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Understanding the Origin of Avian Primordial Germ Cells: Implications for Germ Cell Culture and Transgenesis in Poultry

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Introduction

The production of transgenic poultry is highly desirable for a number of reasons. During the last two decades, considerable advances have been made in our primary understanding of the molecular basis of many genetic traits important to the poultry industry. The application of this knowledge to the global industry through transgenic technology holds considerable promise for improving the profitability and quality of commercial poultry stocks and for the development of novel uses for poultry. Moreover, since the domestic fowl and other species, such as Japanese quail, continue to be model organisms in the basic biological sciences, a routine means of producing transgenic birds will no doubt provide a tool to further enhance our understanding of avian biology.

Among the various approaches toward the development of transgenic poultry, the use of embryonic chimeras has received considerable attention in the last 10 years. Current methods using embryonic chimeras employ early blastodermal cells or primordial germ cells (PGCs) as outlined in Fig. 8.1. This scheme is obviously an adaptation of procedures used for mammalian embryos, particularly the mouse, while taking advantage of the unique features provided by the avian embryo. Briefly, PGCs or precursors to PGCs in the form of blastodermal cells are isolated, cultured, transfected and returned to a recipient embryo to produce a germline chimera, which hopefully contains transgenic gametes that would yield fully transgenic offspring. For the purposes of this discussion, the emphasis will be on the use of primordial germ cells and the current attempts to culture these cells for use in producing transgenic poultry.

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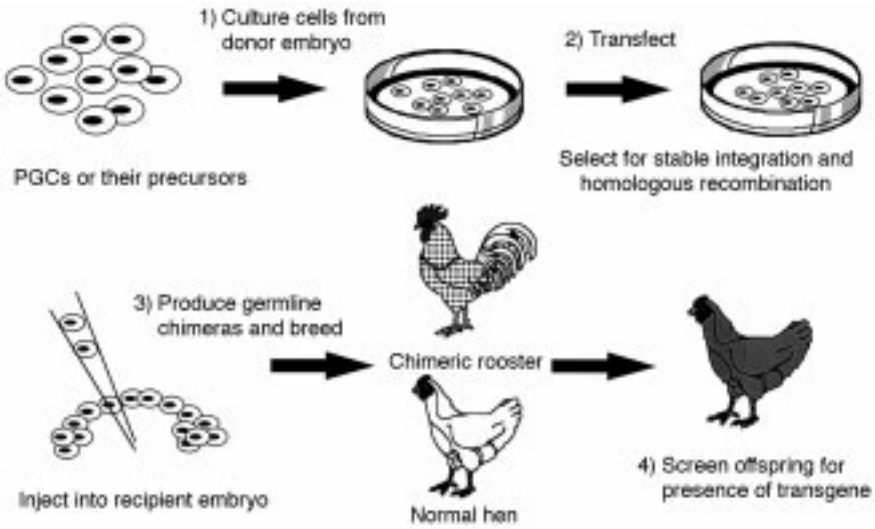

Fig. 8.1. A schematic representation of the steps in the production of transgenic poultry using primordial germ cells (PGCs) or their precursors. 1, PGCs can be obtained from cultured stage X embryo cells, germinal crescent, blood and the gonad (see Fig. 8.2). 2, During culture, the PGCs are transfected with the appropriate DNA construct and selected for stable integration and, if appropriate, evaluated for homologous recombination. 3, The PGCs are then returned to the embryo at the appropriate stage of development to generate a germline chimera. 4, Offspring from the chimera are then screened for the presence of the transgene, and these individuals will become the founding population for a line of transgenic poultry.

Early Development of the Avian Embryo

The two most economically important species of poultry are the domestic fowl (*Gallus gallus domesticus*) and turkey (*Meleagris gallopavo*). Fortunately, domestic fowl have been used in the study of vertebrate development for almost a century and a considerable body of work has amassed regarding the origin of primordial germ cells immediately before and after incubation. Unlike mammals, the main reproductive strategy of the bird entails the packaging of a large vitellus in a calcified eggshell containing the complete nutrient requirement for the development and hatching of a viable chick. Upon ovulation the ovum is fertilized in the distal end of the oviduct, the infundibulum, a process that is clearly polyspermic (Perry, 1987). However, the first cleavage divisions do not begin until about 5–6 h later, after the ovum has received its investment of albumen and the shell membranes and moves into the shell gland for the deposition of the shell (Eyal-Giladi and Kochav, 1976; Perry, 1987). During the approximately 22 h process required for eggshell formation, a considerable amount of cell

division takes place. For several decades, early embryonic development in the shell gland was considered rather amorphous and the staging of embryos began with the onset of incubation (Hamburger and Hamilton, 1951). Subsequently, Eyal-Giladi and Kochav (1976) examined the pre-incubation period of development of the chick. They described and documented a series of well-defined stages from fertilization to the first few hours of incubation based upon significant changes in morphology, which have been shown subsequently to correlate with the early organization of the embryo (Eyal-Giladi, 1991). The staging system of Eyal-Giladi and Kochav (1976) is designated with Roman numerals while that of Hamburger and Hamilton (1951) is designated with Arabic numbers to avoid confusion. Recently, Gupta and Bakst (1993) reported that the early development of the turkey embryo has diverged enough from that of the chick to require a different staging system (for a direct comparison between chick and turkey see Bakst *et al*., 1997).

For the current discussion it is important to understand that by the time the egg is laid the stage X chick embryo consists of a central, translucent cellular area, area pellucida, suspended over a non-yolky cushion of fluid (Fig. 8.2). This region is bordered by a peripheral ring of cells, the area opaca, which is in direct contact with the yolk. Upon incubation, the area pellucida differentiates into two distinct layers, an upper epiblast that will give rise to the embryo proper and a thin hypoblast lying immediately beneath the epiblast (Fig. 8.2). The hypoblast gives rise to portions of the extraembryonic membranes and does not contribute to the embryo proper. The process of hypoblast formation takes place upon incubation between stages XI–XIII. At stage XIV/stage 2, a thickening appears in the posterior end of the embryo and is the first sign of the formation of the primitive streak and the establishment of the three primary germ layers. As the streak elongates the hypoblast is displaced anteriorly by the emerging endoderm (Fig. 8.2). It is the hypoblast that forms the lower layer of the so-called germinal crescent, a region were chick primordial germ cells were first identified (Swift, 1914). This arrangement is important in understanding the origin of the avian germline prior to and after formation of the primitive streak.

Origin of Avian PGCs

For the purpose of producing transgenic poultry, primordial germ cells are the cell type of significance since they will give rise to ova or spermatozoa. The origin of the germline in vertebrates has been a topic of interest for biologists for a very long time. The current scenario for the development of avian germ cells is outlined in Fig. 8.3. PGC development follows a rather circuitous trek beginning with pre-primitive streak development, migration to the germinal crescent, passive migration in the embryonic vasculature, active migration to the presumptive gonad and final residency in the germinal ridge

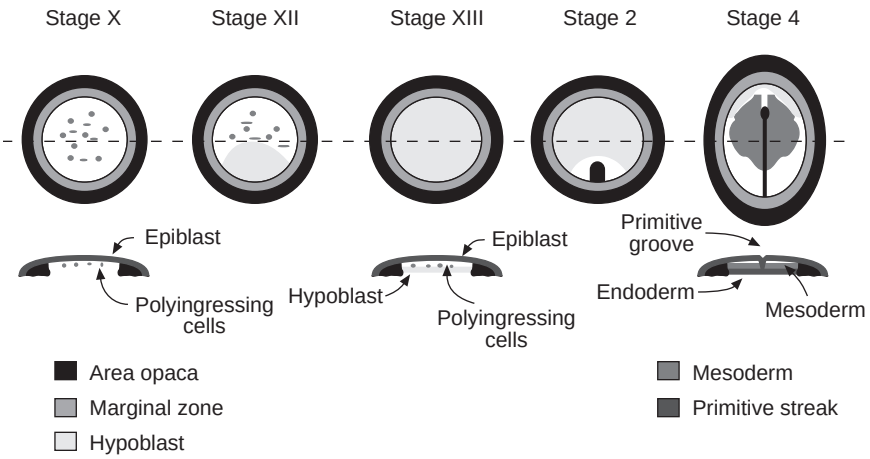

Fig. 8.2. A diagrammatic representation of the structure of the chick embryo from stage X (oviposition) to the formation of the primitive streak at stage 4 (about 12 h of incubation). The stage X embryo is characterized by the area opaca, which is in direct contact with the yolk, and the area pellucida, composed of the marginal zone and the central disk, suspended above the yolk. At this stage, the area pellucida epiblast contains clusters of polyingressing cells on the ventral surface that have moved from the dorsal surface of the epiblast. From stages XI–XIII, the hypoblast forms in a posterior–anterior direction. Some of the polyingressing cells become associated with the hypoblast. During the formation of the primitive streak, beginning at stage 2, the endoderm pushes the hypoblast anteriorly and laterally, taking on a crescent shape.

(Fig. 8.3; see Nieuwkoop and Sutasurya, 1979; Petitte *et al*., 1997). PGCs are ordinarily identified through a combination of morphological characteristics, e.g. large size and eccentric nucleus, coupled with either a histochemical marker such as periodic acid–Schiff (PAS), which stains for glycogen (Meyer, 1960), or with immunohistochemical markers such as EMA-1 and SSEA-1 (stage-specific embryonic antigen 1), which recognize cell-surface carbohydrate epitopes (Urven *et al*., 1988; Loveless *et al*., 1990; Karagenç *et al*., 1996). For the most part, avian PGCs have been identified in primitive streak and older embryos using PAS and in experimental situations where embryo fragments were cultured until PAS staining could be used.

PGCs are derived from the epiblast (Eyal-Giladi *et al*., 1981), a process that begins at stage X (Karagenç *et al*., 1996) before their migration to the germinal crescent (Ginsburg and Eyal-Giladi, 1986), a region in the stage 4–10 embryo located in an anterior, extraembryonic segment bordering the area opaca and area pellucida (Swift, 1914) (Fig. 8.3). Using a series of *in vivo* and *in vitro* techniques, Karagenç *et al*. (1996) utilized antibodies SSEA-1 and EMA-1 to identify a population of about 20 EMA-1/SSEA-1-positive cells that first appear in the stage X epiblast in the area pellucida and that translocate

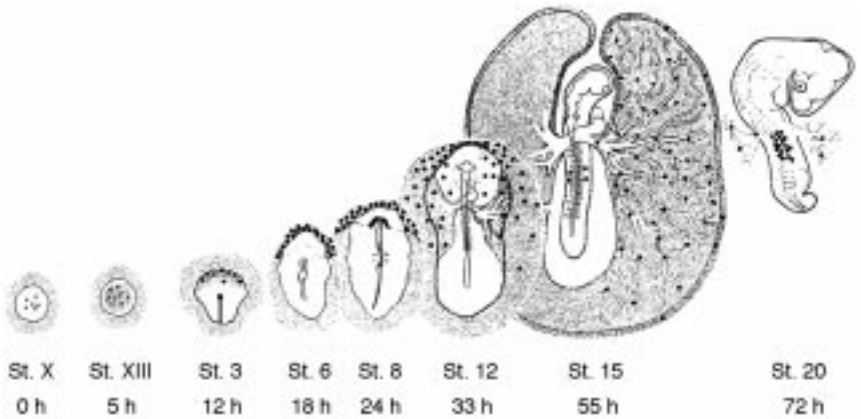

Fig. 8.3. An outline of the origin of primordial germ cells (PGCs) (dark spots in diagram) of the chick embryo from early stages of development through their final residence in the gonadal anlage. The germ cell lineage is first identified by the presence of about 20 SSEA-1 and EMA-1-positive cells in the stage X epiblast at the time of oviposition (Karagenç *et al.*, 1996). Upon incubation, these cells translocate to the hypoblast between stages XI to XIV. With the formation of the primitive streak (stage 3–4) the hypoblast moves in anteriorly to form the germinal crescent (Swift, 1914). From this time onward PGCs can be identified using periodic acid–Schiff (PAS) staining. While in the crescent (stages 6, 8, 12), the germ cells associate with the forming blood islands and then move into the embryonic circulation. Subsequently the germ cells actively leave the blood vessels (stage 15) and migrate along the dorsal mesentery to the gonadal anlage. Most of the germ cells have reached the presumptive gonad by stage 20. The entire process appears to involve passive and active phases of migration, chemotaxis, location of vascular elements and extracellular matrix components. Approximate hours of incubation are shown. (Redrawn after Nieuwkoop and Sutasurya, 1979, with modifications.)

to the hypoblast during stages XII–XIV (Fig. 8.4A, B). Experimentally, it was shown that the SSEA-1-positive cells on the hypoblast have the potential to give rise to PGCs *in vitro* and *in vivo* (Karagenç *et al.*, 1996, and unpublished observations). Additionally, the formation of the area pellucida appears to be a prerequisite for PGC development (Karagenç *et al.*, 1996), although germ cells arise spontaneously in cultured fragments of the central epiblast without normal axial development (Ginsburg and Eyal-Giladi, 1987). Recently, Kagami *et al.* (1997) reported that removing as few as 700 of the 10,000–15,000 cells of the central epiblast in unincubated embryos leads to germ cell-depleted chickens. It is tempting to conclude from this observation that avian germ cell allocation is a one-time event occurring in the geometric centre of the epiblast; however, this hypothesis does not explain the observed gradual allocation of germ cells from the epiblast that occurs from stage XII through to germinal crescent stages (Ginsburg and Eyal-Giladi, 1986;

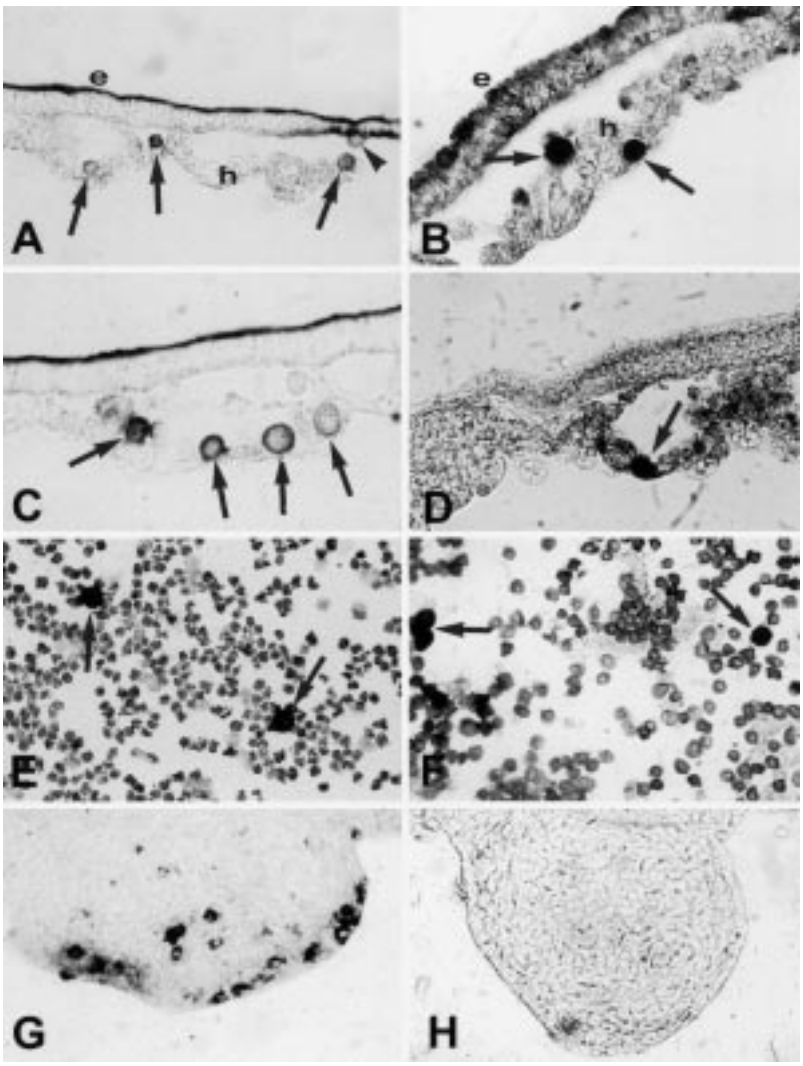

Fig. 8.4. Immunohistochemical staining of primordial germ cells (PGCs) using SSEA-1 in the chick embryo (A, C, E, G) and in the turkey embryo (B, D, F, H). A and B: cross-sections of chicken and turkey embryos, respectively, prior to formation of the primitive streak. Both species contain SSEA-1 labelled cells (arrows) in the hypoblast (h). PGCs originate from the epiblast (e) and translocate to the hypoblast. Arrowhead shows chick PGC emerging from the epiblast. C and D: sections through the germinal crescent of chicken and turkey embryos. Arrows indicate PGCs. E and F: blood smears from chicken and turkey embryos during the period of passive migration of germ cells (arrows) through the blood vessels. G and H: sections through the 5-day chick embryonic gonad and 8-day turkey embryonic gonad (equivalent developmental stages). Clusters of gonadal PGCs can only be identified with SSEA-1 in the chick.

Karagenç *et al*., 1996) nor the body of data showing that PGCs can arise from anterior–posterior or transverse fragments of the stage X embryo and from cells of dispersed stage X embryos (Ginsburg and Eyal-Giladi, 1987, 1989; Karagenç *et al*., 1996). Certainly additional experiments are needed to reconcile these observations.

In any case, once primordial germ cells reach the germinal crescent, they are committed to the germline, and excision or ultra-violet irradiation of the crescent depletes germ cell numbers (Reynaud, 1976, 1977; McCarrey and Abbott, 1978, 1982). While in the germinal crescent, the PGCs associate with the incipient blood islands and enter the embryonic circulation for a period of passive migration (Meyer, 1964; Fujimoto *et al*., 1976a). Subsequently, the PGCs begin an active period of migration and exit the blood vessels to migrate along the dorsal mesentery and collect at the germinal ridges (Fujimoto *et al*., 1976b). By 72 h of incubation most of the germ cells have reached the gonad. This process of passive and active migration is undoubtedly mediated by chemo-attraction, extracellular matrix components and the organization of the vascular system (Dubois and Croisille, 1970; Kuwana *et al*., 1986; Nakamura *et al*., 1988; Urven *et al*., 1989).

All of these periods of PGC development appear to be conserved among various species of birds. However, the successful use of histological markers to identify avian germ cells varies with species (see Table 8.1). For example, while PAS staining is useful for identification of chick PGCs, quail PGCs are PAS-negative. Hence, it cannot be assumed that various markers in one species will be useful in another. Given the importance of the domestic turkey to the poultry industry, we compared the use of SSEA-1 and PAS in the turkey versus the chicken embryo for the identification of PGCs (S. D'Costa and J.N. Petitte, unpublished observations). Figure 8.4 shows that the turkey embryo at stage XI (Gupta and Bakst, 1993), stage 4, and stage 13 is SSEA-1-positive, while the gonad at day 8–9 of incubation is SSEA-1 negative. On the other hand, PGCs of the chick embryo are positive at all the corresponding stages. The role of SSEA-1 in germ cell function is unknown but may be related to migration and cell–cell adhesion, as suggested for mammalian germ cells. If this is the case in birds, the species difference in SSEA-1 expression may prevent turkey germ cells from completing their migration to the gonad. Such a feature would explain, in part, the results obtained by Reynaud (1976) who produced turkey/chick germline chimeras but could not clearly demonstrate functional gametes in the adult. Again, more research in this area is needed to clarify whether interspecies turkey/chick germline chimeras are possible.

Development of Germline Chimeras Using PGCs

The foregoing discussion of germ cell origins and development immediately suggests various intervention points to make germline chimeras using PGCs.

Table 8.1. A comparison of the major histochemical and immunological markers of primordial germ cells (PGCs) among chicken, quail, turkey and the mouse at various stages of germ cell development. Question marks indicate unknown use of the marker and NS indicates non-specific. In practice, the use of these markers must be coupled with the morphological characteristics of PGCs and their location for proper identification. Pertinent references are given.

		Stage of germ cell development					
Species	Marker	Pre-streak embryo	Stage 4–10, germinal crescent	Stage 11–17, blood	Stage 18–22, dorsal mesentery	Stage 23+, gonadal ridge	References
Chicken	AP	NS	NS	NS	+	+	Chiquoine and Rothenberg (1957)
	PAS	NS	+	+	+	+	Meyer(1964), Fujimoto *et al*. (1976b), Swartz (1982)
	EMA-1	+	+	+	+	+	Urven *et al*. (1988), Karagenç *et al*. (1996)
	SSEA-1	+	+	+	+	+	Karagenç *et al*. (1996)
Quail	PAS	NS	–	–	–	–	Nakamura *et al*. (1992)
	QH-1	+	+	?	?	+	Pardanuad *et al*. (1987)
	SSEA-1	+	+	?	?	?	Petitte and Karagenç, unpublished observations
Turkey	PAS	NS	+	+	+	+	Reynaud (1967, 1969)
	SSEA-1	+	+	+	–	–	See text
		6.5 dpc, egg cylinder	8.5 dpc, allantois	No equivalent stage	9–12 dpc, hindgut/dorsal mesentery	12.5–13 dpc, gonadal ridge	
Mouse	AP	+	+	–	+	+	Chiquoine (1954), Mintz and Russel (1957), Ozdzenski (1967), Ginsburg *et al*. (1990)
	EMA-1	?	+	–	+	+	Hahnel and Eddy (1986)
	SSEA-1	?	?	–	+	+	Fox *et al*. (1981)

NS, non-specific; PAS, periodic acid–Schiff; AP, alkaline phosphatase; SSEA-1, stage-specific embryonic antigen-1.

In fact, several groups have used PGCs from the germinal ridge, blood and gonads for this purpose. Reynaud (1969) definitively demonstrated the extragonadal origin of PGCs by injecting germinal crescent cells, which presumably contained PGCs, into the extraembryonic vasculature of sterilized recipient embryos. Subsequently, the donor PGCs populated the host gonad. This observation led to initial attempts to produce germline chimeric chicks or quail through germinal crescent cell transfer or using PGCs from blood (Shuman, 1981; Gonzales, 1989; Wentworth *et al*., 1989; Simkiss *et al*., 1989; Petitte *et al*., 1991). Since that time, the results of other experiments have demonstrated that PGCs from these two sources can be used to produce embryonic germline chimeras as well as adult birds that produce gametes derived from the donor germ cells (Watanabe *et al*., 1992; Yasuda *et al*., 1992; Tajima *et al*., 1993; Vick *et al*., 1993; Naito *et al*., 1994b; Ono *et al*., 1996). In most cases, the depletion of endogenous germ cells improves the frequency of chimerism. Recently, Chang *et al*. (1995b) have shown that PGCs obtained from the germinal ridge can be used to produce germline chimeras when injected into the blood of recipient embryos during earlier stages of development. This is an important observation, since it was assumed that once the PGCs reached the gonadal anlage, their ability to migrate and repopulate the host gonad would be lost. It would be of interest to examine how long gonadal germ cells retain this capability.

Transgenesis Using PGCs

Several methods of inserting DNA into PGCs are available, and the use of each has its advantages and disadvantages. For the most part, only retroviral infection of germinal crescent PGCs has been successful in producing transgenic chickens (Vick *et al*., 1993). This achievement was due mainly to the high integration efficiency of retroviral vectors. DNA complexed with liposomes offers a convenient method both *in situ* and *in vitro* (Brazolot *et al*., 1991; Han *et al*., 1994, 1996; Watanabe *et al*., 1994; Hong *et al*., 1998), and bombardment of the germinal crescent with DNA-coated particles can place DNA within the cells (Li *et al*., 1995). Electroporation of germinal crescent or blood PGCs has yet to be tried because of the large numbers of cells required by most procedures. Recently, electroporation of dispersed gonadal tissue followed by separation of PGCs was tested as a means of transfecting gonadal PGCs (Hong *et al*., 1998). This approach yielded levels comparable to liposome-mediated transfection. Nevertheless, stable integration of exogenous DNA in the PGC genome is normally a rare event using these methods, and the possibility of episomal inheritance of plasmid DNA calls for careful experimentation (Chapters 7 and 9, this volume). The only manner in which to guarantee stable integration of DNA into the germ cells is through the culture of PGCs with the application of some type of selectable marker. Traditionally, antibiotic resistance has been used for this

purpose; however, constructs using jellyfish green fluorescent protein might serve as another means to identify cells where integration events have occurred.

Culture of PGCs

Whatever the source of PGCs and the means of transfection, the production of transgenic poultry would be facilitated immensely by the ability to establish long-term cultures of germ cells or their precursors. In addition to applications in transgenics, the ability to expand primordial germ cell populations could have a major impact on the conservation and preservation of avian genetic resources and endangered wild avian species (Delany and Pisenti, 1998). In this regard, it is noteworthy that cryopreserved chick primordial germ cells have been successfully used to produce chicks though the production of germline chimeras (Naito *et al*., 1994a; Tajima *et al*., 1996). For these applications to achieve their full potential, it is important to have a basic understanding of the complex biology of PGCs at the molecular level. Unfortunately, knowledge in this area is still rudimentary and is often based upon work with mammalian germ cells. Despite this limitation, there have been attempts to culture germ cells from gonadal PGCs for relatively short periods of time, usually less than a week. The first effort to culture chick PGCs was reported by Fritts-Williams and Meyer (1972). In this case, stage 25–26 embryonic gonads were used as the starting material since sexual differentiation was not morphologically apparent. Germ cells were enriched by applying a single-cell suspension of gonadal tissue to a Ficoll gradient. The fraction containing the PGCs was cultured for 7–14 days in hanging drops or roller tubes, but no proliferation was observed. Subsequently, Allioli *et al*. (1994) cultured gonadal PGCs long enough for infection with a retroviral vector and obtained at least 50% of the PGCs expressing *LacZ*. Wentworth *et al*. (1996) mentioned that attempts to culture gonadal PGCs co-cultured with gonadal somatic cells were dependent upon the initial concentration of PGCs, and reported that PGCs could not be regularly subcultured. Likewise, Chang *et al*. (1995a,b, 1997) were able to culture gonadal PGCs on a stromal cell feeder layer for 5 days and then used the cells to produce germline chimeras that transmitted the donor genotype to 1.3–3.5% of the progeny. Such results are quite promising, and testing the effect of longer periods of culture on the ability to produce germline chimeras is an obvious next step.

More culture work has been done using gonadal PGCs than any other source of germ cells because of the relative ease of obtaining sufficient numbers of cells to use for experimental purposes. However, there are some reports of short-term culture of PGCs at other stages. Lee *et al*. (1983) removed the hypoblast from the germinal crescent area of stage 4–5 embryos and observed the migration of PGCs over the hypoblast feeder

layer. Likewise, Kuwana and Fujimoto (1984) have observed the movement of PGCs from the blood *in vitro* on a feeder layer of dorsal mesentery. While these experimental procedures were designed to examine the process of germ cell locomotion, they do suggest that it is possible to culture germ cells from these sources if provided with suitable culture conditions.

Unfortunately, our lack of understanding of what makes avian germ cells survive and proliferate *in vivo* is a genuine problem associated with the culture of avian PGCs. In the mouse, several factors are implicated in the survival and proliferation of PGCs. These include, leukaemia inhibitory factor (LIF), stem cell factor (SCF)/c-kit, transforming growth factor-b, fibroblast growth factor-2 (FGF-2), among others. Unfortunately, Chang *et al.* (1995a) found no observable benefit to adding mammalian SCF or LIF to the culture of gonadal PGCs. In the course of examining the origin of PGCs in the pre-streak embryo, it was observed that the culture of dispersed cells from the area pellucida of stage IX embryos could be induced to give rise to primordial germ cells only when plated on a feeder layer of STO cells (Karagenç *et al.*, 1996; Fig. 8.5). Not only could this culture system yield PGCs, but the numbers obtained were roughly equivalent to those at germinal crescent stages, suggesting that the factors responsible for emergence, proliferation or a combination of the two were being provided by the feeder cells (Fig. 8.6). This observation immediately suggested that the culture of stage X embryo cells on an STO feeder layer could be used to examine the role of various growth factors involved in avian germ cell development.

Among the obvious candidate growth factors is SCF, also called steel factor and mast cell growth factor. SCF plays a pleiotropic role in haematopoiesis, melanocyte development and germ cell development (Bernstein *et al.*, 1990) and is the ligand of the receptor tryosine kinase, c-kit. In mice, mutations in either of these proteins result in impaired germ cell production or migration (Chabot *et al.*, 1988; Geissler *et al.*, 1988;

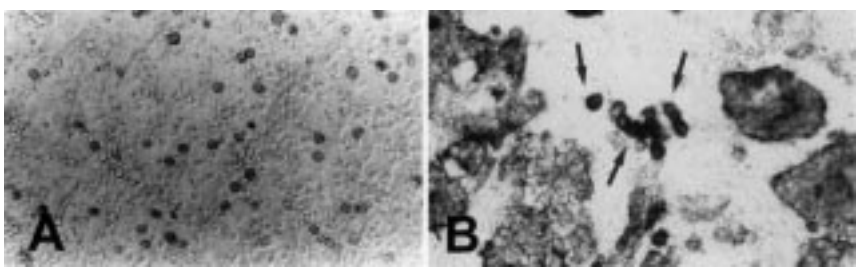

Fig. 8.5. The emergence of PGCs cultured on a feeder layer of STO cells. A, freshly seeded dispersed stage X embryo cells on a feeder layer of mouse STO fibroblasts; B, growth of the culture after 48 h and staining with SSEA-1. The culture consists of SSEA-1-positive and negative patches with PGCs often found in clusters lying above the feeder cells (arrows). Methods according to Karagenç *et al.* (1996).

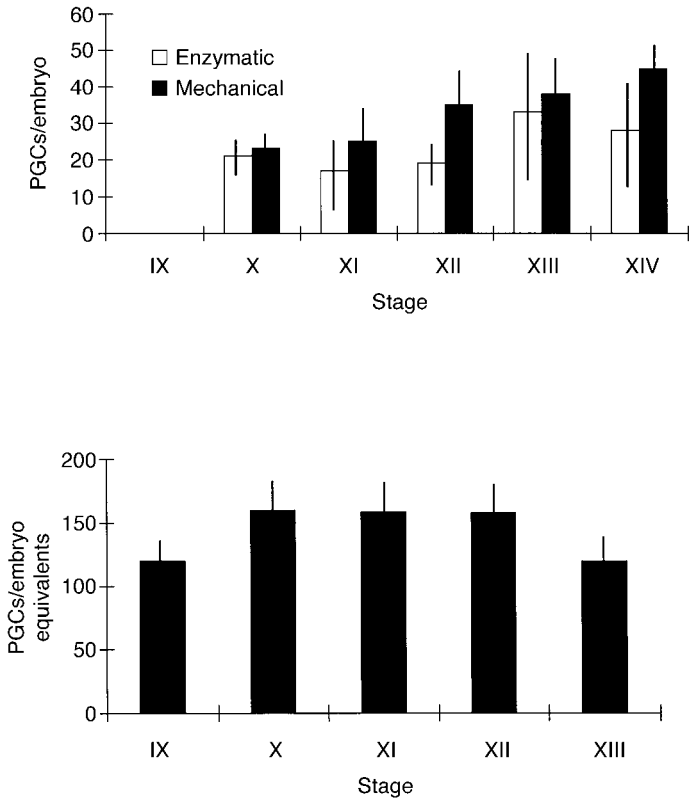

Fig. 8.6. The number of primordial germ cells (PGCs) obtained after 48 h of culture using dispersed chick embryo area pellucida cells without (upper panel) and with (lower panel) an STO feeder layer. Upper panel: whole dispersed embryos were cultured and stained with periodic acid–Schiff (Meyer, 1960). No germ cells were obtained using stage IX embryos. Lower panel: 5000 cells from the area pellucida were seeded on to an STO feeder layer in 96-well plates and stained for primordial germ cells using SSEA-1. Data are presented as number of PGCs per embryo. The number of PGCs obtained was considerably higher than for cultures without a feeder layer at any stage. (Data after Karagenç *et al.*, 1996.)

Copeland *et al.*, 1990). As in mammals, SCF in birds is involved in haematopoiesis and melanogenesis, but little is known about its role in avian germ cell development (Hayman *et al.*, 1993; Lahav *et al.*, 1994; Lecoin *et al.*, 1995). In chicken and quail, the cDNA has been cloned and, like the mammalian homologues, it encodes a transmembrane protein that can exist in two isoforms, a membrane-anchored form and a soluble form (Zhou *et al.*, 1993; Petitte and Kulik, 1996). Homologies between avian and mammalian forms of SCF are about 52%, and this explains the lack of

biological activity of mammalian SCF on avian cells. During the prestreak period of germ cell development, transcripts for both forms of SCF and its receptor c-kit can be detected in whole embryo preparations using reverse transcriptase (RT)-PCR (Petitte and Karagenç, 1996, and unpublished observations). Likewise, preliminary work indicates that c-kit is expressed by avian PGCs at stage 28 when they have reached the germinal ridge. At the same time, the germinal ridge epithelium expresses SCF (J.N. Petitte and L. Karagenç, unpublished observations). All of these observations point to SCF/c-kit participating in avian germ cell development.

Another candidate growth factor that may be involved in germ cell survival and development is FGF-2. Resnick *et al.* (1992) and Matsui *et al.* (1992) reported that FGF-2 stimulates the proliferation of mouse PGCs on a variety of feeder layers. In addition, when combined with a SCF-expressing feeder layer and LIF, FGF-2 is an essential component for the long-term culture of mouse PGCs that resemble embryonic stem cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992; Labosky *et al.*, 1994; Stewart *et al.*, 1994). In the chick embryo, FGF-2 transcripts can be detected in the stage XIII embryo (Mitrani *et al.*, 1990), and the application of FGF-2 changes the behaviour of epiblast cells *in vitro* and inhibits axial development of stage XIII embryos (Cooke and Wong, 1991). Finally, Dono and Zeller (1994) and Riese *et al.* (1995) reported that prior to primitive-streak formation, immunohistochemical staining of FGF-2 was located in the nuclei of all hypoblast cells and in some epiblast cells. At primitive streak stages, FGF-2 then appears in the cytoplasm.

Given the association of SCF/c-kit and FGF-2 expression with early and later stages of germ cell development, it is worthwhile examining their role in the culture of avian PGCs. Therefore, we examined the effect of SCF and FGF-2 in the development of PGCs *in vitro* using dispersed stage X cells. After 48 h of culture, STO cells supported the growth of avian PGCs far better than CV-1 cells, a green monkey kidney fibroblast cell line (Fig. 8.7). However, when STO-conditioned medium was added to the culture using CV-1 cells, the response was better than that observed with CV-1 cells alone, suggesting that soluble factors acting on the embryonic cells were involved. However, the addition of FGF-2 to the culture on CV-1 cells did not yield more PGCs than that with CV-1 cells alone. Nonetheless, when CV-1 cells that express the membrane-anchored form of avian SCF are used to culture dispersed stage X embryo cells, the number of PGCs is higher than that observed using CV-1 cells alone (Fig. 8.7). The addition of FGF-2 to the SCF-expressing CV-1 cells did not potentiate the response (Fig. 8.7). These data suggest that SCF may be involved in the emergence and survival of primordial germ cells early in development. Indeed, avian SCF transcripts and c-kit transcripts can be detected by RT-PCR in cultures of stage X cells on STO feeder layers (J.N. Petitte and L. Karagenç, unpublished observations). FGF-2, on the other hand, does not appear to support the survival and proliferation of PGCs from the stage X embryo. This is somewhat

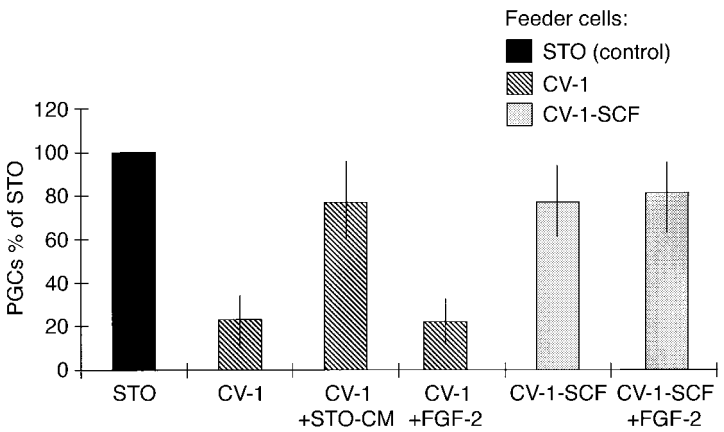

Fig. 8.7. Primordial germ cell culture of dispersed chick embryo cells from the area pellucida on feeder layers of STO, CV-1 and CV-1 cells expressing the membrane-anchored form of stem cell factor (CV-1-SCF). Data are represented as percentage of the response on STO cells alone. CV-1 cells normally do not support the development of PGCs; however, when STO-conditioned medium (STO-CM) is added, the number of germ cells increases relative to culture with CV-1 cells alone. The addition of 100 ng ml^{-1} FGF-2 had no effect on the response. When a CV-1 cell line expressing stem cell factor was used (CV-1-SCF), the percentage of germ cells was equivalent to that observed with STO-CM. Again, the addition of 100 ng ml^{-1} FGF-2 to the culture using CV-1-SCF did not yield more PGCs relative to STO cells alone. Data represent the average of at least three experiments.

unexpected, given the pivotal role of FGF-2 in mammalian PGC culture. However, all of the mammalian culture systems use PGCs from relatively late stages of germ cell development, and the need for FGF-2 in avian germ cell culture may be required for blood or gonadal PGCs. In any case, this culture system should provide a convenient means of examining the effect of other peptide growth factors and cytokines on the proliferation of chick PGCs *in vitro*. Such information could refine current culture methods so that PGCs could then be used for the routine production of transgenic poultry.

Conclusions

The development of robust methods of producing transgenic poultry would greatly facilitate the application of biotechnology to the poultry industry. Despite obstacles, in the last 10 years considerable progress has been made toward using PGCs for the production of transgenic poultry. Several choices are available in starting material and transfection methods. However, the ability to culture avian PGCs for periods long enough to allow selection and

multiplication of cells containing integrated gene constructs requires a better understanding of the molecular requirements of the avian germline. Basic information on the process of germ cell development from their segregation from the somatic lineages to their final residence in the primitive gonad will help in attempts to improve the culture of avian PGCs. The data reported here and in other laboratories offer appreciable optimism for future refinements of avian primordial germ cell culture. This will allow academic and industrial institutions to take full advantage of the usefulness provided by the domestic fowl as a research model and as an agricultural commodity for the next century.

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