paraformaldehyde (pH 7.2), and PGC were identified on the basis of morphology and stage-specific embryonic antigen-1 expression. Bars with different letters are statistically significant (P ≤ 0.05) from one another.](Image)
SCF and its receptor c-kit are present in whole embryos at prestreak stages of development (Petitte and Karagenc, 1996; Karagenc, 1998).

It would also appear from the ongoing discussion that, under the present culture conditions, CNTF could act synergistically with soluble SCF on avian PGC at prestreak stages of development (Figure 4). Nevertheless, the number of PGC obtained on feeder layers of CV-1 cells in the presence of SCF and CNTF is only a fraction of that obtained with STO feeder cells (Figure 4). Therefore, it is most likely that additional factors, other than SCF and CNTF, are involved in the emergence of PGC in cultures carried out on feeder layers of STO cells. Identification of factors that constitute the microenvironment provided by STO feeder cells is a matter of great interest that requires further study.

Taken together, the culture of blastodermal cells on STO and CV-1 cells should provide a useful biological system to examine the molecular basis of avian germ cell development at prestreak stages of development. The information gained from these studies should help to develop strategies for the long-term culture of avian PGC, a prerequisite for the use of PGC in altering the avian genome.

ACKNOWLEDGMENTS

This work was supported, in part, by funds provided under Project Number NC 01868 of North Carolina State University and by the National Science Foundation under Grant Number IBN 9630617.

REFERENCES


Yoshida, K., I. Chambers, J. Nichols, A. Smith, M. Saito, K. Yusu-